Gemcitabine-Induced Activation of Checkpoint Signaling Pathways That Affect Tumor Cell Survival

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Running title: ATM and ATR signaling pathways promote gemcitabine survival

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Abbreviations used are: 9-1-1, Rad9-Hus1-Rad1 complex; ATM, ataxia-telangiectasia mutated kinase; ATR, ATM- and Rad3-related kinase; Chk1, checkpoint kinase 1; Chk2, checkpoint kinase 2; dNTP, 2'-deoxyribonucleotide triphosphate; dCTP, 2'-deoxycytidine triphosphate; PBS, Dulbeccco's calcium- and magnesium-free phosphate buffered saline; siRNA, small inhibitory RNA.

ABSTRACT

Two signaling pathways are activated by antineoplastic therapies that damage DNA and stall replication. In one pathway, double-strand breaks activate ATM and Chk2, two protein kinases that regulate apoptosis, cell cycle arrest and DNA repair. In the second pathway, other types of DNA lesions and replication stress activate the Rad9-Hus1-Rad1 (9-1-1) complex and the protein kinases ATR and Chk1, leading to changes that block cell cycle progression, stabilize stalled replication forks and influence DNA repair. Gemcitabine and cytarabine are two highly active chemotherapeutic agents that disrupt DNA replication. Here we examine the roles these pathways play in tumor cell survival following treatment with these agents. Cells lacking Rad9, Chk1, or ATR were more sensitive to gemcitabine and cytarabine, consistent with the fact that these agents stall replication forks, and this sensitization was independent of p53 status. Interestingly, ATM depletion sensitized cells to gemcitabine and ionizing radiation but not cytarabine. Collectively, these results demonstrate that (1) gemcitabine triggers both checkpoint signaling pathways, (2) both pathways contribute to cell survival following gemcitabine-induced replication stress, and (3) although gemcitabine and cytarabine both stall replication forks, ATM plays differential roles in cell survival after treatment with these agents.

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Introduction

Gemcitabine (2',2'-difluoro 2'-deoxycytidine), a pyrimidine-based antimetabolite, is currently licensed for the therapy of pancreatic cancer. Recent clinical studies have also demonstrated extensive activity of this agent against a variety of additional neoplasms, including carcinomas of the ovary, lung, and breast as well as acute leukemias and refractory lymphomas (Carmichael, 1998; Nabhan et al., 2001). Because of its widespread use, there is considerable interest in understanding factors that affect sensitivity and resistance to this agent. Earlier studies demonstrated that gemcitabine is taken into cells on concentrative nucleoside transporter 1 and phosphorylated to gemcitabine 5'-monophosphate by deoxycytidine kinase (reviewed in Plunkett et al., 1996). Subsequent addition of 5' phosphates results in the formation of gemcitabine diphosphate and gemcitabine triphosphate, both of which contribute to the antiproliferative effects of gemcitabine (reviewed in Plunkett et al., 1996). Gemcitabine diphosphate inhibits ribonucleotide reductase, thereby depleting deoxyribonucleotide levels. Gemcitabine triphosphate is a substrate for replicative DNA polymerases and causes chain termination one base pair beyond the site of incorporation.

Because gemcitabine inhibits replication, this drug is predicted to activate the S phase checkpoint, a series of reactions that inhibits DNA synthesis and enhances survival when cells experience replication stress. According to current understanding, the kinases ATR and Chk1 play critical roles in this checkpoint. When replication forks stall, replication protein A binds areas of single-stranded DNA and facilitates the binding of ATR and its binding partner ATRIP to chromatin (reviewed in O'Connell and Cimprich, 2005). At the same time, a preassembled complex consisting of Rad9, Hus1 and Rad1 (the 9-1-1 complex) (Burtelow et al., 2000; Volkmer and Karnitz, 1999) is loaded onto the damaged chromatin by a clamp loader comprised

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of Rad17 and the small subunits of replication factor C (Bermudez et al., 2003; Zou et al., 2002). The chromatin-bound 9-1-1 complex then facilitates ATR-mediated phosphorylation and activation of Chk1, which in turn enhances survival following replication stress in two ways. First, Chk1 stabilizes stalled replication forks, although the relevant Chk1 substrate is not known. Second, Chk1 phosphorylates Cdc25A, which inhibits and causes proteolytic destruction of this phosphatase, thereby blocking Cdc25A-mediated activation of the Cdk2/Cyclin E and Cdk2/Cyclin A complexes that drive S phase progression (reviewed in Busino et al., 2004).

Several studies have shown that gemcitabine activates the ATR/Chk1 pathway. Shi *et al*. reported that treatment of cells with gemcitabine results in S phase slowing (Shi et al., 2001). More recently, Arlander *et al.* demonstrated that gemcitabine induces Chk1 activation in ML-1 acute myelogenous leukemia and HeLa cervical carcinoma cells (Arlander et al., 2003). This S phase checkpoint activation is abolished when Chk1 is inhibited with UCN-01 (Shi et al., 2001) or depleted by treatment with 17-allylamino-17-demethoxygeldanamycin (Arlander et al., 2003).

A parallel set of studies (Shao et al., 2004; Zhao and Piwnica-Worms, 2001) has examined the action of cytarabine (cytosine arabinoside), a cytosine analog that is administered to patients with acute leukemias and other hematological disorders. Like gemcitabine, cytarabine is taken into cells and phosphorylated to form the 5'-nucleoside di- and triphosphates. In contrast to gemcitabine diphosphate, cytarabine diphosphate has no effect on ribonucleotide reductase. Instead, the cytotoxic effects of cytarabine metabolites are attributed to inhibition of replicative DNA polymerases and chain termination after incorporation into nascent DNA. Consistent with this mechanism, it has been reported that cytarabine activates Chk1 phosphorylation (Loegering et al., 2004; Zhao and Piwnica-Worms, 2001) and that disruption of

the ATR/Chk1 pathway by deletion of the *Rad9* gene or depletion of Chk1 sensitizes cells to cytarabine (Cho et al., 2005; Loegering et al., 2004; Mesa et al., 2005). Additional studies have shown that cytarabine does not activate the ATM/Chk2 pathway, a pathway that typically responds to double-strand DNA breaks (Loegering et al., 2004). In contrast, both the ribonucleotide reductase inhibitor hydroxyurea and high concentrations of the nucleotide thymidine, which blocks replication by specifically depleting dCTP, have been reported to activate ATM and Chk2 (Bolderson et al., 2004). At the present time the role of the ATM/Chk2 pathway in cells treated with gemcitabine and cytarabine is unknown.

Experiments in the present study evaluated the relative importance of the ATR/Chk1 and ATM/Chk2 pathways in determining the fate of gemcitabine- and cytarabine-treated cells. Consistent with the role of the ATR/Chk1 pathway in replication fork stalling, both nucleoside analogs were more cytotoxic when this checkpoint pathway was disabled. Surprisingly, however, inactivation of ATM sensitized tumor cells to gemcitabine but not cytarabine.

