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Cisplatin-induced DNA damage activates replication checkpoint signaling components that differentially affect tumor cell survival

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Running title: Rad9 and ATR, but not Chk1, reduce cisplatin tumor killing

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Abbreviations

9-1-1, Rad9-Hus1-Rad1; TLS, Translesion Synthesis; FA, Fanconi's Anemia; HR, Homologous Recombination; ss, single-stranded; RPA, Replication Protein A;

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ABSTRACT

Cisplatin and other platinating agents are some of the most widely used chemotherapy agents. These drugs exert their antiproliferative effects by creating intrastrand and interstrand DNA crosslinks, which block DNA replication. The crosslinks mobilize signaling and repair pathways, including the Rad9-Hus1-Rad1 (9-1-1)•ATR•Chk1 pathway, a pathway that helps tumor cells survive the DNA damage inflicted by many chemotherapy agents. Here we show that Rad9 and ATR play critical roles in helping tumor cells survive cisplatin treatment. Unexpectedly, however, depleting Chk1 with siRNA or inhibiting Chk1 with AZD7762 did not sensitize these cells to cisplatin, oxaliplatin, or carboplatin. Moreover, when Rad18, Rad51, BRCA1, BRCA2, or FancD2 were disabled, Chk1 depletion did not further sensitize the cells to cisplatin. In fact, Chk1 depletion reversed the sensitivity seen when Rad18 was disabled. Collectively, these studies suggest that the pharmacological manipulation of Chk1 may not be an effective strategy to sensitize tumors to platinating agents.

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Introduction

The platinum-based chemotherapy drugs cisplatin, carboplatin, and oxaliplatin are among the most active and widely used agents for the treatment of malignancies, including testicular, head and neck, ovarian, lung, colorectal, and bladder cancers (Kelland, 2007). It is generally accepted that these agents kill tumor cells primarily by creating DNA lesions, which are most cytotoxic during S phase, likely because the lesions are potent inhibitors of DNA replication (Donaldson et al., 1994).

When a replication fork is stalled by either intra- or interstrand crosslinks, sophisticated repair and signaling pathways are called into action. In the case of bulky adducts such as intrastrand crosslinks (which comprise the majority of platin-induced lesions), the stalled replication fork triggers the monoubiquitylation of PCNA (Lehmann, 2006). Ubiquitylated PCNA then recruits one or more translesion synthesis (TLS) polymerases, which have active sites that can accommodate bulky lesions, thereby allowing error-prone bypass of the lesion. In contrast, interstrand crosslinks, which account for a few percent of cisplatin-induced DNA lesions but are far more cytotoxic, cannot be simply bypassed. Instead, their repair involves a complex interplay between a series of DNA repair pathways, including the TLS, Fanconi's anemia (FA), and homologous repair (HR) pathways (Dronkert and Kanaar, 2001). While the complete mechanistic details of how these pathways accomplish this repair remain unknown, it is clear that defects in these pathways dramatically sensitize cells to agents that cause interstrand crosslinks, including the platinating agents (Dronkert and Kanaar, 2001).

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Notably, defects in these pathways are frequently found in tumor cells, raising the possibility that these repair deficiencies contribute to enhanced sensitivity of tumor cells to platinating agents (Gossage and Madhusudan, 2007; Gudmundsdottir and Ashworth, 2006; Lyakhovich and Surralles, 2006; Miyagawa, 2008).

In addition to triggering repair pathways, stalled replication forks also activate the Rad9-Hus1-Rad1 (9-1-1)•ATR•Chk1 signaling pathway (Cimprich and Cortez, 2008). The pathway is initiated when the replicative helicase that unwinds the double-stranded DNA continues advancing in front of the stalled DNA polymerase. This creates extensive regions of single-stranded (ss) DNA that are coated with the RPA (Replication Protein A) complex. The RPA-coated ssDNA then triggers the Rad17-mediated loading of the Rad9-Hus1-Rad1 (9-1-1) clamp complex and the binding of the ATR-ATRIP complex. The chromatin-bound 9-1-1 clamp, which associates with the ATR activator TopBp1 then triggers ATR activation (Burrows and Elledge, 2008). Activated ATR phosphorylates multiple substrates that regulate DNA repair and cell cycle arrest, including Chk1, which helps cells survive replication stress by preventing the firing of origins of replication, delaying G2 exit, stabilizing the stalled replication forks, and regulating DNA repair (Cimprich and Cortez, 2008).

Consistent with the multiple roles of the 9-1-1•ATR•Chk1 pathway in regulating cell cycle arrest, DNA repair, and replication fork stability, much work has now shown that the pathway plays a pivotal role in helping cells survive a wide range of genotoxic stresses, including radio- and chemotherapies. These findings have provoked intense

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interest in pharmacologically targeting this pathway as a means to increase the cytotoxicity of genotoxic cancer therapies, with most of these efforts focused on identifying small molecule inhibitors of Chk1, the most "druggable" component in the signaling pathway. Consistent with that prediction, recent work has shown that Chk1 inhibitors potentiate the activity of nucleoside analogs and topoisomerase I inhibitors in cell lines and xenografts; and these inhibitors are now in early stage clinical trials in combination with gemcitabine and irinotecan (Ashwell and Zabludoff, 2008).

Although platinating agents are among the most widely used chemotherapy agents, little is known about what checkpoint signaling pathways are activated by these agents or how these pathways affect the survival of tumor cells treated with these agents. To that end, we performed a stepwise analysis and examined the role the 9-1-1•ATR•Chk1 pathway in cells treated with platinating agents to gain insight into which aspects of this signaling pathway are important for tumor cell survival and to assess whether Chk1 plays an important role in facilitating tumor cell survival following treatment with platinating agents.

