The Radiomimetic Enediyne C-1027 Induces Unusual DNA Damage Responses to Double-Strand Breaks†

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ABSTRACT: Cells lacking the protein kinase ataxia telangiectasia mutated (ATM) have defective responses to DNA double-strand breaks (DSBs), including an inability to activate damage response proteins such as p53. However, we previously showed that cells lacking ATM robustly activate p53 in response to DNA strand breaks induced by the radiomimetic enediyne C-1027. To gain insight into the nature of C-1027-induced ATM-independent damage responses to DNA DSBs, we further examined the molecular mechanisms underlying the cellular response to this unique radiomimetic agent. Like ionizing radiation (IR) and other radiomimetics, breaks induced by C-1027 efficiently activate ATM by phosphorylation at Ser1981, yet unlike other radiomimetics and IR, DNA breaks induced by C-1027 result in normal phosphorylation of p53 and the cell cycle checkpoint kinases (Chk1 and Chk2) in the absence of ATM. In the presence of ATM, but under ATM and Rad3-related kinase (ATR) deficient conditions, C-1027 treatment resulted in a decrease in the level of Chk1 phosphorylation but not in the level of p53 and Chk2 phosphorylation. Only when cells were deficient in both ATM and ATR was there a reduction in the level of phosphorylation of each of these DNA damage response proteins. This reduction was also accompanied by an increased level of cell death in comparison to that of wild-type cells or cells lacking either ATM or ATR. Our findings demonstrate a unique cellular response to C-1027-induced DNA DSBs in that DNA damage response proteins are unaffected by the absence of ATM, as long as ATR is present.

The successful detection of and response to DNA DSBs[1] is crucial for cellular maintenance of genomic stability. Therefore, cells utilize a complex network of DNA strand break sensors, signal transmitters, and effectors to optimize DNA repair prior to mitosis, helping to prevent the consequences of genomic damage such as apoptosis, mitotic catastrophe, and irreversible growth arrest (*1*). Our understanding of DNA DSB damage response mechanisms is primarily based upon IR studies. Following IR treatment, cells activate the phosphatidylinositol 3-kinase-like protein kinase (PIKK) ATM through an autophosphorylation event (*2*). Activated ATM initiates a kinase cascade leading to cell cycle checkpoint responses and recruitment of repair proteins to sites of damaged DNA. For example, Chk2, a protein kinase directly phosphorylated by ATM at Thr68, mediates phosphorylation of Cdc25C and Cdk1, resulting in a G_2/M checkpoint (*3*). Another important downstream target of ATM following IR treatment is p53-Ser15 phosphorylation, which mediates the G_1/S checkpoint (*4*−*6*). Cells without ATM function cannot efficiently activate checkpoint responses to IR-induced DNA DSBs and are hypersensitive to IR treatment in regard to cell death and growth inhibition (*7*−*9*).

A complementary approach to studying PIKK responses to DNA DSBs utilizes radiomimetics such as enediynes. In comparison to IR, which induces multiple types of DNA damage [e.g., thymidine dimers, purine deamination, single-strand breaks (SSBs), and DSBs], enediynes generally induce only DNA strand breaks (both SSBs and DSBs) (*10*, *11*). Despite differences in the nature of the DNA damage, cellular responses to treatment with enediynes such as neocarzinostatin (NCS) share hallmarks of the IR-induced damage response, including ATM-dependent activation of p53-Ser15, Nbs1-Ser343, and Chk2-Thr68 (*12*−*14*), and cells lacking the ATM kinase are more readily killed by radiomimetic treatment than ATM-wild-type cells (*11, 15*). Furthermore, enediynes have helped to define the nature of the ATM response to DNA breaks. For example, a study comparing damage induced by IR and calicheamicin, an enediyne known to induce almost exclusively DSBs, has shown that ATM activation strongly correlates with DSBs and not SSBs (*16*). Enediyne studies have also led to the discovery of DNA DSB response pathways that do not strictly follow the IR model. We have shown that C-1027 treatment induces activation of p53-Ser15 in the absence of ATM, C-1027-induced cell growth inhibition does not increase in magnitude in cells lacking ATM, and radioresistant DNA synthesis is not observed (*17*).

Damage response mechanisms have also been identified that show while ATM is the primary PIKK to respond to

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[1] Abbreviations: ATM, ataxia-telangiectasia mutated; ATR, ATM and Rad3-related; Chk, checkpoint kinase; DSBs, double-strand breaks; IR, ionizing radiation; kd, kinase dead; NCS, neocarzinostatin; PIKK, phosphatidylinositol 3-kinase-like protein kinase; SSBs, single-strand breaks.

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DNA DSBs, ATR can play a role, albeit the response is typically much more limited. The ATM-Chk2 and ATR-Chk1 pathways both can activate G_2 checkpoints by inducing inhibition of Cdc25C phosphatase (*3*, *18*). A recent study has now confirmed that either ATM or ATR can phosphorylate p53-Ser15 as well as the Chk kinases in response to IR, although generally the induced phosphorylation signals are significantly lower when cells are deficient in either of the kinases (*8*). In contrast, after C-1027 treatment, cells lacking either ATM or ATR can still fully phosphorylate p53-Ser15 (*17*).