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Materials and Methods

Materials. Reagents were purchased from the following suppliers: 2-mercaptoethanol from Sigma; siRNAs from Dharmacon (Layfayette, CO); OptiMEM, Lipofectamine 2000, and L-glutamine from Invitrogen (Carlsbad, CA); and ES-GRO leukemia inhibitory factor from Chemicon (Temecula, CA). Antisera that recognize various antigens were obtained as follows: rabbit anti-phospho-Thr^{68}-Chk2 and anti-phospho-Ser^{345}-Chk1 from Cell Signaling Technology (Beverly, MA) or R & D Systems (Minneapolis, MN); rabbit anti-ATR from Oncogene Research Products; murine anti-Chk1, anti-Cdc25A, and anti-ATM as well as goat anti-actin from Santa Cruz Biotechnology (Santa Cruz, CA). Other reagents were obtained as described previously (Loegering et al., 2004; Roos-Mattjus et al., 2003; Ward and Chen, 2001).

Cell lines and tissue culture. Parental and *Chk2*$^{-/-}$ HCT116 cells (Jallepalli et al., 2003), HeLa cells, and K562 cells were cultured in RPMI-1640 containing 10% heat-inactivated fetal bovine serum and 2 mM L-glutamine. *Rad9*$^{-/-}$ embryonic stem (ES) cells (Hopkins et al., 2004) and stable *Rad9*$^{-/-}$ ES cell clones expressing human wild-type Rad9 (Roos-Mattjus et al., 2003) were propagated and used in clonogenic assays as described previously (Loegering et al., 2004; Roos-Mattjus et al., 2003).

GM847 and GM847/kdATR cells, which were derived from GM847 by transfection with cDNA encoding kinase-inactivated ATR under the control of a doxycycline-responsive promoter (Cliby et al., 1998), were cultured in Dulbecco's modified Eagle's medium with 4.5 g/L glucose, 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin G, 100 µg/ml streptomycin, and 2 mM L-glutamine (medium A). At the start of each experiment, cells were propagated in medium A for 48 h in the absence or presence of 1 µg/ml doxycycline. Aliquots containing 7500 cells

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were plated in triplicate 35-mm dishes in the continued presence of doxycycline or diluent. Cells were allowed to adhere overnight, exposed to varying gemcitabine concentrations for 24 h, washed twice in serum- and drug-free medium, and incubated for 12-14 d in medium A (in the absence or presence of doxycycline) to allow colony formation.

To perform clonogenic assays in parental and *Chk2*$^{-/-}$ HCT116 cells, aliquots containing 500 cells were plated in 35-mm dishes, allowed to adhere for 14-16 h, and treated with the indicated concentrations of gemcitabine for 24 h. Following drug exposure, cells were washed with RPMI 1640 and cultured for 7-8 days in drug-free medium to allow colonies to form.

siRNA transfections and clonogenic assays. On day 1, HeLa or A549 cells (1 x 10^6) were plated in 35-mm tissue culture dishes and incubated overnight. One day 2, cells were washed twice with OptiMEM medium, and 2 mL OptiMEM was added to each plate. As negative controls, a luciferase siRNA (CTT ACG CUG AGU ACU UCG A)(Elbashir et al., 2001) or control siRNA #1 (Dharmacon product # D-001210-01-05) were used. The Chk1 (Zhao et al., 2002), ATM (Wang and Hays, 2002) and ATR (Casper et al., 2002) siRNAs have been described previously. Four hundred nmol of each siRNA were complexed with 10 µl Lipofectamine 2000 in 0.5 ml OptiMEM for 20 min. Following addition of the lipid-siRNA complexes to the cells, the cultures were incubated for 4-7 h, at which time 1 ml of OptiMEM containing 30% fetal bovine serum was added. The transfections were repeated on day 3. On day 4 the cultures were trypsinized and replated in 150-mm tissue culture dishes containing medium A. On day 5, cells were released by trypsinization. For clonogenic assays, 300 cells were plated in triplicate into 60-mm dishes, allowed to attach for 4 h, treated for 24 h with the indicated drugs, washed, and incubated for 7 days to allow colony formation. Plates were stained with Coomassie Blue; and colonies with ≥50 cells were counted. Survival was calculated as the ratio of colonies in dishes

treated with drug compared to diluent. To assess the effect of siRNA on levels of target proteins, the cells remaining after setting up the clonogenic assay were lysed and prepared for SDS-polyacrylamide gel electrophoresis and immunoblotting as described previously (Volkmer and Karnitz, 1999).

Analysis of checkpoint signaling pathway activation. Chromatin binding of 9-1-1 complexes was analyzed as previously described (Burtelow et al., 2000) following treatment of cells with gemcitabine or exposure to ultraviolet light. Ultraviolet light exposure was carried out by washing cells with PBS, aspirating the buffer, and irradiating the monolayers with 30 J/m^2 254-nm ultraviolet light . Following irradiation, prewarmed medium (37˚C) was added; and the cells were cultured for 1 h prior to harvest.

Chk1 and Chk2 phosphorylation were assessed following treatment of HeLa, K562, or ES cells with the indicated concentrations of gemcitabine. Alternatively, the cells were treated with ultraviolet light as described above or with γ-radiation using a ^{137}Cs source and harvested 1 h later. Following incubation for the indicated times, cells were lysed; and soluble proteins were sequentially immunoblotted for anti-phospho-Ser^{345}-Chk1 followed by Chk1 or phospho-Thr^{68}-Chk2 followed by Chk2 as described previously (Roos-Mattjus et al., 2003). Competition experiments examined the ability of 1 μg/ml phosphorylated or unphosphorylated 15-mer peptide centered on Ser^{345} of Chk1 or Thr^{68} of Chk2 to block binding of phospho-epitope-specific antibodies to these immunoblots.

Assays to assess genotoxin-induced changes in Cdc25A levels (Arlander et al., 2003) and formation of phospho-Ser^{139}-H2AX foci (Ward and Chen, 2001) were also performed by previously described methods following treatment with the indicated agents.

Apoptosis assays. Adherent cells were released by trypsinization, pooled with nonadherent cells, sedimented at 200 x g for 10 min, washed once with ice-cold PBS, fixed in 3:1 methanol:acetic acid, and deposited on glass slides. After air drying, samples were stained with 1 μg/ml Hoechst 33258 and examined by fluorescence microscopy as previously described (Loegering et al., 2004). A minimum of 400 cells/sample was scored for apoptotic changes (chromatin condensation or nuclear fragmentation) using a Zeiss Axioplan microscope equipped with a N.A. 1.40 63X objective, a 365-nm excitation filter, and a 420-nm emission filter.

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Results

Gemcitabine activates genotoxin-induced signaling cascades. As a first step to assess the activation of checkpoint signaling pathways by gemcitabine, we determined whether gemcitabine induced the phosphorylation of histone H2AX, an event that occurs rapidly following genotoxic stress and is mediated by ATM and/or ATR (Burma et al., 2001; Ward and Chen, 2001). Gemcitabine induced phospho-H2AX foci (Fig. 1A and B) nearly as effectively as did 1 Gy of γ-radiation (Fig. 1C), a potent activator of the ATM-Chk2 pathway. In contrast, the replication inhibitor hydroxyurea, which activates the ATR-Chk1 pathway (Cho et al., 2005; Liu et al., 2000; Roos-Mattjus et al., 2003; Zhao and Piwnica-Worms, 2001), induced fewer foci.

To further discern which signaling pathways were activated by gemcitabine, cells were treated with varying concentrations of gemcitabine for up to 24 h and harvested for immunoblotting with antisera that specifically recognize activating phosphorylations of the checkpoint kinases Chk1 and Chk2. For these studies, specificity of the antisera was demonstrated by confirming that binding to the species identified as phosphorylated Chk1 and Chk2 in Fig. 1D-G, was attenuated in the presence of phosphorylated peptides but not unphosphorylated peptides (data not shown).