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Materials and Methods

Reagents. Cisplatin and carboplatin were from NovaPlus. Oxaliplatin was from Sigma-Aldrich Corp. Gemcitabine was obtained from Eli Lilly. Antibodies that recognize the indicated proteins were obtained as follows: Chk1 and ATM (Santa Cruz Biotechnology); Rad18 (Novus Biologicals); Rad51 (Thermo Fisher Scientific); Cdc25A (Neomarkers); phospho-Ser^{345}-Chk1 and BRCA1 (Cell Signaling); Rad9 (Volkmer and Karnitz 1999); ATR and BRCA2 (Calbiochem); FancD2 (GeneTex, Inc); Hsp90 (David Toft, Mayo Clinic); and ß-actin (Sigma). The Chk1/Chk2 inhibitor AZD7762 was purchased from Axon Medchem BV.

Cell culture, siRNA transfections, clonogenic assays, and drug treatment. HeLa, HCT-116, and U2OS cells were grown in RPMI-1640 supplemented with 10% fetal bovine serum. Stable clones of *Rad9*$^{-/-}$ mouse embryonic stem (ES) cells (Hopkins et al., 2004) transfected with empty vector or expressing wild-type Rad9 were derived and cultured as previously described (Roos-Mattjus et al., 2003). The following siRNAs (synthesized by Thermo Fisher Scientific) were used: luciferase, CUUACGCUGAGUACUUCGA (Elbashir et al., 2001); ATR, CCUCCGUGAUGUUGCUUGA (Casper et al., 2002); Chk1, GCGUGCCGUAGACUGUCCA, (Zhao et al., 2002); BRCA1, GUGGGUGUUGGACAGUGUA (Bartz et al., 2006); BRCA2, GACUCUAGGUCAAGAUUUA (Bartz et al., 2006); Rad51, CUCCUAGAGGUGAAACCCU (Lio et al., 2004); ATM, GCACCAGUCCAGUAUUGGC (Wang and Qin, 2003); Rad9, ACUUGAAUCUUAGCAUUCA; Rad18,

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GCUCUCUGAUCGUGAUUUA; and FancD2, GGUCAGAGCUGUAUUAUUC. On day 1, siRNA (900 ng) was combined with 12 µL HiPerFect reagent (Qiagen), incubated at room temperature for 5 min, and added to cells in the well for a final siRNA concentration of 30 nM. Transfections were repeated on day 2. On day 3, cells were re-plated in 100-mm tissue culture dishes. On day 4, cells were trypsinized, used to set up clonogenic assays and lysed for immunoblotting. Clonogenic assays were performed as described previously (Karnitz et al., 2005) using 24-h drug treatments. Cell lysis and immunoblotting were performed as described (Volkmer and Karnitz, 1999), and blots were developed with SuperSignal West Pico chemiluminescent substrate (Pierce).

Cell cycle analysis. Trypsinized cells were permeabilized with ice-cold 70% ethanol in PBS, stored at -20˚C for 1 h, centrifuged, resuspended in phosphate-buffered saline containing 50 µg/ml propidium iodide and 100 µg/ml RNase, incubated at 30˚C for 30 min, and analyzed by flow microfluorimetry (FACScan, Becton Dickenson).

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Results

Cells lacking Rad9 are sensitive to the antiproliferative effects of cisplatin. To begin a stepwise assessment of the role(s) of 9-1-1•ATR•Chk1 pathway in tumor cells treated with cisplatin, initial experiments focused on Rad9, a key participant in DNA repair and Chk1 signaling. Using a previously described model system of mouse *Rad9*$^{-/-}$ ES cells stably transfected to express wild-type Rad9 or transfected with empty vector (Hopkins et al., 2004; Roos-Mattjus et al., 2003), we assessed the impact of Rad9 status on the ability of these cells to form colonies after a 24-h treatment with graded concentrations of cisplatin. As shown in Fig. 1A, cells lacking Rad9 were exceptionally sensitive to the antiproliferative effects of this crosslinking agent.

Rad9 and ATR depletion sensitizes HeLa cells to cisplatin. To further evaluate the role of Rad9 and ATR in resistance to cisplatin (and to also demonstrate that the results of a Rad9 deficiency were not specific to mouse ES cells), we analyzed the effects of depleting Rad9 and ATR from HeLa cells using siRNAs (Fig. 1B). Because we had shown previously that Rad9 and ATR play critical roles in helping tumor cells (including HeLa cells) survive treatment with gemcitabine, a nucleoside analog that disrupts DNA replication (Karnitz et al., 2005), we also treated the cells with gemcitabine. Depletion of either Rad9 or ATR sensitized HeLa cells to cisplatin (Fig. 1C) as well as gemcitabine (Fig. 1D), thus demonstrating that these checkpoint proteins play critical roles in facilitating the survival of cisplatin-treated tumor cells.

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Disrupting Chk1 signaling does not sensitize HeLa cells to platinating agents. An important target substrate for activated ATR is Chk1, a protein kinase that participates in blocking cell cycle progression and regulating DNA repair following DNA damage or replication stress (Ashwell and Zabludoff, 2008). Given the central role of Chk1 in ATR signaling and the fact that Chk1 inhibition sensitizes many tumor cell lines to genotoxic chemotherapies, including gemcitabine (Fig. 1D and Karnitz et al., 2005), we asked whether Chk1 depletion (Fig. 1B) affected HeLa cell clonogenicity following treatment with cisplatin, oxaliplatin or carboplatin (Fig. 1C, 1E, and 1F, respectively). Surprisingly, even though Chk1 depletion sensitized cells to gemcitabine (Fig. 1D), Chk1 depletion did not sensitize HeLa cells to any of the platinating agents.

To further probe the role of Chk1 in cisplatin cytotoxicity, we used AZD7762, a small molecule that inhibits both Chk1 and Chk2 with similar potency (Zabludoff et al., 2008). While this agent dramatically sensitized HeLa cells to gemcitabine (Fig. 2A), it did not sensitize the cells to cisplatin (Fig. 2B). This result suggested that neither Chk1 nor Chk2 plays an important role in helping cells survive cisplatin treatment. Consistent with this finding, co-depletion of Chk1 and Chk2 with siRNAs (Fig. 2C) did not sensitize HeLa cells to cisplatin (Fig. 2D). Taken together, these results demonstrate that while ATR is important for tumor cell survival after treatment with platinating agents, Chk1 is not, even when Chk2 is also depleted.