This study elaborates on the nature of C-1027-induced DNA DSB damage responses in an attempt to identify additional cellular responses to DNA DSBs that are not solely dependent upon ATM. We have found that, like the cellular responses to IR or NCS treatment, ATM-Ser1981 is rapidly phosphorylated after C-1027 treatment, yet contrary to the IR-mimic NCS, DNA damage induced by C-1027 does not require ATM to phosphorylate Chk2-Thr68 and Chk1-Ser345. Furthermore, in response to C-1027-induced DNA DSBs, we found that ATR status does not affect Chk2 phosphorylation and only partially affects Chk1 phosphorylation. However, the C-1027-induced damage response requires either ATM or ATR, as simultaneous loss of both kinases results in a lower level of p53 phosphorylation and a nearly complete loss of Chk1 and Chk2 phosphorylation. Additionally, an increased level of cell death in response to C-1027 treatment is only observed when both PIKKs are compromised.

MATERIALS AND METHODS

Chemicals. C-1027, a gift from Taiho Pharmaceuticals Co. (Saitama, Japan), was stored at 40 μM and 4 °C in ddH_2O, and neocarzinostatin, a gift from Bristol-Myers Squib Co. (Syracuse, NY), was stored at 200 μM and 4 °C in ddH_2O.

Cells. Isogenic ATM-null (AT169A) and ATM-restored (YZ510B) human fibroblast cell lines, a gift from P. Lu and Y. Shiloh, were grown at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 50 μg/mL hygromycin to maintain selective pressure on the transfected constructs (*19*). Wild-type GM00536 lymphoblast cells[2] were grown at 37 °C and 5% CO_2 in RPMI 1640 medium supplemented with 10% fetal bovine serum.

siRNA. Oligofectamine (Invitrogen, Carlsbad, CA) was incubated at 30 °C for 20 min in the presence or absence of siRNA targeted against ATR (Dharmacon Inc., Lafayette, CO) before being added to cells that had been incubated in serum-deficient medium. The siRNA was targeted to CCTC-CGTGATGTTGCTTGA, which corresponds to nucleotides 296−314 of the ATR gene (*20*), and has previously been shown to be effective and selective against ATR in these cells (*21*). Control siRNA sequences were also examined to ensure that the knockdown was specific for ATR. After 4 h, an equal volume of medium supplemented with 20% fetal bovine serum was added to return serum levels to normal. Forty-eight hours after siRNA treatment, cells were treated for 1 h with C-1027 and used in subsequent assays.

Immunoblotting. Cells with and without siRNA treatment were incubated with drugs at 37 °C for 1 h, harvested, and washed in phosphate-buffered saline. Total cellular extracts were prepared by directly adding SDS−PAGE buffer or by incubating cells on ice in lysis buffer [50 mM Hepes (pH 7.6), 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 0.5% Triton X-100, 50 mM sodium fluoride, 1 mM sodium *o*-vanadate, 1 mM β-glycerophosphate, 1 mM phenylmethanesulfonyl fluoride, and protease inhibitors] for 15 min. The cell lysates were cleared by centrifugation, and the protein content was determined using the Bradford method (Bio-Rad). Equal amounts of protein were electrophoresed on SDS−polyacrylamide gels and transferred to a PVDF membrane. The membranes were probed with primary antibodies, followed by secondary antibodies conjugated with horseradish peroxidase. The following primary antibodies were used: anti-phospho-p53-Ser15, anti-phospho-Chk1-Ser345, and anti-phospho-Chk2-Thr68 (Cell Signaling Technology, Beverly, MA), anti-phospho-ATM-Ser1981 (Rockland Immunochemicals, Gilbertsville, PA), anti-ATR (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-β-actin (Sigma, St. Louis, MO). Protein bands were visualized by enhanced chemiluminescence, and phosphorylation levels were measured using a Personal Densitometer SI (Amersham Biosceinces, Piscataway, NJ).

Growth Inhibition Assay. Cells in six-well dishes were seeded at a density of 2.5×10^5 cells/well and were treated with drug 24 h later. Following a 3 day incubation, dishes were washed and cells were counted with a coulter counter (Beckman Coulter Inc., Fullerton, CA). The degree of cell growth inhibition was calculated by comparing the number of treated to nontreated control cells.

Trypan Blue Exclusion. Cells growing in six-well dishes were seeded at a density of 2.5×10^5 cells/well. After 24 h, the cells were treated with drug. The cells were then incubated for 24 h, at which point the medium and cells were collected, spun, and incubated with a 0.4% solution of trypan blue for 10 min at room temperature. Cell death was based on the percentage of trypan-stained cells in samples containing $\geq$300 cells.