Treatment of HeLa cells with 100 nM gemcitabine, a concentration that kills approximately 70% of cells in a 24-h treatment (see below), resulted in detectable phosphorylation of Chk1 on Ser^{345} after 2 h (Fig. 1D, left panel). This phosphorylation increased at 4 and 8 h, persisted for 24 h, and was as strong or stronger than the phosphorylation induced by ultraviolet light, a potent Chk1 activator (Liu et al., 2000). To determine the approximate fold increase in Chk1 phosphorylation, we probed a lysate prepared from untreated cells and serial dilutions of lysates prepared from gemcitabine-treated cells with anti-phospho-Ser^{345}-Chk1. As

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shown in Fig. 1F, gemcitabine triggered at least a 4-fold increase in Chk1 phosphorylation. Similarly, in K562 cells, Chk1 phosphorylation was detectable within 2 h at gemcitabine concentrations as low as 10 nM, whereas 200 and 500 nM gemcitabine triggered extensive Chk1 phosphorylation (Fig. 1E, left panel). As was seen in HeLa cells, Chk1 phosphorylation persisted for at least 24 h.

Analysis of Chk2 phosphorylation on Thr^{68} in HeLa cells revealed that low levels of Chk2 phosphorylation occurred within 2 h at 100 nM gemcitabine, with more pronounced phosphorylation at later time points (Fig. 1D, right panel). Notably, these levels of phosphorylation were similar and in some cases equal to the level of phosphorylation seen in cells treated with 20 Gy γ-radiation. Analysis of serial dilutions from gemcitabine-treated cells revealed that Chk2 phosphorylation was increased approximately 4-fold (Fig. 1G). A similar pattern was observed in K562 cells, where low levels of Chk2 phosphorylation were detected at 1 and 4 h (Fig. 1E, right panel). In both cell lines Chk2 phosphorylation was evident after a 24-h drug exposure at the lowest concentrations tested.

Collectively, the results in Fig. 1 suggest that gemcitabine activates both the ATR/Chk1 and ATM/Chk2 signaling pathways. Because this activation occurs within 1 h (Fig. 1), whereas apoptosis does not increase for at least 8 h even after treatment with the highest of the gemcitabine concentrations (data not shown), activation of these pathways appears to be an early response of cells to this agent. To determine whether both of these pathways play roles in tumor cell survival following gemcitabine treatment, cells deficient in one or the other pathway were compared to isogenic control cells in a series of subsequent assays.

Role of Rad9 in survival after gemcitabine treatment. Although previous studies have suggested that the ATR/Chk1 pathway plays a protective role after treatment with gemcitabine

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(see Introduction), it is important to realize that UCN-01 and 17-allylamino-17-demethoxygeldanamycin, the two agents used to interrupt Chk1 signaling, also inhibit additional signaling pathways (Komander et al., 2003; Workman, 2004). To more definitively assess the effect of the ATR/Chk1 pathway, we examined paired cell lines that had defined alterations in pathway components, focusing initially on Rad9.

Many DNA lesions induce the chromatin binding of the 9-1-1 complex (Burtelow et al., 2000), which plays an important role in Chk1 activation and has been implicated in DNA repair (reviewed in O'Connell and Cimprich, 2005). Consistent with a role for this complex in the response to gemcitabine, this agent induced Rad9 chromatin binding (Fig. 2A) at a concentration that activated Chk1 (Fig. 2B). Examination of *Rad9*$^{-/-}$ cells revealed a defect in Chk1 activation (Fig. 2C) that was paralleled by a defect in gemcitabine-induced Cdc25A degradation (Fig. 2D), an event that involves Chk1-induced phosphorylation of Cdc25A (reviewed in Busino et al., 2004). We also analyzed Chk2 activation in the ES cells. Because the anti-phospho-Thr^{68}-Chk2 antibody employed in Fig. 1 does not recognize phosphorylated murine Chk2, a mobility shift assay was used with these cells. As was seen in the HeLa and K562 cells, gemcitabine also induced Chk2 phosphorylation in the ES cells (Fig. 2E).

Additional experiments then assessed the role of the 9-1-1 complex on survival after gemcitabine treatment. *Rad9*$^{-/-}$ cells exhibited increased apoptosis (Fig. 2F) and decreased colony formation (Fig. 2G) when compared to wild-type cells. These results were similar to the effect of *Rad9* deletion on cytarabine sensitivity (Loegering et al., 2004). The role of Rad9 in this effect was confirmed by showing that the gemcitabine sensitivity of *Rad9*$^{-/-}$ ES cells was reversed by expressing wild-type human Rad9 (Fig. 2G). In contrast, *Rad9* deletion did not sensitize cells to the farnesyl transferase inhibitor tipifarnib (Fig. 2H), ruling out the possibility

that the increased sensitivity to gemcitabine reflected increased sensitivity to non-genotoxic cellular stresses. Taken together, these results suggest that the 9-1-1 complex plays an important role in cell survival following gemcitabine-induced replication by participating in DNA repair and/or checkpoint activation.

Roles of ATR and Chk1 in gemcitabine-induced cytotoxicity. In view of the results observed when *Rad9* was deleted, the roles of ATR and Chk1 in protecting cells from gemcitabine were assessed. Because *ATR*$^{-/-}$ cells are not viable (Brown and Baltimore, 2000), we used the parental GM847, an SV-40-transformed cell line, and it derivative, GM847/kdATR, which contains a doxycycline-inducible dominant negative kinase-inactive *ATR* allele (Cliby et al., 1998), to assess the role of ATR. Exposure of the parental cells to doxycycline did not affect sensitivity to gemcitabine when cells were treated with increasing gemcitabine concentrations for 24 h and assayed for clonogenic survival (Fig. 3A). In contrast, induction of kinase-inactive ATR sensitized cells to gemcitabine (Fig. 3B), confirming that the ATR pathway plays a role in survival after treatment with gemcitabine. Further experiments demonstrated that ATR depletion by siRNA in HeLa (inset, Fig. 3C) and A549 cells (inset, Fig. 3D) also enhanced gemcitabine sensitivity in both cell lines (Fig. 3C, D), arguing against the possibility that the enhanced sensitivity observed in GM847/kdATR cells reflects a unique feature of this model system.

One response that requires the concerted action of the 9-1-1 complex and ATR is Chk1 activation (reviewed in O'Connell and Cimprich, 2005). Therefore, we assessed the role of Chk1 by depleting Chk1 with siRNA in HeLa cells, which lack a functional p53 pathway, and in A549 cells, which express wild-type p53 (insets, Fig. 4A and B, respectively). As shown in Fig. 4A and B, Chk1-depleted HeLa and A549 cells displayed enhanced sensitivity to gemcitabine, indicating that Chk1 is responsible, at least in part, for the effects of Rad9 and ATR on

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gemcitabine sensitivity. Similar results were also obtained after treatment with cytarabine (Fig. 4C), confirming that Chk1 depletion sensitizes cells to cytarabine (Cho et al., 2005; Mesa et al., 2005). In contrast, there was no reduction in survival in Chk1-depleted cells treated with γ-radiation (Fig. 4D), again ruling out the possibility that the enhanced sensitivity to gemcitabine and cytarabine reflects a nonspecific sensitization to all genotoxic stresses.