Cisplatin activates Chk1. In view of the unexpected finding that Chk1 depletion did not sensitize HeLa cells to platinating agents, we asked whether the DNA damage induced

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by cisplatin could activate Chk1. HeLa cells were treated with cisplatin concentrations that reduced clonogenicity by 10% (250 nM) and 90% (600 nM), and Chk1 phosphorylation on Ser^{345}, a site phosphorylated by ATR and required for Chk1 activation (Liu et al., 2000), was assessed (Fig 3A). In addition, to demonstrate that the phosphorylated Chk1 was relaying signals to downstream targets, we analyzed Cdc25A, a Chk1 substrate that is targeted for proteasomal degradation after Chk1-mediated phosphorylation. Consistent with previous results, cisplatin induced Chk1 phosphorylation under all conditions tested; and there was a corresponding decrease in the levels of Cdc25A (Fig. 3A, lanes 2-5). As a control for this experiment, we initially treated cells with concentrations of gemcitabine that also reduced clonogenicity by 10% (30 nM) and 90% (100 nM), but we observed nearly undetectable Chk1 phosphorylation (Fig. 3A, lanes 6 and 8, and data not shown); notably, however, a high concentration of gemcitabine (1000 nM) induced robust Chk1 phosphorylation and Cdc25A degradation (Fig. 3A, lane 7). Taken together, these results suggest that cisplatin – at isotoxic concentrations – is a better inducer of Chk1 phosphorylation than gemcitabine; however Chk1 only plays a role in helping cells survive gemcitabine but not cisplatin treatment.

Depleting Chk1 disrupts the cisplatin-induced S-phase arrest. A major function of Chk1 following genotoxic stress is to block origin firing and S-phase progression. To assess whether the Chk1 activated in cisplatin-treated HeLa cells was indeed promoting an S phase arrest, we examined the cisplatin-induced cell cycle arrest in control and Chk1-depleted HeLa cells. For these assays cells were treated for 20 h with 1 and 4 μM cisplatin. (These comparatively high concentrations of cisplatin were used because

we did not observe robust S-phase accumulation in the control cells at lower concentrations.) Consistent with previously published results, 1 μM cisplatin induced mid-S-phase accumulation in control cells, with the higher concentration of cisplatin causing an early S-phase accumulation (Fig. 3B). In contrast, in Chk1-depleted cells, this S-phase arrest was partially disrupted and the cells accumulated in late S phase or G2/M. Taken together, these results suggest that Chk1-mediated inhibition of S-phase progression does not play an important role in helping HeLa cells survive cisplatin treatment.

Multiple tumor cell lines are not sensitized to cisplatin by Chk1 depletion. To further explore the surprising finding that Chk1 depletion does not sensitize HeLa cells to cisplatin, we examined the effect of depleting Chk1 in additional cell lines. HCT-116 and U2OS cells, which were derived from a colorectal carcinoma and an osteosarcoma, respectively, were selected for these studies because patients with these tumors are often treated with platinating agents. Consistent with the results for HeLa cells (Fig. 1), Chk1 depletion did not sensitize either HCT-116 or U2OS cells to cisplatin, whereas both cells lines were sensitized to gemcitabine (Fig. 4A-D). Similarly, Chk1 depletion did not sensitize HCT-116 cells to oxaliplatin, an agent that is often used to treat colon cancer, or the lung cancer cell line A549 to cisplatin (data not shown). Collectively, these results show that Chk1 does not play a rate-limiting role in preventing the antiproliferative effects of platinating agents in multiple cell types, including cell lines derived from tumors that are routinely treated with these drugs.

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Disabling DNA repair pathways does not make cisplatin-treated tumor cells reliant on Chk1. We reasoned that Chk1 signaling pathways might assume increased importance if the pathways that repair platinum-induced lesions were disabled. Many of the tumors that are treated with cisplatin harbor defects in repair pathways for cisplatin-induced lesions. Thus, if Chk1 depletion sensitized a tumor cell with a defect in a specific repair pathway, then Chk1 inhibitors might be useful to sensitize these tumors to platinating agents. To test this idea, we first depleted HeLa cells of Rad51, BRCA1, Rad18, FancD2, or BRCA2 (supplemental figure 1), all of which participate in the repair of cisplatin-induced lesions. In all cases, knockdown of any single repair protein increased the sensitivity of the cells to cisplatin (Fig. 5A-E). When the effects of simultaneously depleting Chk1 with each individual repair protein were examined, we observed that in no case did co-depletion of Chk1 and the repair protein further sensitize the cells to cisplatin. To the contrary, simultaneous depletion of Rad18 or FancD2 with Chk1 rendered cells less sensitive to cisplatin than depletion of Rad18 or FancD2 alone (Fig. 5A and 5E).

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Discussion

In the present study, we have examined the role of the 9-1-1•ATR•Chk1 pathway in protecting a series of tumor cell lines from the antiproliferative effects of cisplatin and other platinating agents. Previously published studies, using small molecule Chk1 inhibitors and RNA interference approaches, demonstrated variable sensitization of some tumor cell lines to platinating agents when Chk1 is disabled (Blasina et al., 2008; Ganzinelli et al., 2008; Gao et al., 2006). However, none of these studies addressed the role of the entire 9-1-1•ATR•Chk1 pathway, nor did they examine the effects of disabling specific DNA repair pathways in the context of Chk1 inhibition. Our studies demonstrate that cells lacking Rad9 and ATR are exquisitely sensitive to platinating agents. In stark contrast, however, Chk1 depletion did not enhance the antiproliferative effects of cisplatin in multiple cell lines, even though Chk1 was activated and relayed a checkpoint signal that caused Cdc25A degradation and slowed S-phase progression in cisplatin-treated cells. Additionally, we showed that depleting key repair proteins, which are part of DNA repair pathways that are frequently disabled in a variety of tumor cells, did not render cells more dependent on Chk1. In fact, in some cases depleting Chk1 from cells lacking specific repair proteins reversed the sensitivity caused by the deficiency of the repair protein.