Genomic DNA Damage. Following a 30 min drug treatment and harvest, cells were resuspended in PBS at a concentration of 1×10^7 cells/mL. Cells were then diluted into 0.5% low-melting temperature agarose, placed on a frosted slide, and covered with a coverslip for 2 h at 4 °C. The slide was then immersed in alkaline lysis buffer [2.5 M NaCl, 0.1 M EDTA, 50 mM Tris (pH 10.0), 10% DMSO, 1% Sarcosyl, and 1% Triton X-100] in water overnight at 4 °C. The slides were then placed in electrophoresis buffer [30 mM NaOH and 10 mM EDTA (pH 13.0)] and equilibrated for 20 min before electrophoresis at 27 V for 25 min at 4 °C. The slides were then placed in 0.4 M Tris (pH 7.5) three times for 5 min, followed by immersion in 100% methanol, and then ethanol. The slides were dried and stained with 15 μg/mL ethidium bromide and covered with a coverslip. DNA damage was assessed as described elsewhere (*22*).

[2] In testing ATM phosphorylation, we used an intrinsically ATM-wild-type cell line as opposed to the ATM-restored fibroblasts that were used in other experiments, although both cell lines exhibited ATM-Ser1981 phosphorylation in response to C-1027 treatment.

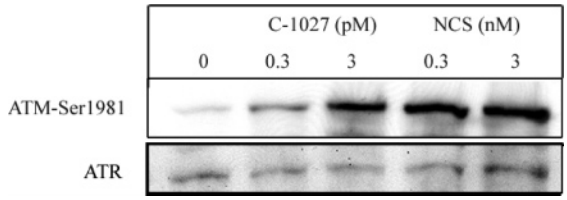

FIGURE 1: C-1027 treatment induces ATM activation. Wild-type human lymphoblast cells were treated with 0, 0.3, or 3 pM C-1027 or 0.3 and 3 nM NCS for 15 min at 37 °C, and the cellular extracts were analyzed by Western blotting. Immunoblots were then probed with an antibody specific for phosphorylated ATM-Ser1981.

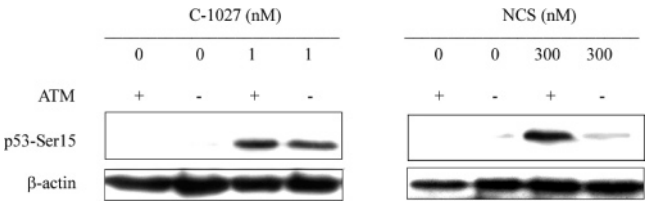

FIGURE 2: C-1027 induces ATM-independent p53-Ser15 phosphorylation. ATM-null and restored human fibroblast cells were treated with 0 or 1 nM C-1027 or 300 nM NCS for 1 h at 37 °C, and the cellular extracts were analyzed by Western blotting. Immunoblots were then probed with an antibody specific for phosphorylated p53-Ser15.

RESULTS

ATM Is Activated by C-1027-Induced DNA Breaks. ATM-regulated DNA damage responses after IR and radiomimetic treatment are well-characterized. To test whether the unusual ATM-independent damage responses to C-1027 are related to a weakened ability to activate ATM, we tested whether low levels of C-1027 resulted in ATM-Ser1981 autophosphorylation (Figure 1). Like IR-induced responses, ATM was activated within minutes using exceedingly low levels (300 fM) of C-1027 treatment. NCS, another enediyne known to induce IR-like DNA damage responses, also activated ATM at concentrations that induce low levels of DNA breaks, consistent with the findings of others (*14*).

Verification that C-1027 Can Induce p53-Ser15 Phosphorylation in a Manner Independent of ATM. In our previous study using IR or C-1027 treatment, p53-Ser15 phosphorylation was significantly attenuated in an ATM-null line for the former but not the latter, when compared to nonisogenic ATM-wild-type cells (*17*). Also, unlike IR, C-1027 treatment resulted in a similar cytotoxicity regardless of ATM status (*17*). However, since the cell lines were not isogenic, we could not rule out the possibility that the observed p53 phosphorylation in the ATM-null cells was affected by the differing genotypes. To confirm that C-1027-induced DNA damage responses could be activated in the absence of ATM, p53-Ser15 phosphorylation was assessed in isogenic ATM-wild-type and null cells after C-1027 treatment. We found little difference in p53-Ser15 phosphorylation between the two cell lines in response to C-1027 treatment, while as expected, relative to the ATM-wild-type line, the level of p53-Ser15 phosphorylation in the ATM-null cells was substantially reduced following NCS treatment (Figure 2). Similarly, we found that growth of the isogenic ATM-null cells when compared to that of ATM-wild-type cells was more inhibited by NCS but not by C-1027 (*17*, *19*) (Figure 3).

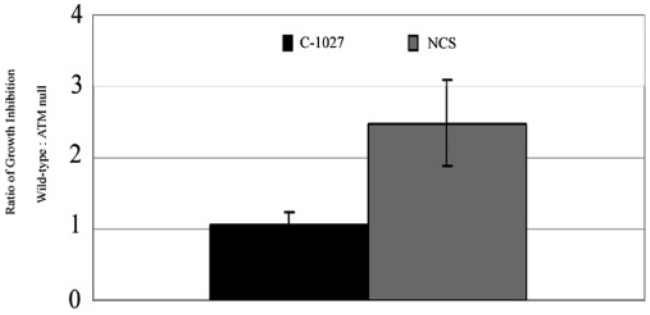

FIGURE 3: ATM-null cells are not hypersensitive to C-1027 treatment. ATM-null and restored human fibroblast cells were treated with 0 or 30 pM C-1027 or 30 nM NCS for 72 h at 37 °C, and the cells were harvested. The number of cells was counted and normalized as a percentage of the untreated control from three independent experiments. From these percentages, the ratio of growth inhibition between wild-type and ATM-null cells was determined.