Effect of ATM depletion on genotoxin sensitivity. Results in Fig. 1 show that gemcitabine also induces Chk2 activation, a response that occurs primarily in response to ATM activation. Accordingly, we assessed whether ATM and Chk2 play a role in determining cell survival following treatment with gemcitabine. The role of Chk2 was assessed using parental and *Chk2*$^{-/-}$ HCT116 cells (Jallepalli et al., 2003), which were treated with varying concentrations of gemcitabine for 24 h and examined for the ability to subsequently form colonies. As indicated in Fig. 4E, *Chk2* deletion had no effect on gemcitabine sensitivity. A similar conclusion has been reached regarding the role of Chk2 in the response to other genotoxic stresses (Jallepalli et al., 2003).

To assess the role of ATM in gemcitabine sensitivity, we depleted ATM in HeLa (inset, Fig. 5A) and A549 (inset, Fig. 5B) cells using siRNA. Consistent with the known role of ATM in the response to γ-radiation, ATM depletion sensitized both cells lines to γ-radiation (Fig. 5A, B). Importantly, ATM depletion also sensitized these cell lines to gemcitabine (Fig. 5C, D). However, this response did not extend to other replication inhibitors, as ATM depletion had a much smaller effect effect on cytarabine sensitivity in the two cell lines (Fig. 5E, F).

Discussion

Experiments described above were initiated to examine the respective roles of the ATR/Chk1 and ATM/Chk2 pathways in the response to the nucleoside analogues gemcitabine and cytarabine. Previous studies with a number of nucleoside analogues, including cytarabine and gemcitabine, suggested that inhibiting the ATR/Chk1 pathway might sensitize cells to replication stress, providing precedence for the effects we observed after disruption of Rad9, ATR or Chk1. Surprisingly, however, we also observed that ATM depletion sensitized cells to gemcitabine but not cytarabine. These results have potentially important implications for current understanding of the cytotoxic action of gemcitabine.

Gemcitabine metabolites not only inhibit ribonucleotide reductase and DNA polymerases, but also block DNA replication when incorporated into DNA (reviewed in Sampath et al., 2003). In the face of these types of replication stress, the ATR/Chk1 signaling pathway is known to promote cell survival by blocking additional origin firing and stabilizing stalled replication forks (reviewed in O'Connell and Cimprich, 2005). Consistent with a role for Chk1 in the response to gemcitabine and cytarabine, previous reports demonstrated that the Chk1 inhibitor UCN-01 sensitizes cells to gemcitabine and cytarabine (Shi et al., 2001; Wang et al., 1997). UCN-01, however, inhibits many kinases in addition to Chk1 (Komander et al., 2003), raising the possibility that the effects of UCN-01 might reflect inhibition of kinases other than Chk1. Here we used a variety of complementary approaches, including targeted gene deletion, siRNA and expression of dominant negative constructs, to demonstrate more directly that disruption of the Chk1 activation pathway at the level of the 9-1-1 complex (Fig. 2), ATR (Fig. 3), or Chk1 itself (Fig. 4) does indeed sensitize tumor cells to gemcitabine. Additionally, our results demonstrated that Chk1 depletion sensitizes tumor cells to gemcitabine independent of an

intact p53 signaling pathway (Fig. 4A and B). Taken together, these results not only suggest that gemcitabine-induced replication fork stalling activates that Chk1 signaling pathway, which in turn facilitates cell survival, but also provide further support for the view that inhibitors of the Chk1 signaling pathway might be useful agents to sensitize tumor cells to nucleoside analogs.

Although the ATM pathway has classically been implicated in the response to DNA double-strand breaks rather than nucleoside analogues, we found that ATM also plays an important role in gemcitabine-treated cells (Fig. 5C and D), again in a manner that does not depend on p53. The role of ATM in replication stress has not received much previous attention. A study published while the present experiments were in progress (Bolderson et al., 2004) reported that ATM deficiency sensitizes cells to high concentrations of thymidine, which allosterically inhibits ribonucleotide reductase leading to the accumulation of dCTP, but not to hydroxyurea, which inactivates ribonucleotide reductase and blocks the production of all dNTPs (Bianchi et al., 1986). In the present study, we found that ATM depletion sensitized cells to gemcitabine but not cytarabine (Fig. 5C-F), providing another example of the differential involvement of ATM in the effects of some antimetabolites but not others.

Because ATM facilitated survival of gemcitabine-treated cells, we also examined the role of Chk2 in gemcitabine cytotoxicity. In mice, deletion of *Chk2* and *ATM* produces very different phenotypes. ATM-deficient mice, like ATM-deficient humans, are highly sensitive to ionizing radiation (Barlow et al., 1996). In contrast, *Chk2* deletion promotes radioresistance by blocking ionizing radiation-induced apoptosis in the thymus, intestine, and central nervous system (Takai et al., 2002). Similar to these findings, we observed that ATM depletion sensitized cells to gemcitabine (Fig. 5C and D), but *Chk2* deletion did not (Fig. 4E), suggesting that the effect of ATM on survival is mediated by an ATM substrate other than Chk2.

The demonstration that gemcitabine activates parallel signaling pathways, both of which contribute to survival, might have implications for the clinical use of this agent. Although many factors undoubtedly influence the sensitivity of tumor cells to nucleoside analogs such as gemcitabine, it is tempting to speculate that differences in the types of damage produced, the checkpoint signaling pathways activated, and the repair pathways subsequently set in motion play important roles in the varied activities of these agents. The efficacy of gemcitabine in solid tumors, for example, might reflect the ability of this agent to induce lesions in cells outside of S phase. Additionally, the results presented here suggest that tumors lacking ATM might be more sensitive to gemcitabine. Recent work has shown that certain neoplasms, such as mantle cell lymphoma and B-cell chronic lymphocytic leukemia, often harbor somatically mutated *ATM* alleles that affect ATM function (reviewed in Boultwood, 2001). In view of data showing that gemcitabine has clinical activity against both neoplasms (Dumontet et al., 2001; Savage et al., 2000; Waselenko et al., 2001), it will be interesting to see whether the subset of these or other neoplasms with *ATM* mutations might be preferentially responsive to gemcitabine.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Checkpoint signaling pathway activation by gemcitabine. **A-B**, HeLa cells grown on coverslips were treated with vehicle (A) or 100 nM gemcitabine (B) for 3 hr prior to fixation. Cells were then stained for phospho-Ser^{139}-H2AX (green) and counterstained with 1 μg/ml Hoechst 33258 (blue) to visualize nuclei, and examined by confocal microscopy. **C**, Cells were treated with vehicle (control) or gemcitabine as in A and B. Alternatively, cells were exposed to 100 cGy γ-radiation 1 h prior to fixation or were treated with 300 μM hydroxyurea for 3 h before fixation and staining for phospho-H2AX. Results obtained in 6 experiments with each treatment are summarized. Closed bar indicates mean number of cells staining positive for phospho-Ser^{139}-H2AX after the indicated treatment. Error bar, ± 1 standard deviation. **D-E,** HeLa (D) or K562 cells (E) were incubated with the indicated concentrations of gemcitabine for the indicated times. For ultraviolet light exposure, PBS-washed HeLa cell monolayers were exposed to 15 J/m^2 ultraviolet (UV) light. Following the addition of prewarmed (37˚C) medium, the cells were incubated for 1 h. Alternatively, cells were exposed to 20 Gy γ-radiation (IR) and cultured for 1 h. At the end of the incubations, cell lysates were separated by SDS-PAGE (10% gel) and sequentially immunoblotted for phospho-Ser^{345}-Chk1 and total Chk1 (left panels) or phospho-Thr^{68}-Chk2 and total Chk2 (right panels). **F-G,** Cell lysates were prepared from HeLa cells that were untreated or treated with 500 nM gemcitabine for 6 h. The indicated amounts of protein were separated by SDS-PAGE (12.5% gel) and immunoblotted for phospho-Ser^{345}-Chk1 and total Chk1 (F) or phospho-Thr^{68}-Chk2 and total Chk2 (G). * denotes a non-specific bands detected by the anti-phospho-specific antibodies. The non-specific band detected in Panel F is visible with some lots of phospho-Ser^{345}-Chk1 but not others (e.g., D and E, left panels).