Multiple studies have shown that Chk1 depletion and Chk1 inhibitors potently sensitize tumor cells to the damage induced by S phase-active agents such as gemcitabine, hydroxyurea, or 5-fluorouracil (Blasina et al., 2008; Cho et al., 2005; Karnitz et al., 2005; Matthews et al., 2007; Morgan et al., 2006; Robinson et al., 2006).

During S phase, Chk1 contributes to cell survival by blocking the firing of unfired origins of replication, preventing cells from exiting G2, stabilizing stalled replication forks, and regulating DNA repair (Enders, 2008). Because the intrastrand and interstrand crosslinks caused by cisplatin are also potent inhibitors of DNA replication, we expected that Chk1 would also facilitate tumor cell survival after cisplatin treatment. Surprisingly, however, even though cisplatin provoked robust Chk1 activation and this activation was important in blocking progression through S phase, Chk1 depletion did not sensitize these tumor cell lines to platinating agents.

Such results strongly suggest that not all stalled replication forks require Chk1 to maintain their stability. Moreover, they also indicate that the Chk1-mediated block of origin firing does not contribute to increased cell survival. One possible explanation is that the Chk1-mediated suppression of origin firing is most important when continued replication would actually create additional DNA damage — such as when additional gemcitabine is incorporated into the genome. In contrast, when the damage is preexisting, as with cisplatin, additional origin firing would not incorporate further damage into the genome. This later point is of particular interest since a recent study has shown that the repair of interstrand crosslinks is initiated only when two opposing replication forks converge on the lesion (Raschle et al., 2008), thus raising the possibility that the repair of these lesions might depend on the activation of additional replication origins.

Chk1, in addition to regulating origin firing and replication fork stability, also positively regulates DNA repair pathways that are important for the repair of interstrand crosslinks in a least two ways. First, Chk1 promotes HR, in part by phosphorylating Rad51 (Huang et al., 2008; Sorensen et al., 2005). Second, Chk1 phosphorylates FancE (Wang et al., 2007), which stimulates the repair of interstrand crosslinks through the FA pathway (Wang, 2007). Because our results clearly demonstrate that the HR and FA pathways are important in HeLa cells treated with cisplatin, the lack of an effect on cell survival when Chk1 is depleted suggests that Chk1 does not play a major regulatory role in these repair pathways in the cell lines examined.

We also explored the possibility that Chk1 might only become important in cisplatin-treated cells when specific DNA repair pathways were disrupted. This is of particular relevance because tumors often have defective DNA repair pathways, and the defects in these pathways likely contribute to the sensitivity of the tumor to chemotherapy regimens (Garcia and Benitez, 2008; Powell and Kachnic, 2008). For example, patients with defects in *BRCA1* and *BRCA2* have better overall responses (including longer survival) to platinum-based therapies (Foulkes, 2006; Sakai et al., 2008), likely because BRCA1 and BRCA2 play critical roles in repairing the cisplatin-induced damage. If Chk1 was important in such cells, then tumors that harbor these defects might be good candidates for clinical trials that combine cisplatin and a Chk1 inhibitor. We did not observe such an outcome. Instead, we found that Chk1 depletion actually reduced the sensitivity of cells with disabled FA (i.e., FancD2) and TLS (i.e., Rad18) pathways. Not only do these results further suggest that Chk1 inhibitors might

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not be useful agents to sensitize tumors to platinating agents, they also suggest that the addition of a Chk1 inhibitor to combination therapies containing cisplatin should be undertaken with great caution.

The present findings suggest that Chk1 inhibitors may be of limited utility to sensitize tumor cells to platinum-induced damage. In fact, given that Chk1 depletion actually reversed the sensitivity of cells with defects in repair pathways that are often defective in tumors treated with cisplatin, the use of such inhibitors may be counterproductive in some patients. In contrast, because both Rad9 and ATR depletion cause profound sensitization to cisplatin, the identification of small molecule inhibitors that disrupt this portion of the pathway may be effective agents to sensitize tumors to platinating agents.

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Footnotes

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This article has not been copyedited and formatted. The final version may differ from this version.

Figure Legends

Figure 1. Rad9 and ATR but not Chk1 are important for tumor resistance to platinating agents. A, *Rad9*$^{-/-}$ murine ES cells stably transfected with empty vector or expressing wild-type Rad9 (inset) were exposed to the indicated cisplatin concentrations for 24 h and analyzed for clonogenic survival. B-F, HeLa cells were transfected with luciferase (Con), ATR, Chk1, or Rad9 siRNAs, and 48 h later lysates were sequentially immunoblotted (B) for ATR, Rad9, Chk1, and ß-actin of the cells were treated with the indicated concentrations of cisplatin (C), gemcitabine (D), oxaliplatin (E), or carboplatin (F) for 24 and analyzed for clonogenicity.

Figure 2. Chk1 inhibition and Chk2 co-depletion do not affect cisplatin cytotoxicity. A-B, HeLa cells were treated with the indicated concentrations of DMSO or AZD7762 (AZD) simultaneously with the indicated concentrations of gemcitabine or cisplatin for 24 h and analyzed for clonogenicity. C-D, HeLa cells were transfected with luciferase (Luc), Chk1, Chk2, or Chk1 and Chk2 siRNAs and 48 h later were analyzed for Chk1, Chk2, and Hsp90 expression (A) and for clonogenic survival (B) following 24-h treatment with the indicated concentrations of cisplatin.