C-1027 Induces Robust p53 Phosphorylation in Cells Deficient in either ATM or ATR but Not in Cells Deficient in Both Kinases. Previously, we showed that ATM-null cells respond to C-1027-induced damage like ATM-wild-type cells (Figures 2 and 3) (*17*). Prior studies in our laboratory have also revealed that the amount of p53-Ser15 phosphorylation observed after treatment with C-1027 or with IR was unaffected by ATRkd expression (*17*). We have now extended these results by utilizing ATM-wild-type and -null cells in combination with siRNA targeted against ATR (Figure 4A,B). ATR levels averaged 15% of the control level after siRNA knockdown (Figure 4A), consistent with the findings of others using this siRNA sequence in this and in other mammalian cell lines (*20*, *21*). Reduction of ATR in ATM-wild-type cells had little effect on C-1027-induced phosphorylation of p53-Ser15 (Figure 4A,B). In contrast, ATM-null cells with ATR depleted by siRNA exhibited an approximate 50% decrease in the level of p53-Ser15 phosphorylation in response to C-1027 treatment (Figure 4A,B). Thus, unlike IR and other radiomimetics such as NCS, C-1027-induced phosphorylation of p53-Ser15 is only decreased in cells deficient in both ATM and ATR.

C-1027 Induces Robust Chk2 Phosphorylation in Cells Deficient in either ATM or ATR but Not in Cells Deficient in Both Kinases. Chk2 is upstream of p53 and is directly phosphorylated by ATM in response to IR or enediyne-induced DNA breaks (*23*). The amount of Chk2-Thr68 phosphorylation following incubation of cells for 1 h with 1 nM C-1027 was measured by Western blotting. Similar to the p53 results and unlike IR, NCS, and calicheamicin treatment, Chk2-Thr68 was similarly phosphorylated in response to C-1027 treatment regardless of ATM status (Figure 5A,B). Also, the diminishing ATR kinase activity caused by siRNA targeting of ATR in ATM-wild-type cells had little effect on Chk2 phosphorylation in response to C-1027-induced DSB (Figure 5A,B), which is also in contrast to the IR model (*8*). Moreover, a diminished level of phosphorylation of Chk2 was also observed following NCS treatment of ATR-reduced ATM-wild-type cells (data not shown). However, only ATM-null cells with siRNA-depleted ATR exhibited an almost complete loss of Chk2-Thr68 phosphorylation in response to C-1027 (Figure 5A,B). Thus, the ATM-independent DNA damage responses induced by C-1027 extend to Chk2-Thr68 phosphorylation.

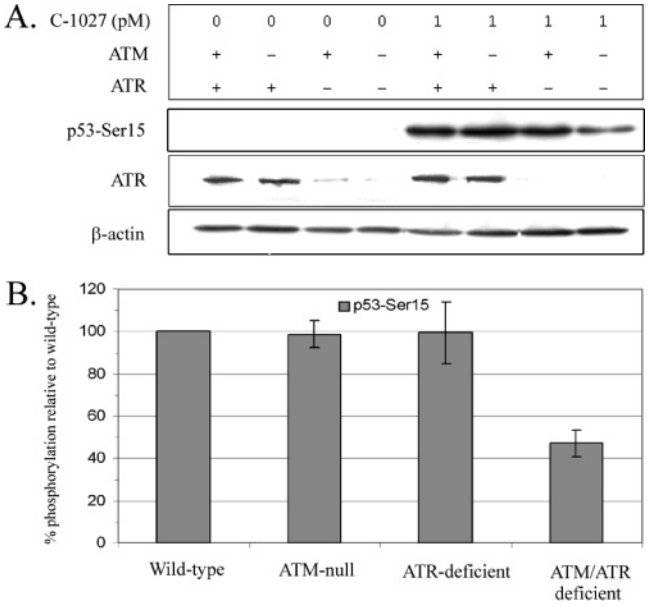

FIGURE 4: Induction of p53-Ser15 phosphorylation by C-1027 is partially inhibited only in the absence of both ATM and ATR. (A) ATM-null and restored human fibroblast cells were pretreated for 48 h with mock siRNA (ATR +) or siRNA directed toward ATR (ATR −) before being treated with 0 or 1 nM C-1027 for 1 h at 37 °C. The cellular extracts were analyzed by Western blotting. Immunoblots were then probed with an antibody specific for phosphorylated p53-Ser15 and quantitated using image quant software. (B) Each lane was normalized to a loading control (*β*-actin), and then the normalized value of each untreated signal was subtracted from the C-1027-treated signal under each kinase condition. Since the signal intensities can vary between experiments, the values of five independent experiments were converted to a percentage of the wild-type signal to estimate the loss of p53-Ser15 phosphorylation.