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Figure 2. Effect of 9-1-1 complex disruption on gemcitabine sensitivity. **A**, HeLa cells were incubated with 500 nM gemcitabine or treated with 30 J/m^2 ultraviolet (UV) light and incubated for 1 h. Following incubation, the cells were differentially extracted to first release unbound Rad9 and to then release chromatin-bound Rad9. **B**, Parental Rad9 ES cells were incubated with the indicated concentrations of gemcitabine for 1 or 4 h and lysed. The lysates were separated by SDS-PAGE (10% gel) and sequentially immunoblotted for phospho-Ser^{345}-Chk1 and total Chk1. **C,** Parental or *Rad9*$^{-/-}$ ES cells were treated with the indicated concentrations of gemcitabine for 4 h and lysed. Lysates were separated by SDS-PAGE (10% gel) and sequentially immunoblotted for phospho-Ser^{345}-Chk1 and total Chk1. Dashed lines indicate juxtaposition of non-adjacent lanes from the same gel. **D**, Parental or *Rad9*$^{-/-}$ ES cells were incubated with the indicated concentrations of gemcitabine for 4 h and lysed. Cdc25A was immunoprecipitated from the lysates. The immunoprecipitates were separated by SDS-PAGE (10% gel) and immunoblotted for Cdc25A. **E**, Parental ES cells were treated with indicated concentrations of gemcitabine for 4 or 24 h and lysed. The lysates were separated by SDS-PAGE (10% gel) and immunoblotted for total Chk2. **F**, Parental and *Rad9*$^{-/-}$ cells were treated with the indicated concentrations of gemcitabine and incubated for 24 h. Cells were harvested, stained with Hoechst 33258, and assessed for apoptotic morphology by fluorescence microscopy. **G,H**, Parental and *Rad9*$^{-/-}$ cells were exposed to the indicated concentrations of gemcitabine (G) for 24 h, washed, and cultured for 7 d. Alternatively, parental and *Rad9*$^{-/-}$ cells were exposed to the indicated concentrations of tipifarnib (H) for the entire 7-d incubation period. Colonies were then stained with Coomassie Blue and counted.

Figure 3. Effect of the ATR on genotoxin sensitivity. **A-B**, Parental GM847 cells (A) or GM847/kdATR cells (B) were treated with vehicle or 1 μg/ml doxycycline (ATR_{KD} induced in panel B) for 2 d, treated with the indicated concentrations of gemcitabine for 24 h, and cultured for 12-14 d. Colonies were stained with Coomassie Blue and counted. **C-D**, HeLa (C) or A549 (D) cells were transfected with control siRNA or ATR siRNA. Forty-eight h after transfection, cells were harvested by trypsinization. The cells were split into two samples. One sample was lysed and the lysates were sequentially immunoblotted for ATR and Hsp90, as a loading control (insets). Cells from the other sample were replated, exposed to gemcitabine for 24 h, and cultured for 7 d. Colonies were then stained with Coomassie Blue and counted. Dashed lines in immunoblots indicate the juxtaposition of non-adjacent lanes from the same gel.

Figure 4. Effect of Chk1 and Chk2 on cytotoxicity. **A-D,** HeLa (A, C, D) or A549 (B) cells were transfected with control siRNA or Chk1 siRNA. Forty-eight h after transfection, cells were harvested by trypsinization. The cells were split into two samples. One sample was lysed and subsequently blotted for Chk1 and, as a loading control, β-actin or Hsp90 (insets). Cells from the other sample were replated, exposed to gemcitabine (A, B) or cytarabine (C) for 24 h, and cultured for 7 d. Alternatively, replated cells were subjected to the indicated doses of ionizing radiation (D) and culture for 8 d. Colonies were then stained with Coomassie Blue and counted. **E,** Parental or *Chk2*$^{-/-}$ HCT116 cells were treated with the indicated concentrations of gemcitabine for 24 h and cultured for an additional 7 d. Colonies were stained with Coomassie Blue and counted.