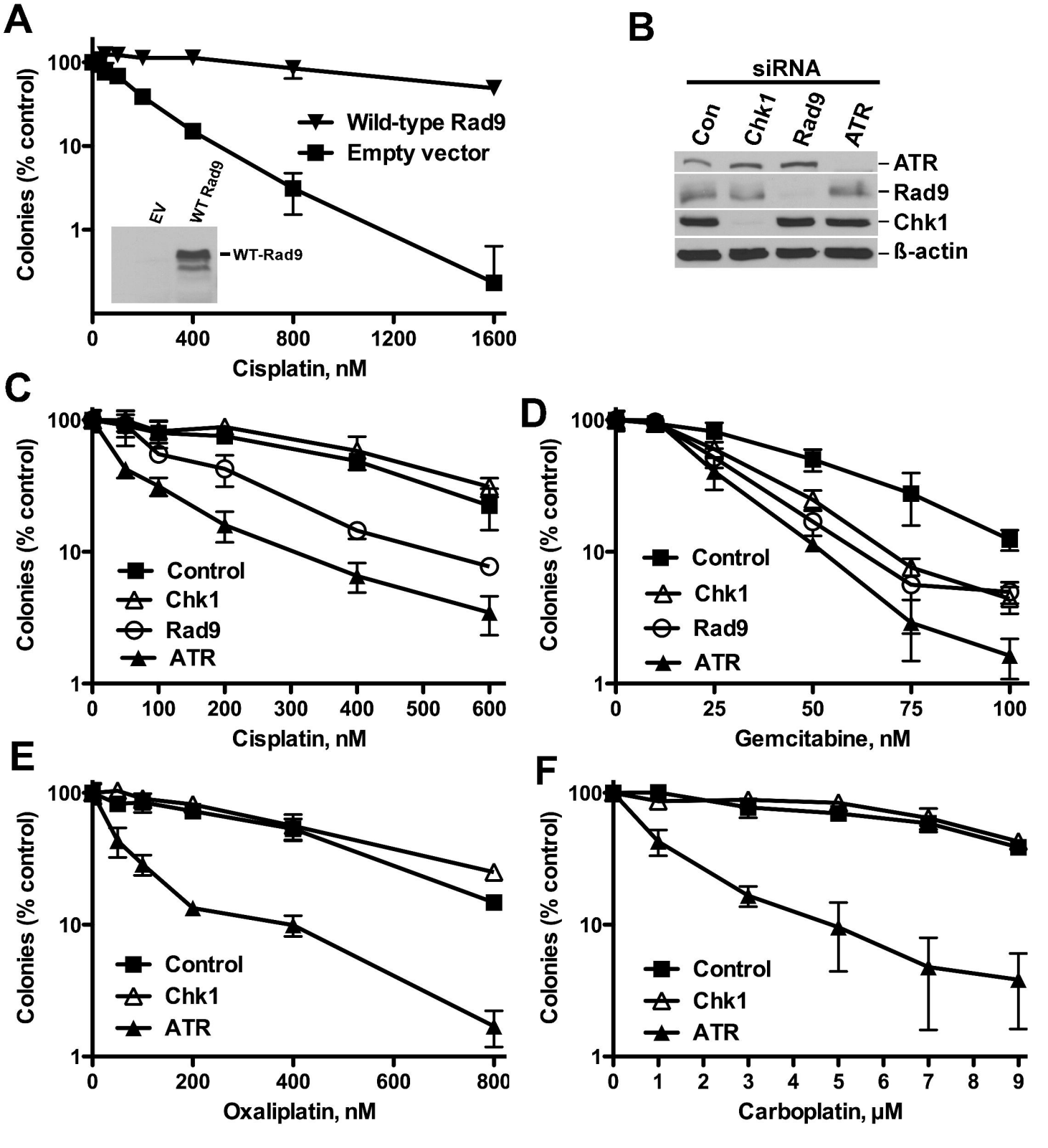
Figure 3. Cisplatin induces activating Chk1 phosphorylation and causes Chk1-dependent S-phase arrest. A, HeLa cells were exposed to vehicle (-) or the indicated concentrations of cisplatin or gemcitabine for 4 or 24 h. Cell lysates were then sequentially immunoblotted for phospho-Ser^{345}-Chk1 (P-Chk1), total Chk1, and Cdc25A. *, indicates non-specific band. B, HeLa cells transfected with luciferase

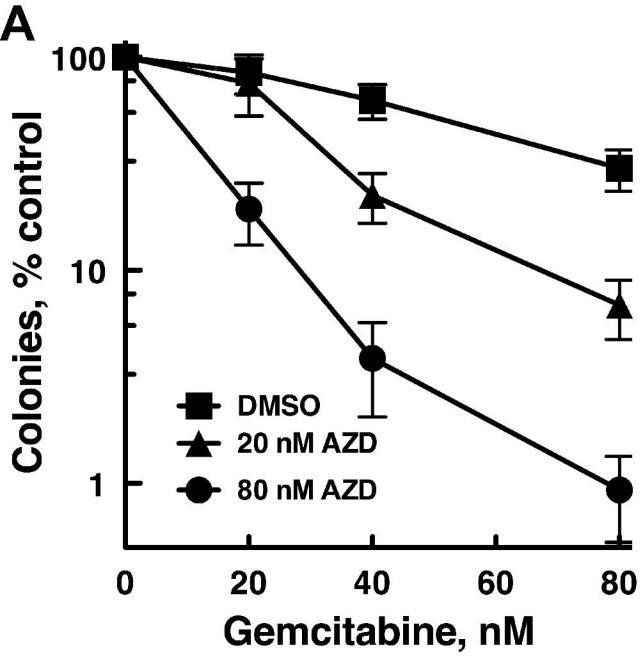
(Control) or Chk1 siRNAs were treated with indicated concentrations of cisplatin for 24 h, stained with propidium iodide, and analyzed by flow microflourimetry.