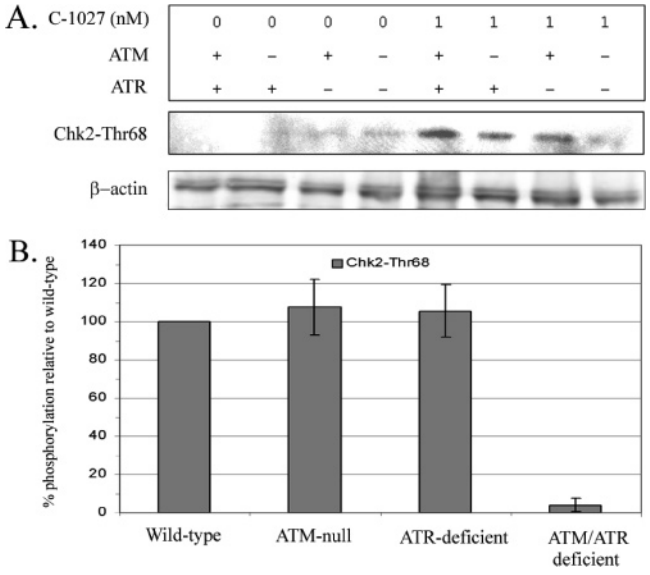

FIGURE 5: Induction of Chk2-Thr68 phosphorylation by C-1027 is greatly inhibited only in the absence of both ATM and ATR. (A) ATM-null and restored human fibroblast cells were pretreated for 48 h with mock siRNA (ATR +) or siRNA directed against ATR (ATR −) before being treated with 0 or 1 nM C-1027 for 1 h at 37 °C. The cellular extracts were analyzed by Western blotting. Immunoblots were then probed with an antibody specific for phosphorylated Chk2-Thr68 and quantitated using image quant software. (B) Each lane was normalized to a loading control (*β*-actin), and then the normalized value of each untreated signal was subtracted from the C-1027-treated signal under each kinase condition. Since the signal intensities can vary between experiments, the values of five independent experiments were converted to a percentage of the wild-type signal to estimate the loss of Chk2-Thr68 phosphorylation.

C-1027 Induces Chk1 Phosphorylation in Cells Deficient in either ATM or ATR but Not in Cells Deficient in Both Kinases. Loss of C-1027-induced activation of DNA damage response proteins seems to occur only when both ATM and ATR are depleted, in contrast to other radiomimetics and IR, where loss of ATM is sufficient to cause a substantial reduction in activation of these proteins. Phosphorylation of Chk1-Ser345 could be a possible exception since it is activated directly by ATR following DNA damage (*18*). The level of Chk1 phosphorylation is also known to be significantly decreased in the absence of ATM after IR treatment (*8*); thus, the level of Chk1 phosphorylation would be expected to decrease with a loss of either ATM or ATR. In contrast, C-1027 treatment of cells lacking ATM did not result in any decrease in the level of Chk1 phosphorylation (Figure 6A,B). Moreover, in ATM-wild-type cells that are deficient in ATR, C-1027 treatment resulted in an only partial loss of Chk1-Ser345 phosphorylation (Figure 6A,B). Only cells deficient in both ATM and ATR exhibited a nearly complete loss of Chk1-Ser345 phosphorylation after C-1027 treatment (Figure 6A,B).

C-1027-Induced DNA Breaks Are Not Affected by ATM or ATR Status. To test if the amount of C-1027-induced breaks was similar under each kinase deficient condition, DNA breaks were measured by Comet analysis.[3] SiRNA-treated (either mock or ATR) ATM-wild-type or -null cells were incubated with C-1027 under conditions used for Western analysis (1 nM C-1027 for 1 h) and then analyzed for induction of DNA breaks (Figure 7). PIKK status had little effect on the amount of DNA breaks observed after C-1027 treatment, consistent with the idea that the difference in DNA damage response signaling is not related to the amount of DNA breaks induced.

The Level of C-1027-Induced Cell Death Increases in Cells Deficient in both ATM and ATR. Cells lacking ATM are unable to induce DNA damage checkpoint responses to DNA DSBs induced by IR and radiomimetics (except C-1027) and, as such, are more readily killed by these agents (*6*). Since reduction of C-1027-induced DNA damage response checkpoints for the most part requires loss of both ATM and ATR, we sought to determine whether hypersensitivity in regard to cell death would vary accordingly. We examined siRNA-treated (either mock or ATR) ATM-wild-type or -null cells for an increased level of cell death in response to C-1027 treatment. Cells were treated with 0.3 and 1 nM C-1027 for 24 h and examined for their ability to exclude trypan blue. As in 3 day growth inhibition (Figure 3) (*17*), there was no difference in C-1027-induced cell death in ATM-wild-type and ATM-null cells (Figure 8). Also consistent with our previous study was the fact that there was no change in drug-induced cell death between ATM-wild-type cells treated with mock siRNA or SiRNA targeted against ATR. In contrast,

[3] Though Comet detects both SSBs and DSBs, C-1027 induces almost exclusively DSBs (*17*), and our comet results are consistent with neutral pulse field gel electrophoresis estimates of DNA DSBs from C-1027-treated cells (Dr. M. McHugh, personal communication).

