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14. ABSTRACT
The major goal of this project is to identify small inhibitory peptides that can interfere with critical DNA damage responsive pathways in order to develop novel therapeutic agents for prostate cancer radiotherapy. During the grant funding period, we have identified two DNA damage responsive pathway targets that can be explored for radiosensitization. We have demonstrated that small peptides containing SMC1 phosphorylation or NBS1-ATM binding sequences can abrogate optimal DNA damage responses in vitro. Further we have shown that these peptides can decrease prostate tumor cell clonogenic survival after radiation, indicating these peptides function as powerful radiosensitizers. In order to test in vivo radiosensitization activities, we generated a series of tumor homing peptides containing these sequences and proved tumor specific targeting of the peptides. Prostate cancer xenograft models have been explored though limited radiosensitization effects have been observed. In addition to in vitro and in vivo studies, we have also elucidated the mechanistic insights of inhibitory peptides. Completion of this project will have significant impact on developing molecular targeted radiosensitizers for prostate cancer treatment.

15. SUBJECT TERMS
ATM, SMC1, NBS1, DNA damage, radiotherapy, peptides

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Table of Contents

INTRODUCTION

Prostate cancer is the most common cancer and the second leading cause of cancer-related death among North American men. Currently available therapeutic modalities include surgery, radiotherapy, hormone therapy and chemotherapy. Delivery of radiotherapy has failed to cure patients because of tumor resistance and toxicity of radiation to adjacent tissues. Developing radiosensitizers continues to be a major challenge to radiation oncologists and radiobiologists. The goal of this project has been to identify and characterize small inhibitory peptides that can sensitize prostate tumor cells to radiation therapy both *in vitro* and *in vivo*. We have aimed to target critical DNA damage responsive pathways. In the original proposal, we proposed to test only one type of small inhibitory peptides targeting protein phosphorylation. During the performance period, we have actually identified two types of peptides that that can interfere with ATM-mediated pathways. ATM is a protein kinase critical for the cellular survival after radiotherapy. ATM phosphorylates a series targets including the Structural Maintenance of Chromosome protein one (SMC1)(Kim et al., 2002; Kitagawa et al., 2004; Yazdi et al., 2002) and Nijmegen Breakage Syndrome 1 (NBS1) (Lim et al., 2000) to limit the amount of radiation sensitivity. We have developed several peptides that function as radiosensitizers *in vitro* by targeting ATM phosphorylation of SMC1 and NBS1-ATM interaction (Cariveau et al, 2007).

BODY

Task 1. To further characterize the inhibitory effect of the THM-SMC1 peptide on cellular response to IR.

The goal of Task 1 was to investigate the magnitude and specificity of the THM-SMC1 peptides on radiation responses such as radiation induced cell survival, cell cycle checkpoint etc.

A. Determine cellular toxicity of the peptides on normal prostate cells and prostate tumor cells.

Since our goal was to evaluate a potential radiosensitizer, the first thing we would like to test was the cytotoxicity of the peptides to normal and tumoral prostate cells. To achieve this goal, we utilized normal prostate epithelial cells (provided by Dr. Shahriar Koochekpour), PC-3, DU-145 and LNCap cells. ID_{50} of the peptides was determined by cell viability assays, which were measured by the MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay. Both dose response and time course of exposure to the peptides were conducted. As shown in **Figure 1**, the three peptides, when doses were lower than 50 μM for 2-hour exposure, showed minimal effects on cell survival. Meanwhile, when doses were higher than 50 μM, all three peptides have moderate toxicity to the cells. A positive control

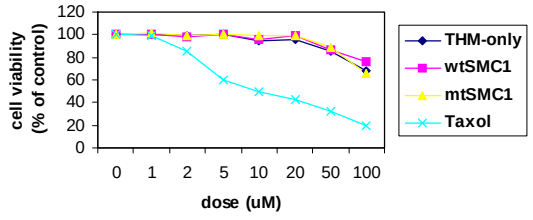

Figure 1. The THM-SMC1 fusion peptides possess no cytotoxicity at doses lower than 50 μM. PC-3 cells were treated with indicated concentrations of the peptides or Taxol for 2 hrs. Cellular viability was assessed by the MTT assay.