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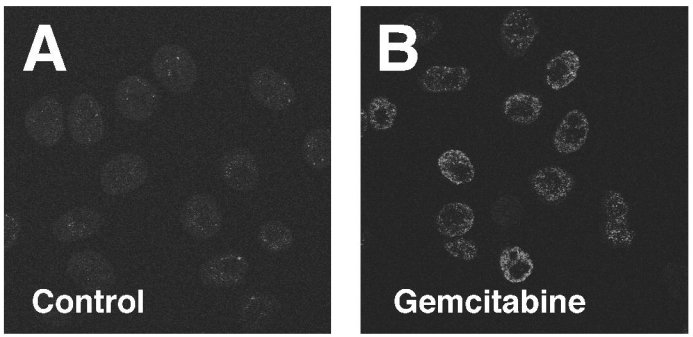
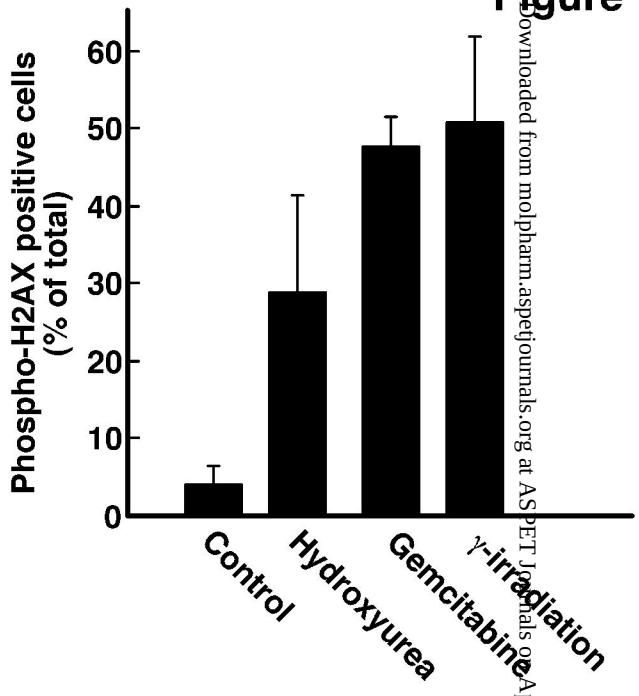
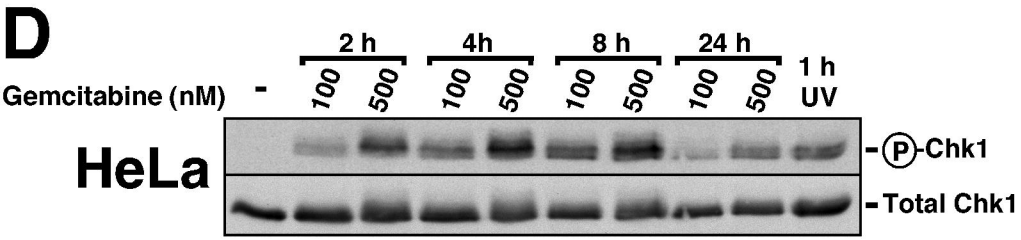
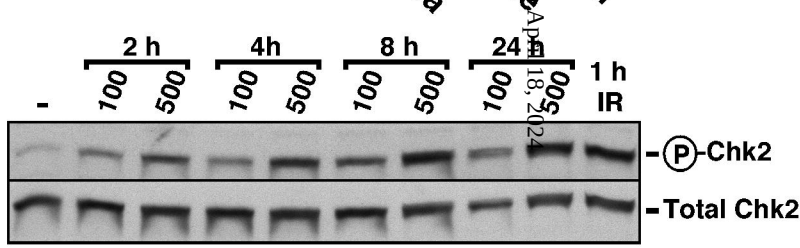
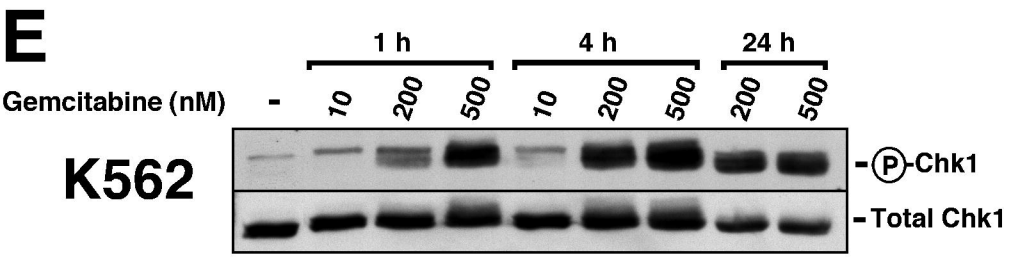
Figure 5. Effect of ATM on cytotoxicity. A549 (A, C, E) or HeLa (B, D, F) cells were transfected with control or ATM siRNA. Forty-eight h after transfection, cells were harvested by trypsinization. The cells were split into two samples. One sample was lysed and subsequently blotted for ATM and, as a loading control, Hsp90 (insets). Cells from the other sample were replated and treated with γ-irradiation (A, B). Alternatively, cells were treated with gemcitabine (C, D) or cytarabine (E, F) for 24 h. Colonies were then allowed to grow for 7 d, stained with Coomassie Blue, and counted.