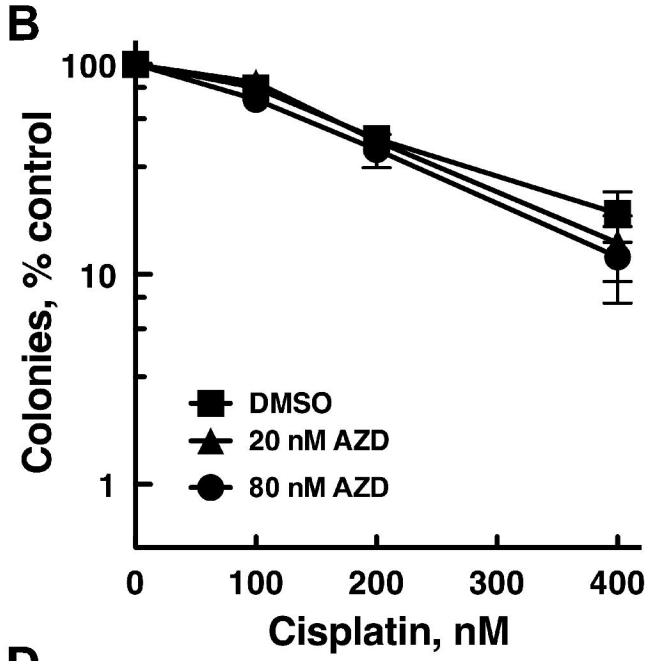
Figure 4. Chk1 depletion does not sensitize HCT-116 and U2O2 cells to cisplatin. A-D, HCT-116 cells (A,B) or U2OS (C,D) transfected with luciferase (Control), ATR, or Chk1 siRNAs were treated with the indicated concentrations of cisplatin or gemcitabine for 24 h and clonogenic assays were performed.

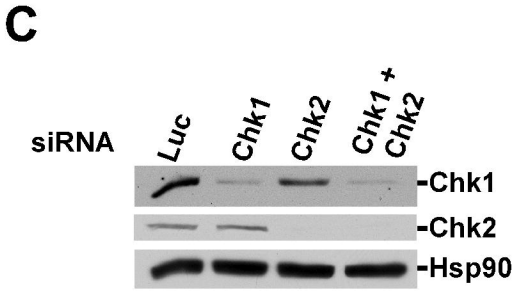
Figure 5. Chk1 depletion does not further sensitize cells with defects in pathways that repair cisplatin-induced DNA lesions. A-E, HeLa cells transfected with siRNAs to FancD2 (A), Rad51 (B), BRCA1 (C), BRCA2 (D), or Rad18 (E), alone or in combination with Chk1 siRNA were treated for 24 h with the indicated concentrations of cisplatin and analyzed for clonogenicity.

Figure 1


A
Colonies (% control)
100
10
1
Wild-type Rad9
Empty vector
EV
WT Rad9
WT-Rad9
0
400
800
1200
1600
Cisplatin, nM
B
siRNA
Con
Chk1
Rad9
ATR
ATR
Rad9
Chk1
ß-actin
C
Colonies (% control)
Control
Chk1
Rad9
ATR
0
100
200
300
400
500
600
Cisplatin, nM
D
Colonies (% control)
Control
Chk1
Rad9
ATR
0
25
50
75
100
Gemcitabine, nM
E
Colonies (% control)
Control
Chk1
ATR
0
200
400
600
800
Oxaliplatin, nM
F
Colonies (% control)
Control
Chk1
ATR
0
1
2
3
4
5
6
7
8
9
Carboplatin, µM


A
100
10
1
Colonies, % control
DMSO
20 nM AZD
80 nM AZD
0
20
40
60
80
Gemcitabine, nM


B
100
10
1
Colonies, % control
DMSO
20 nM AZD
80 nM AZD
0
100
200
300
400
Cisplatin, nM


C
siRNA
Luc
Chk1
Chk2
Chk1 + Chk2
Chk1
Chk2
Hsp90

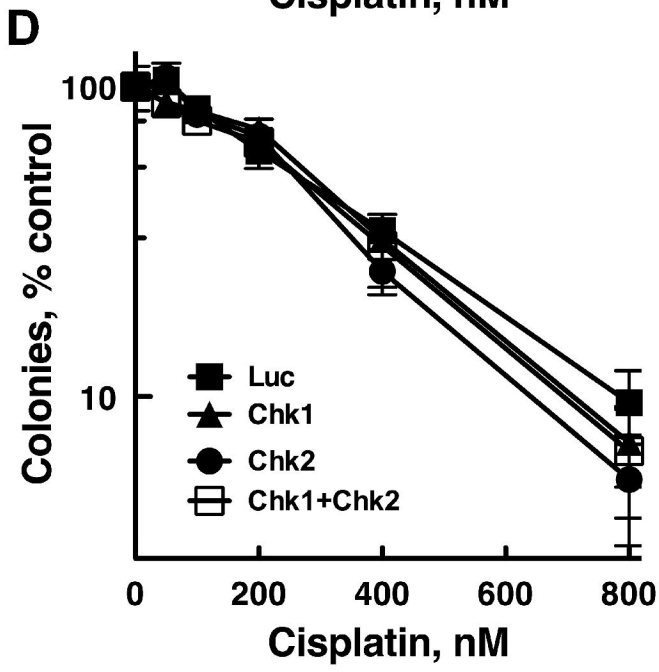

D
100
10
Colonies, % control
Luc
Chk1
Chk2
Chk1+Chk2
0
200
400
600
800
Cisplatin, nM