(Taxol) was utilized in these experiments. In the time course experiments (**Figure 2**), different time exposure, with up to 24 hours and at a dose of 10μM, posed minimal toxicity to the cells. The effect of the peptides on cell cycle progression was also investigated. Cells treated with peptides were harvested, fixed in 70% of ethanol, and stained with propidium iodide, followed by flow cytometric analysis. No change of basal cell cycle distribution is detected (data not shown). These observations suggest that the THM-fusion peptides have minimal cytotoxicity to normal prostate cells and prostate tumor cells; therefore they can be tested as pure radiosensitizers.

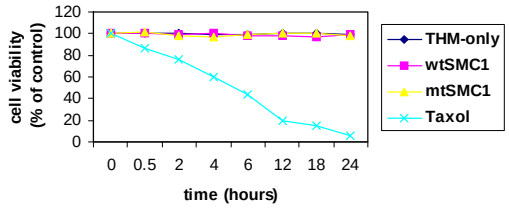

Figure 2. The THM peptides have limited cytotoxicity (time course). Cells were treated with 10 μM of the peptides or Taxol for indicated time. The viability was assessed by the MTT assay.

B. Investigate the effect of the THM-SMC1 peptide on ATM activation and SMC1 phosphorylation after IR.

Our hypothesis is that the wt SMC1 peptide can target the ATM-SMC1 pathways. To test this hypothesis, we investigated the effect of these peptides on ATM activation and SMC1 phosphorylation in response to IR. Western blot analyses were performed to assess ATM autophosphorylation (using a commercially available antibody recognizing phosphorylation of Serine 1981 from Rockland) and SMC1 phosphorylation (using a commercially available antibody recognizing phosphorylation of Serine 966 from Bethyl Laboratories Inc.) after IR in PC-3 cells treated with the peptides. Total amount of ATM and SMC1 were immuno-blotted and used as loading controls. As shown in **Figure 3**, while the THM only peptide does not change IR-induced ATM activation and SMC1 phosphorylation, the wtSMC1 peptide significantly diminished ATM autophosphorylation and SMC1 phosphorylation. It is interesting to observe that the mtSMC1 peptide functioned similar to THM-only with no noticeable effect on ATM and SMC1 phosphorylation. This is consistent with our

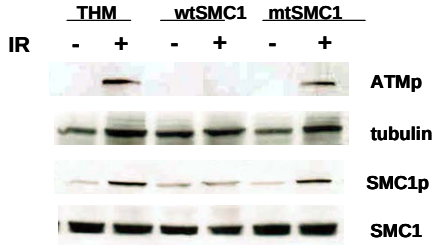

Figure 3. The THM-wtSMC1 peptide abogates ATM activation and SMC1 phosphorylation n response to IR. PC-3 cells were treated with indicated peptides, followed by 6 Gy of IR. Whole cell lysates were harvested and immunoblotted with indicated antibodies.

previous observation that the mtSMC1 has minimal effects on activation of the S-phase checkpoint after IR.

C. Investigate the effect of the THM-SMC1 peptide on the IR-induced G2/M checkpoint.

One of the endpoints to evaluate the THM peptides is to study the effect on the G2/M checkpoint. The G2/M checkpoint is dependent on the ATM and BRCA1 proteins. ATM phosphorylation of BRCA1 regulates the checkpoint, whereas ATM phosphorylation of SMC1 is independent of the checkpoint (Xu et al., 2002; Kim et al., 2002). If the THM-wtSMC1 peptide only interferes with the ATM-SMC1 signal, they should have minimal effect on activation of the G2/M checkpoint. However, if the THM-wtSMC1peptide target ATM kinase activity, then the G2/M checkpoint should be abrogated by the peptide. We previously looked at the G2/M checkpoint in PC-3 cells and concluded a normal response to IR in the cells. When treated with the THM only peptide, the cells maintain an intact G2/M checkpoint as measured by histone H3 phosphorylation, an event required for chromosome condensation and mitotic entry. However, the THM-wtSMC1 peptide partially abrogates the checkpoint (**Figure 4**). Similar to its effect on the S checkpoint, the THM-mtSmc1 peptide has less effect on the process.