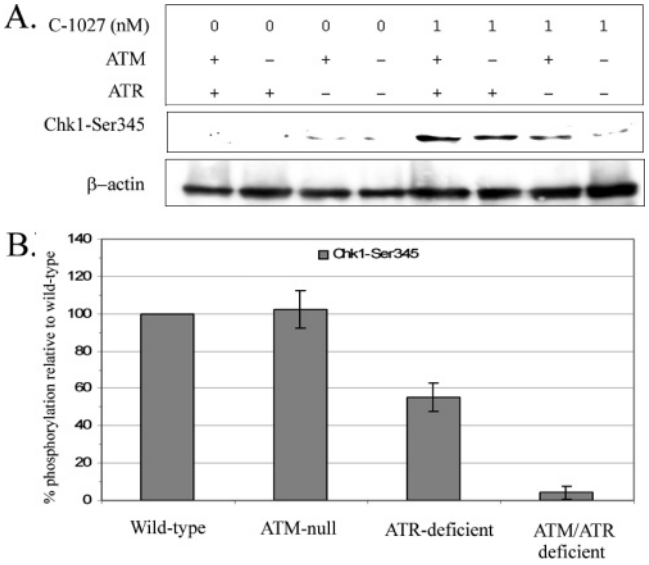

FIGURE 6: Induction of Chk1-Ser345 phosphorylation requires either ATM or ATR. (A) ATM-null and restored human fibroblast cells were pretreated for 48 h with mock siRNA (ATR +) or siRNA directed against ATR (ATR −) before being treated with 0 or 1 nM C-1027 for 1 h at 37 °C. The cellular extracts were analyzed by Western blotting. Immunoblots were then probed with an antibody specific for phosphorylated Chk1-Ser345 and quantitated using image quant software. (B) Each lane was normalized to a loading control (*β*-actin), and then the normalized value of each untreated signal was subtracted from the C-1027-treated signal under each kinase condition. Since the signal intensities can vary between experiments, the values of five independent experiments were converted to a percentage of the wild-type signal to estimate the loss of Chk1-Ser345 phosphorylation.

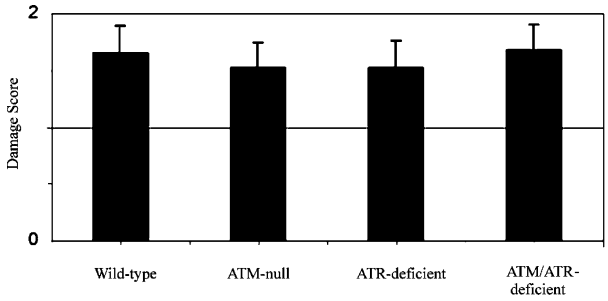

FIGURE 7: Amount of C-1027-induced DNA strand breaks are not affected by PIKK status. Under the conditions used for Figures 4−6, the amount of DNA breaks induced by 1 nM C-1027 was examined under varying PIKK deficient conditions. Cells were harvested and placed on a microscope slide. Following cell lysis, electrophoresis, and the addition of ethidium bromide, the size of the DNA tail was viewed by fluorescence microscopy and DNA breaks were quantitated.

ATM-null cells that were treated for 48 h with siRNA targeted against ATR exhibited a substantial increase in the level of cell death following C-1027 treatment (Figure 8). Thus, unlike IR and other radiomimetics, where loss of ATM leads to an increased level of cell death, loss of both ATM and ATR is required for C-1027 to recapitulate this response.

DISCUSSION

Cells lacking ATM are hypersensitive with regard to cytotoxicity induced by IR or radiomimetic treatment, consistent with their inability to activate DNA damage response proteins (*6*). This study further examined and expanded findings from this laboratory showing that after induction of DNA breaks by the enediyne C-1027, ATM-null cells robustly activated p53-Ser15 and did not display hypersensitive growth inhibition in comparison to ATM-wild-type cells (*17*). Recent studies have demonstrated that phosphorylation of ATM-Ser1981 occurs within minutes after low levels of IR treatment or treatment with radiomimetics such as NCS and calicheamicin (*2*, *14*, *16*). This study revealed that the ATM-independent nature of C-1027-induced DNA damage responses is not due to an inability to activate ATM, as very low levels of C-1027 treatment resulted in ATM-Ser1981 phosphorylation (Figure 1). These results demonstrate a traditional role for ATM in response to C-1027-induced breaks.