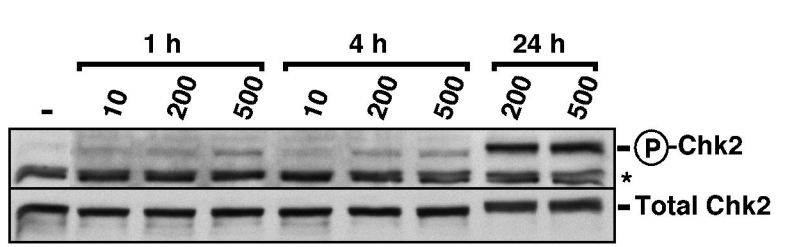


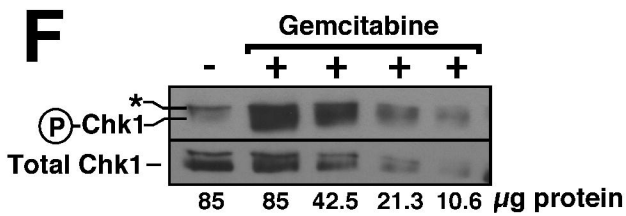












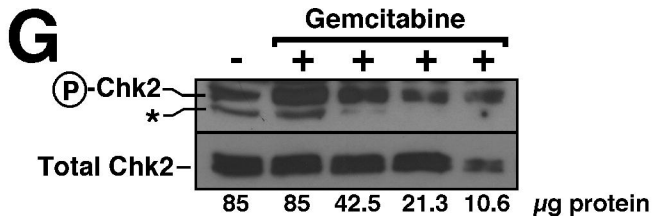


Figure 2

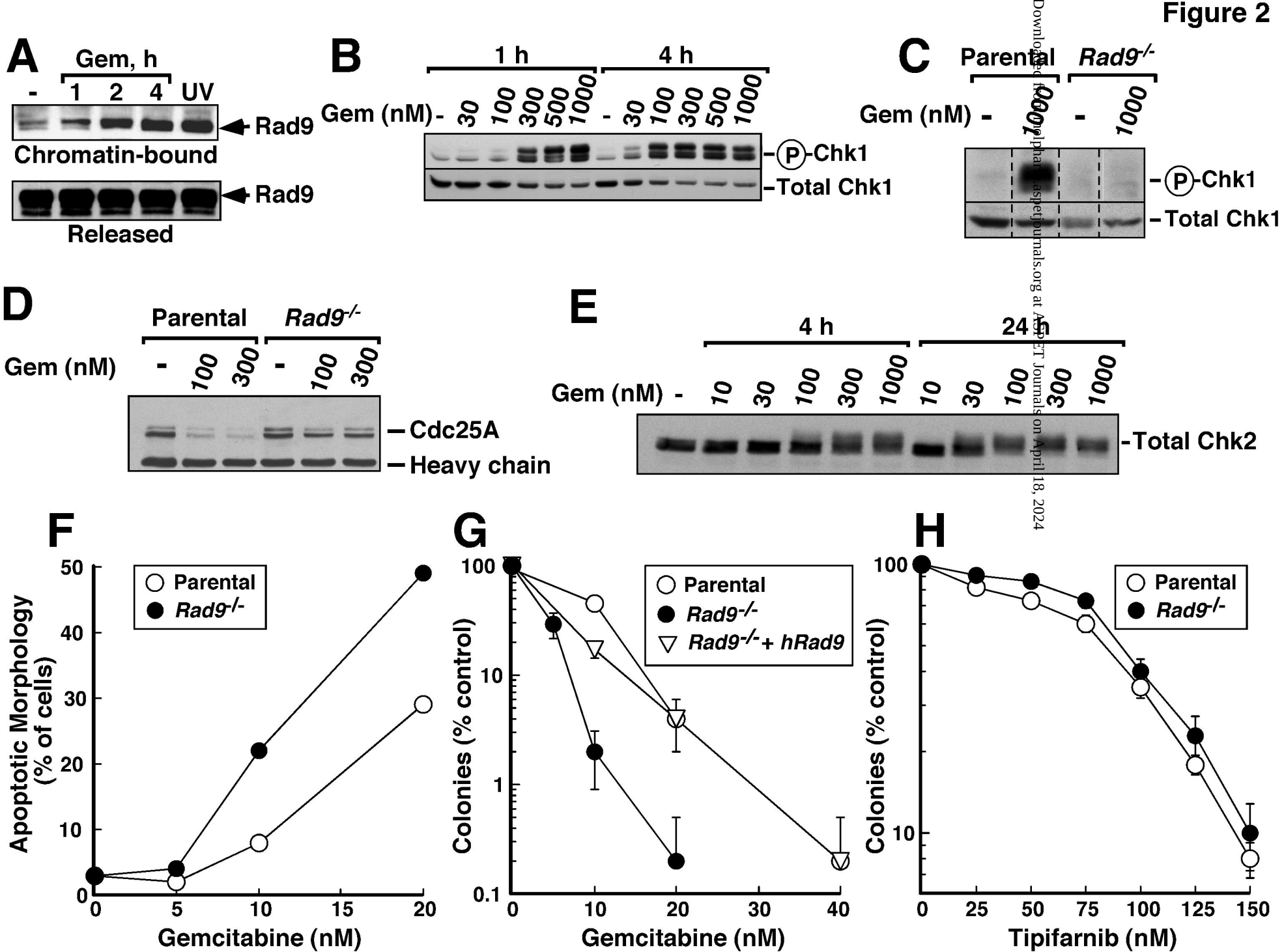
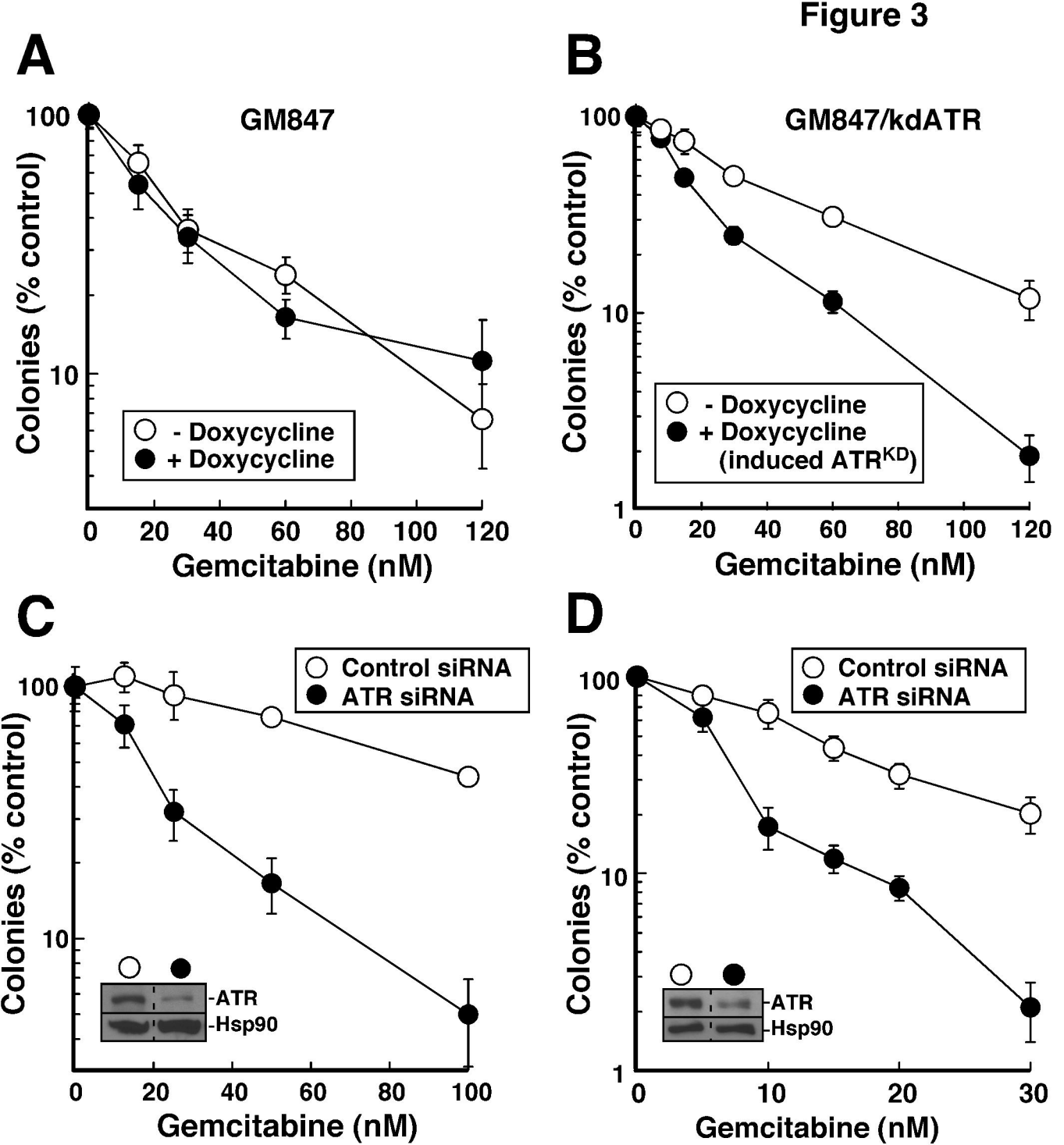


Figure 3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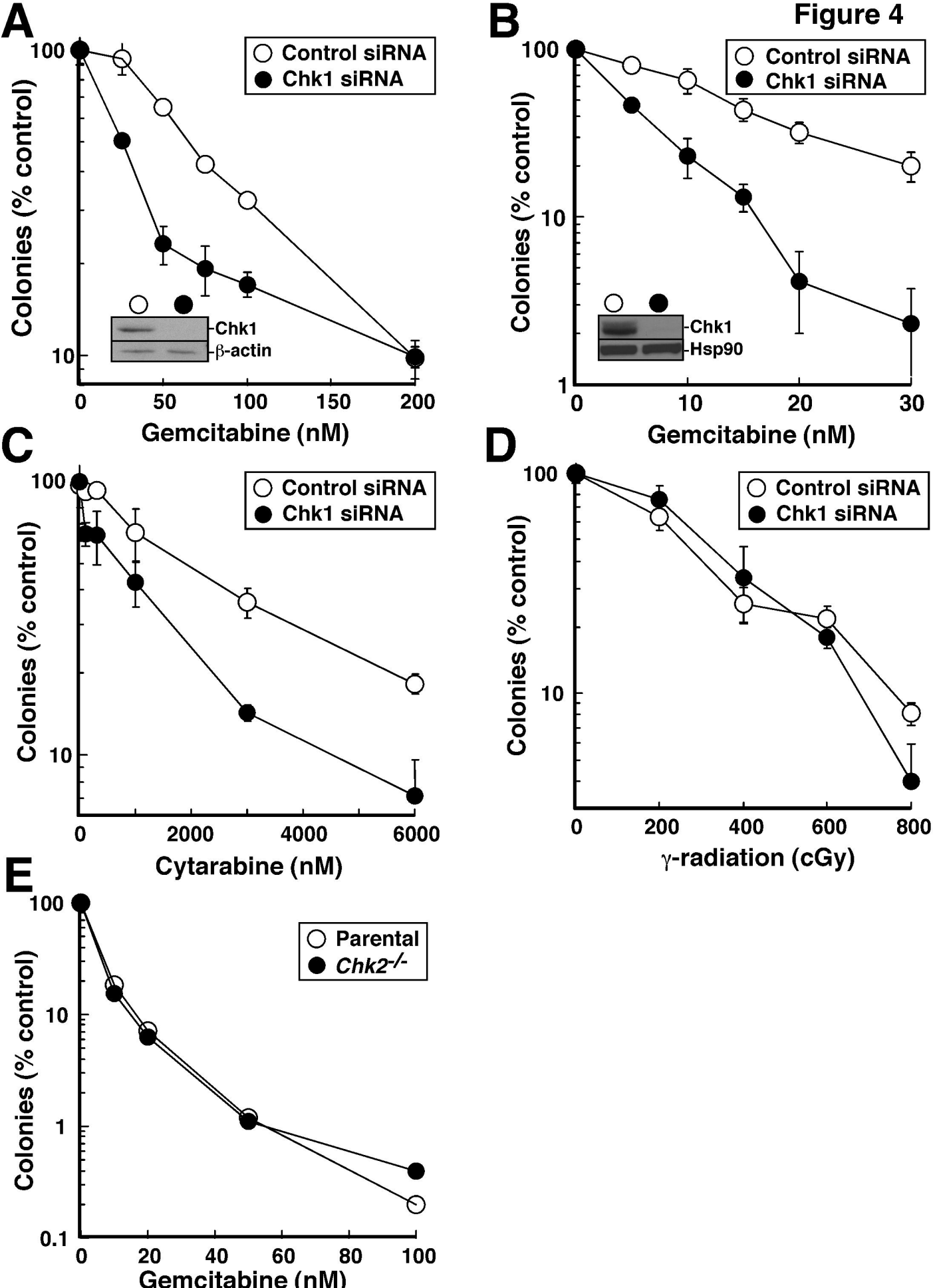
GM847
100
10
Colonies (% control)
- Doxycycline
+ Doxycycline
0
20
40
60
80
100
120
Gemcitabine (nM)
B
GM847/kdATR
100
10
1
Colonies (% control)
- Doxycycline
+ Doxycycline
(induced ATR^KD)
0
20
40
60
80
100
120
Gemcitabine (nM)
C
Control siRNA
ATR siRNA
100
10
Colonies (% control)
-ATR
-Hsp90
0
20
40
60
80
100
Gemcitabine (nM)
D
Control siRNA
ATR siRNA
100
10
1
Colonies (% control)
-ATR
-Hsp90
0
10
20
30
Gemcitabine (nM)