Figure 3

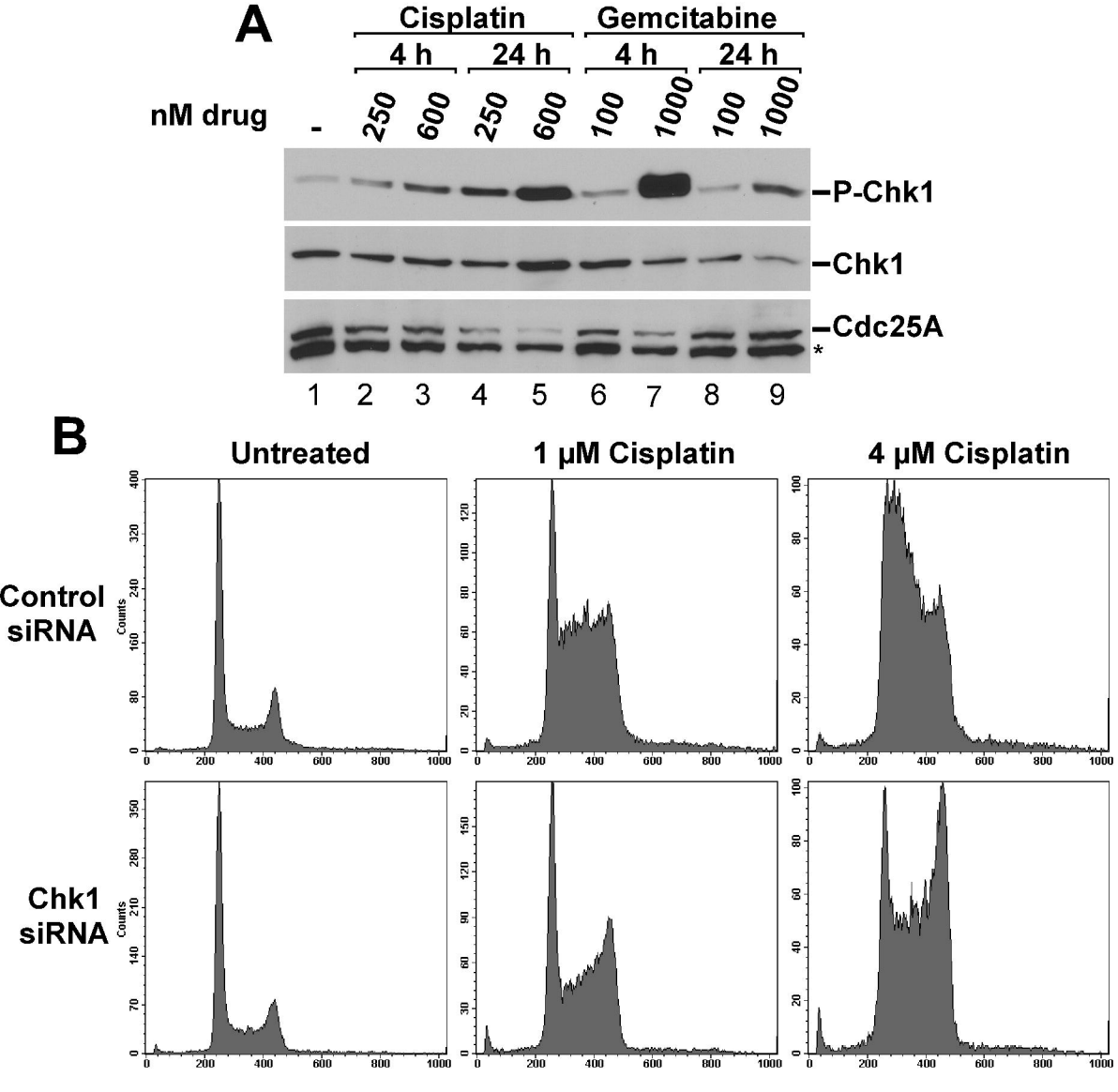
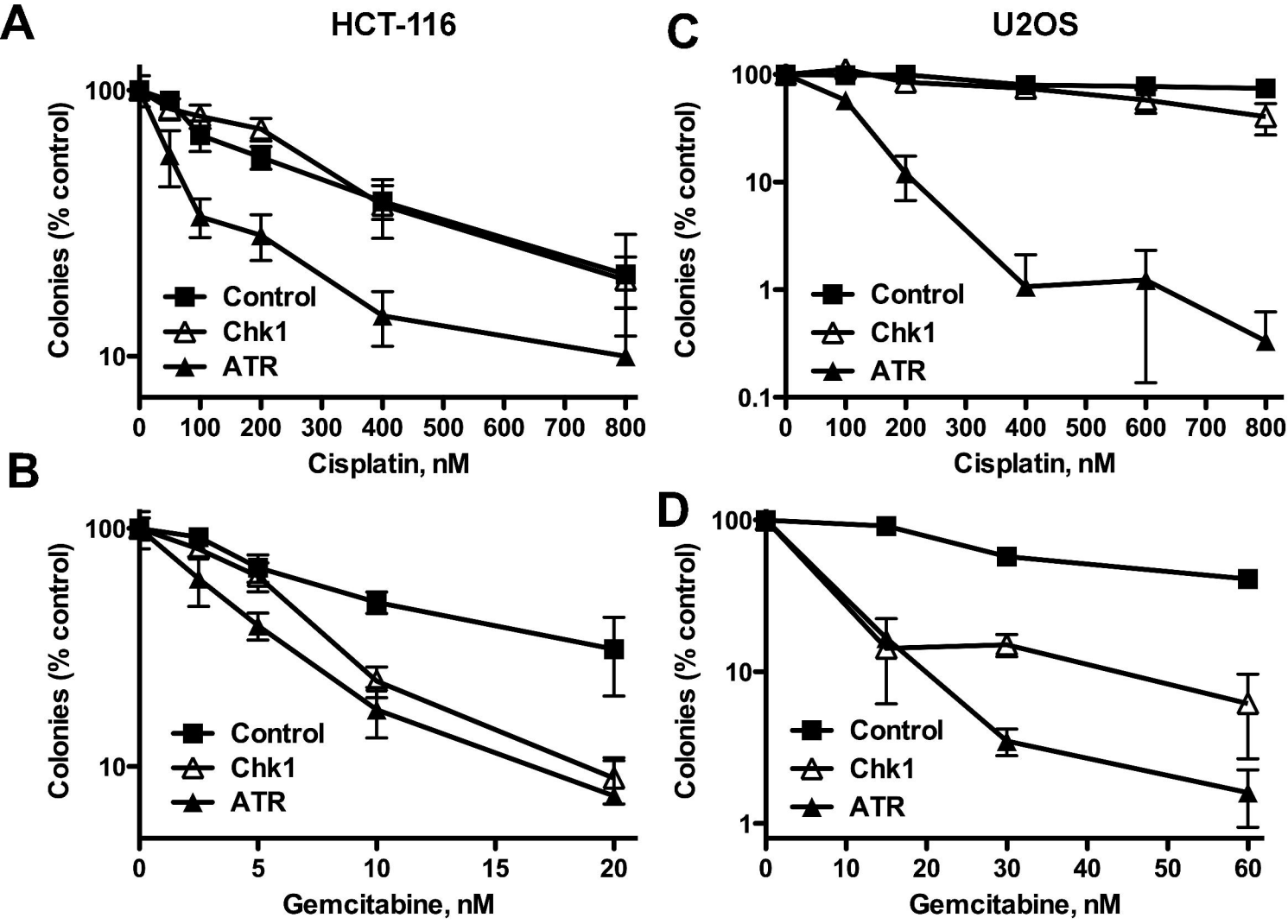
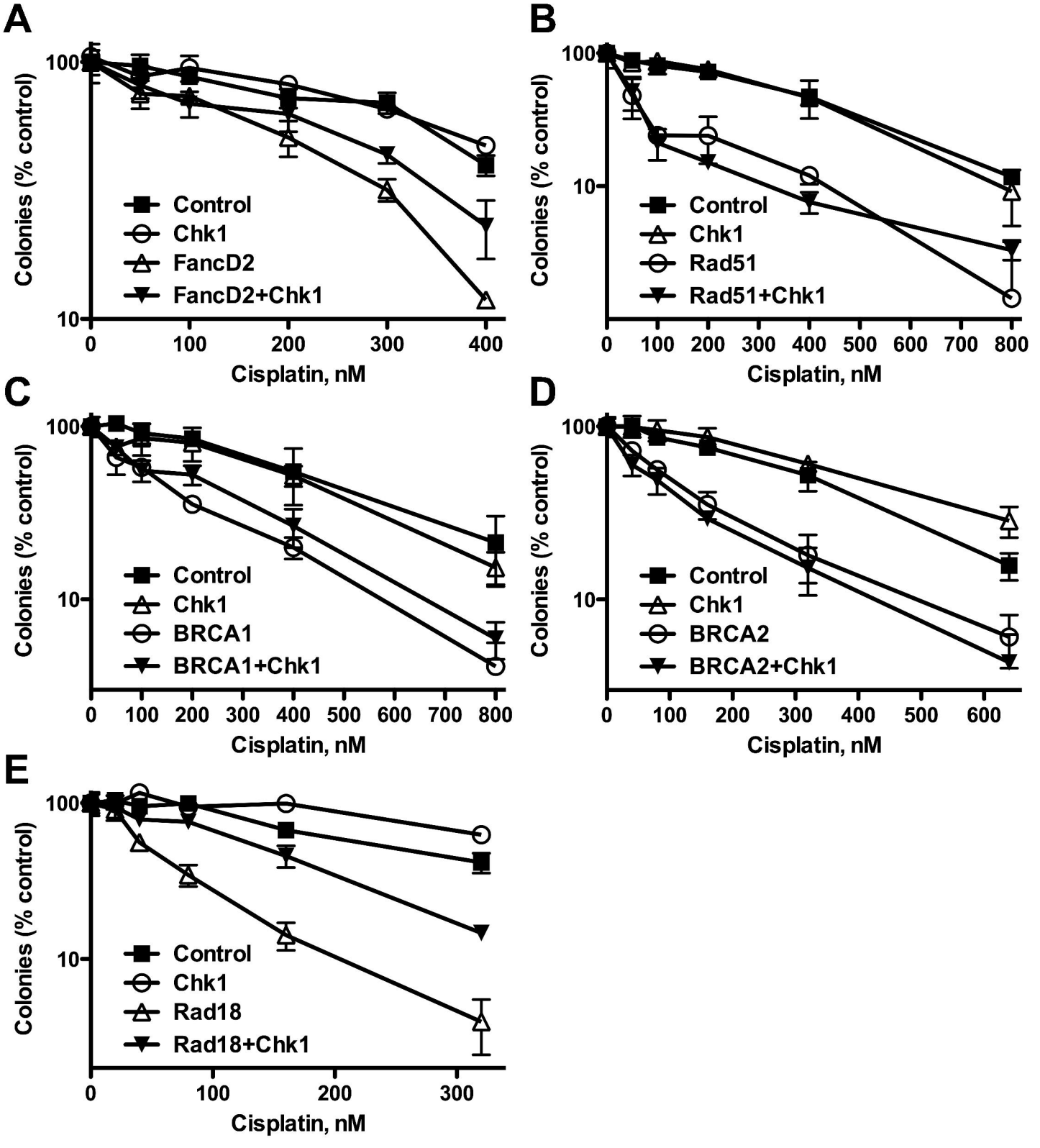