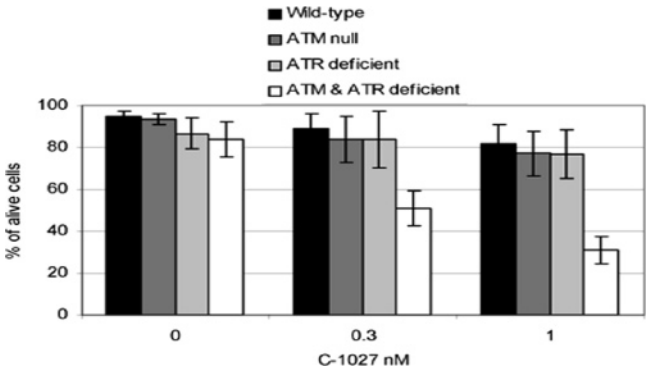

FIGURE 8: Only cells deficient in both ATM and ATR exhibit increased levels of cell death in response to C-1027 treatment. ATM-null and restored human fibroblast cells were pretreated for 48 h with mock siRNA or siRNA directed against ATR before being treated with 0, 0.3, or 1 nM C-1027 for 24 h at 37 °C. Cells were harvested from three independent experiments and assessed for the ability to exclude trypan blue. Three hundred cells from each sample were counted, and the percentage of cells excluding trypan blue was determined.

In general, cells lacking ATM show a weakened response to DNA damage induced by IR and a wide variety of radiomimetics, including enediynes such as calicheamicin and NCS, as well as glycopeptides such as bleomycin (*11*). The notable exception to this scenario is C-1027, which induced similar p53 phosphorylation in nonisogenic ATM-wild-type and ATM-null cell lines (*17*) and, now in this study, isogenic ATM-wild-type and -null cells, while the level of NCS-induced activation was greatly reduced in the cells lacking ATM (Figure 2). Similarly, the Chk kinase transducer proteins, located immediately downstream of ATM, are also activated differently after C-1027 treatment when compared to treatment with IR or other radiomimetics. In response to C-1027 treatment, Chk2 and Chk1 are robustly phosphorylated (Figures 5 and 6), while after treatment with IR or other radiomimetics, cells lacking ATM exhibited markedly reduced levels of phosphorylation of Chk2 and Chk1 (*8*).

While all three damage response proteins were fully activated in the absence of ATM after C-1027-induced DNA DSBs, each protein was activated differently in cells deficient in ATR or both ATM and ATR. We found that after C-1027 treatment as long as ATM is present when ATR is suppressed, both p53 and Chk2 were robustly phosphorylated while the level of Chk1 phosphorylation was reduced by ∼50% (Figures 4−6). In contrast, ATR activation of DNA

damage response proteins in response to IR or other radiomimetics in ATM-null cells is not robust and occurs after longer periods of time and/or at high levels of DSBs when compared against ATM-wild-type cells (*16*, *24*). For example, ATR is thought to be responsible for Chk2 phosphorylation in response to high levels of IR or calicheamicin-induced DSBs in the absence of ATM (*16*). After IR treatment, the level of phosphorylation of both Chk kinases is diminished in cells deficient in ATR, although ATR status did not affect p53-Ser15 phosphorylation (*8*, *17*). We obtained similar results after ATR deficient cells were treated with NCS (data not shown). While Chk1 phosphorylation is known to require ATR, the reason Chk2 activation is lost under these conditions is less clear (*6*). Since ATM-mediated phosphorylation of Chk2 in response to IR requires other damage response proteins such as Nbs1 and DNA-PK, perhaps activation of these proteins also involves ATR (*21*, *25*). Taken together, these findings suggest that unlike the observed ATR-dependent activation of the Chk kinases in response to DSBs induced by IR and NCS, ATM can compensate for the loss of ATR when the breaks are induced by C-1027.

The notion that cells can readily use either ATM or ATR to respond to C-1027-induced DSBs is supported by the finding that the level of phosphorylation of p53 is partially reduced, while the levels of Chk1 and Chk2 are drastically reduced, only when both PIKKs are lost (Figures 4−6). While there was almost no phosphorylation of either of the Chk kinases, p53-Ser15 phosphorylation levels were still significant, consistent with a previous observation that p53 can be activated by additional PIKK kinases such as DNA-PK and Smg1 (*26*, *27*). Further study is needed to determine the reason for these differences in phosphorylation and whether other PIKKs are involved in the C-1027-induded damage response.

Our findings also revealed that PIKK status dictates not only DNA damage responses but also cell survival in response to DNA breaks. Thus, only cells lacking both ATM and ATR were hypersensitive (with regard to cell death) to C-1027 treatment compared to wild-type cells and cells deficient in ATM or ATR (Figure 8). In contrast, after IR or NCS treatment, deficiency in either ATM or ATR results in greater cell death (*17*, *19*). Thus, activation of cellular damage responses by C-1027 is unique on the basis of PIKK status, and these responses correlate with cellular hypersensitivity with regard to cell death.