Figure 4


A
Control siRNA
Chk1 siRNA
Colonies (% control)
100
10
Chk1
β-actin
0
50
100
150
200
Gemcitabine (nM)
B
Control siRNA
Chk1 siRNA
Colonies (% control)
100
10
1
Chk1
Hsp90
0
10
20
30
Gemcitabine (nM)
C
Control siRNA
Chk1 siRNA
Colonies (% control)
100
10
0
2000
4000
6000
Cytarabine (nM)
D
Control siRNA
Chk1 siRNA
Colonies (% control)
100
10
0
200
400
600
800
γ-radiation (cGy)
E
Parental
Chk2-/-
Colonies (% control)
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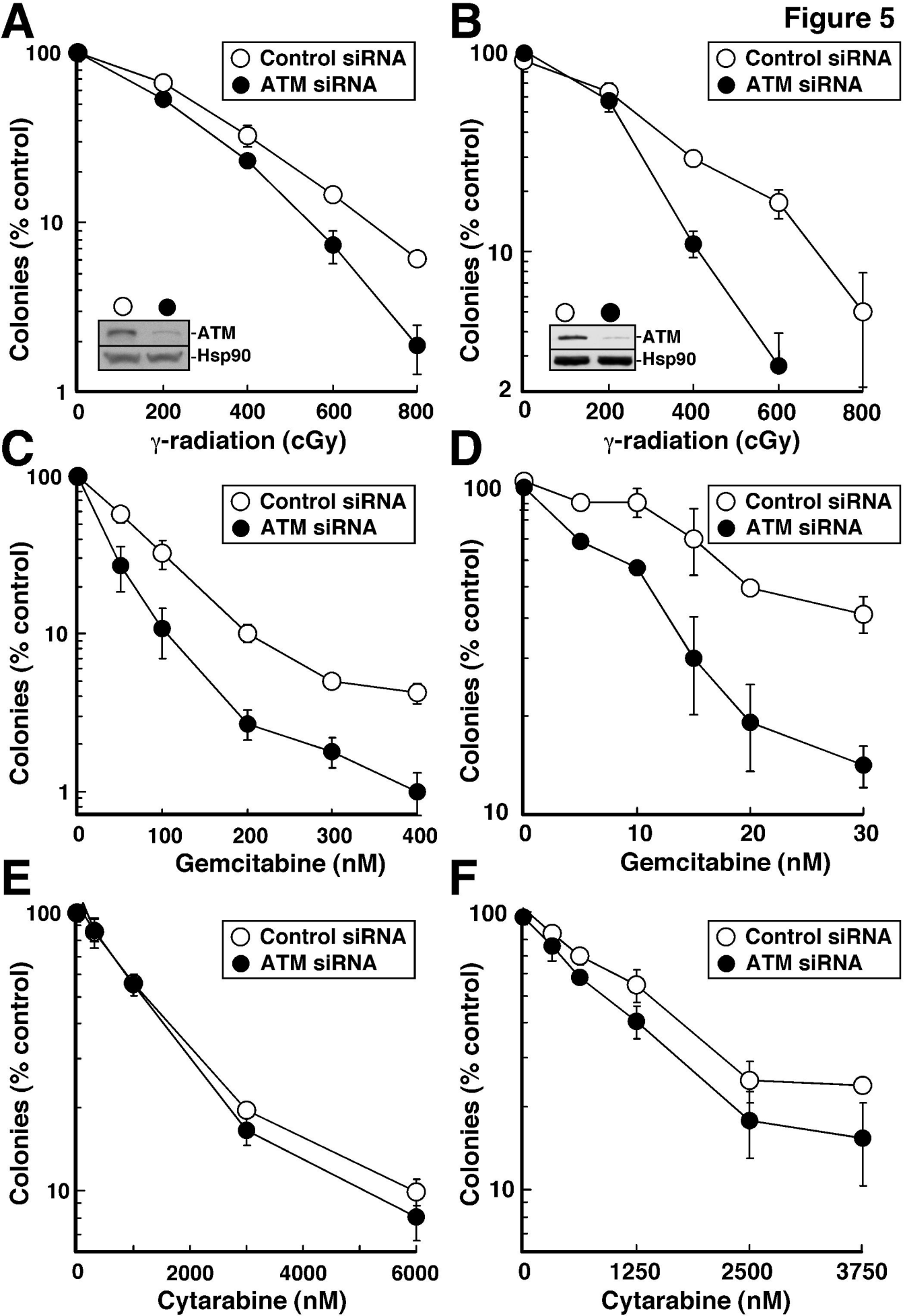
1
0.1
0
20
40
60
80
100
Gemcitabine (nM)

Figure 5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ATM siRNA
Colonies (% control)
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10
1
ATM
Hsp90
0
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400
600
800
γ-radiation (cGy)
B
Control siRNA
ATM siRNA
Colonies (% control)
100
10
2
ATM
Hsp90
0
200
400
600
800
γ-radiation (cGy)
C
Control siRNA
ATM siRNA
Colonies (% control)
100
10
1
0
100
200
300
400
Gemcitabine (nM)
D
Control siRNA
ATM siRNA
Colonies (% control)
100
10
0
10
20
30
Gemcitabine (nM)
E
Control siRNA
ATM siRNA
Colonies (% control)
100
10
0
2000
4000
6000
Cytarabine (nM)
F
Control siRNA
ATM siRNA
Colonies (% control)
100
10
0
1250
2500
3750
Cytarabine (nM)