A
Cisplatin
Gemcitabine
4 h
24 h
4 h
24 h
nM drug
-
250
600
250
600
100
1000
100
1000
P-Chk1
Chk1
Cdc25A
*
1 2 3 4 5 6 7 8 9
B
Untreated
1 μM Cisplatin
4 μM Cisplatin
Control siRNA
Chk1 siRNA
Counts

Figure 4


A
HCT-116
Colonies (% control)
100
10
Control
Chk1
ATR
0
100
200
300
400
500
600
700
800
Cisplatin, nM
B
Colonies (% control)
100
10
Control
Chk1
ATR
0
5
10
15
20
Gemcitabine, nM
C
U2OS
Colonies (% control)
100
10
1
0.1
Control
Chk1
ATR
0
100
200
300
400
500
600
700
800
Cisplatin, nM
D
Colonies (% control)
100
10
1
Control
Chk1
ATR
0
10
20
30
40
50
60
Gemcitabine, nM


A
Colonies (% control)
100
10
0
100
200
300
400
Cisplatin, nM
Control
Chk1
FancD2
FancD2+Chk1
B
Colonies (% control)
100
10
0
100
200
300
400
500
600
700
800
Cisplatin, nM
Control
Chk1
Rad51
Rad51+Chk1
C
Colonies (% control)
100
10
0
100
200
300
400
500
600
700
800
Cisplatin, nM
Control
Chk1
BRCA1
BRCA1+Chk1
D
Colonies (% control)
100
10
0
100
200
300
400
500
600
Cisplatin, nM
Control
Chk1
BRCA2
BRCA2+Chk1
E
Colonies (% control)
100
10
0
100
200
300
Cisplatin, nM
Control
Chk1
Rad18
Rad18+Chk1

Supplemental Figure 1