An important question is why C-1027-induced DNA damage responses differ from those induced by IR and other radiomimetics. Radiomimetics can be classified into two major groups, glycopeptides such as bleomycin, which interact with activated oxygen and a metal cation to damage DNA, and enediynes, which damage DNA by generation of free radicals that undergo a Bergman cycloaromatization reaction (*11*). Furthermore, enediynes can consist of a 10-membered ring chromophore such as calicheamicin or a nine-membered ring chromophore surrounded by a hydrophobic protein such as that with NCS and C-1027 (*28*). However, with the exception of C-1027, these various types of radiomimetics all produce DNA breaks that require ATM to robustly activate DNA damage responses and to prevent an increased level of cell death and growth inhibition. Thus, the particular characteristics of the C-1027 protein chromophore structure are not likely to be the basis for induction of ATM-independent DNA damage responses.

While the major DNA lesions produced by enediynes are strand breaks, under anaerobic conditions in cell free systems, both interstrand cross-links and monoadducts have also been observed (*29*). In these studies, C-1027 produced mainly covalent drug−DNA interstrand cross-links, NCS produced mostly monoadducts, and calicheamicin was less efficient at introducing either type of lesion (*29*). Possibly, C-1027 treatment of cells may also result in DNA interstrand cross-links that activate ATR in addition to DSBs, which activate ATM. Recent studies using mitomycin C, a known DNA interstrand cross-linker, have shown that cells utilize ATR to respond to DNA interstrand cross-links (*30*). There are also studies showing that enediynes can damage proteins or cleave RNA, which could also contribute to C-1027's uniqueness as an enediyne (*31*, *32*).

It is also possible that the type of DNA strand break (either single or double) produced by IR or radiomimetics influences PIKK regulation of DNA damage responses. Enediyne treatments induce DNA strand breaks by abstracting hydrogen from the C-1′, C-4′, and/or C-5′ atoms of the DNA deoxyribose backbone (*33*, *34*). However, as the efficiency of hydrogen abstraction from each DNA strand differs with the agent, so does the frequency of single- to double-strand breaks. Even though IR and radiomimetics have ratios of SSBs to DSBs that vary from 100 SSBs for every one DSB for IR to almost exclusively DSBs for calicheamicin, all induce ATM-dependent damage responses (*11*, *34*−*36*). Thus, the SSB:DSB ratio of 2:1 induced by C-1027 would likely not be a factor in the ATM dependence of the DNA damage response (*37*).

Could the types of DNA ends generated from the strand scission have an impact on the ATM dependence of the DNA damage response? C-1027-induced DNA DSBs have a 2 bp 3′ overhang and produce a combination of 3′-phosphoglycolate and base propenal ends on one strand, and either a nucleoside 5′-aldehyde, 2-deoxyribonolactone, or 4′-hydroxylated abasic site on the other (*38*). Similarly, NCS-induced damage also produces a 2 bp 3′ overhang and forms all of the DNA ends mentioned above (*11*). Therefore, it is unlikely that it is solely the products of C-1027-induced damage that can explain the observed responses to DNA DSBs in the absence of ATM. However, more subtle differences in DNA damage can exist between NCS and C-1027. For example, NCS reacts with bulges in the DNA structure associated with mismatch base pairing not seen with C-1027, which might influence how the damaged DNA triggers PIKK-dependent cell cycle checkpoint responses (*39*, *40*).

The ability of radiomimetics to focus DNA damage to particular DNA sequences or regions in the genome might also influence the nature of the DNA damage response. In contrast to IR, radiomimetics such as NCS and bleomycin can preferentially cleave particular subsets of genomic DNA, such as actively transcribing regions (*41*). However, since these enediynes also induce ATM-dependent damage responses, such regional DNA targeting is also unlikely to be a factor (*41*). Unlike IR, which induces undirected DNA damage, radiomimetics induce breaks by cleaving DNA at site-specific sequences. Although the highly preferred target sequences vary greatly, all of the agents besides C-1027

follow the IR model of ATM-dependent activation of damage responses (*11*, *33*, *39*, *42*).

Interestingly, one of C-1027's preferred target sequences, GTTA, is contained in the telomere repeat GGGTTA (*37*). Telomeres are a repetitive DNA sequence 8−15 kb in length, found at the ends of chromosomes, and are responsible for maintaining cell viability and genomic stability (*43*). We recently showed that at equal levels of DSBs, C-1027 treatment caused telomere erosion accompanied by a degree of chromosomal aberrations and genomic instability significantly higher than that produced by IR treatment (*44*). Recent studies have also shown that telomere dysfunction activates repair responses in a partially ATM-independent manner (*45*). While it is unknown if ATR is involved in this telomere dysfunction response, the ATM and ATR yeast homologues are known to work together in the regulation of repair of damaged telomeres (*46*). Although more study is needed, preferential targeting of telomeres by C-1027 could be contributing to the unique PIKK dependence of its induced damage response.

In conclusion, this study adds to the growing body of evidence which shows that DNA damage responses to DNA DSBs are not uniformly regulated by ATM. This study shows for the first time that loss of both ATM and ATR can be required to weaken activation of DNA damage response proteins and for an increase in the level of cell death. Future studies will determine if the ATM and ATR kinases have overlapping functions in response to C-1027-induced DNA damage and examine whether preferred targeting of telomere DNA and/or formation of DNA interstrand cross-links contributes to the unusual nature of the cellular responses to C-1027-induced breaks.

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