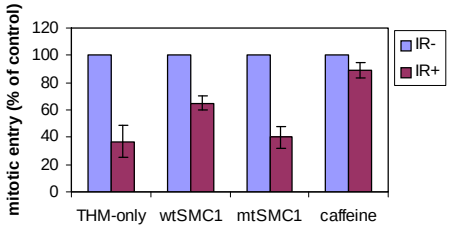

Figure 4. The THM-wtSMC1 peptide can partially abrogate the G2/M checkpoint. PC-3 cells treated with the peptides were radiated with 0 or 6 Gy of IR. The G2/M checkpoint was assessed by histone H3 staining followed by flow cytometry analysis. Error bars represent averages of at least three samples.

D. Investigate the effect of the THM-SMC1 peptide on radiation sensitivity.

We then tested radiation sensitivity in PC-3 cells. Colony formation assays in soft agar were performed in six-well plates using $1X10^4$ cells per well. The bottom layer contains 2 ml of 0.6% Noble agar (Difco, Becton Dickinson), penicillin (100U/ml), streptomycin (100µg/ml), 10% FBS in DMEM medium. The top layer contains 1 ml of 0.3% Nobel agar, penicillin (100Ug/ml), streptomycin (100µg/ml), 10% of FBS, DMEM and 10,000 cells. Plates were treated with the peptides and then be radiated at 0-6Gys. After 2-

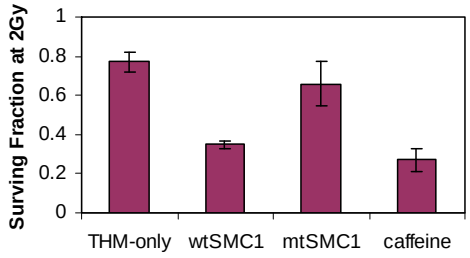

Figure 5. The THM-wtSMC1 peptide increases PC-3 cell radiosensitivity. PC-3 cells were treated with indicated peptides followed by mock or up to 6 Gy of IR. Colony formation assay was conducted and the surviving fraction was calculated. Shown are surviving fractions at 2Gy (SF2). Error bars represent averages of at least three samples.

week incubation, the numbers of colonies (those with more than 50 cells will be counted as one surviving colony) were counted and the surviving fractions were calculated. Shown in **Figure 5** is the surviving fraction at 2Gy of IR (SF2). The THM-only peptide and the mtSMC1 peptide did not change SF2, while THM-wtSMC1 significantly decreases the SF2. More colony formation experiments are underway and some critical parameters of radiation sensitivity will be obtained.

To establish statistical significance of peptide induced radiosensitivity, Student's t-test (Paired 2 sample for means) was incorporated. The data were first fit to each experimental group over a dose range of 0-6Gy. Significant differences in clonogenic survival were observed between cells treated with wtSMC1, and those treated with PBS, THM only or THM-mtSMC1 treated cells. Collectively, these results have provided strong evidence that the wtSMC1 peptide containing the phosphorylation sequence of SMC1 indeed sensitizes prostate tumor cells to ionizing radiation.

E. Identification of NBS1 inhibitory peptides for prostate cancer radiosensitization.

In addition to the SMC1 peptides we have characterized, we have continued to identify novel peptides that can target the DNA damage responsive pathways. A recent study revealed that the C-terminal NBS1 domain is critical for binding to ATM (Falck et al., 2005). An NBS1 truncated derivative lacking the C-terminal 20 residues does not

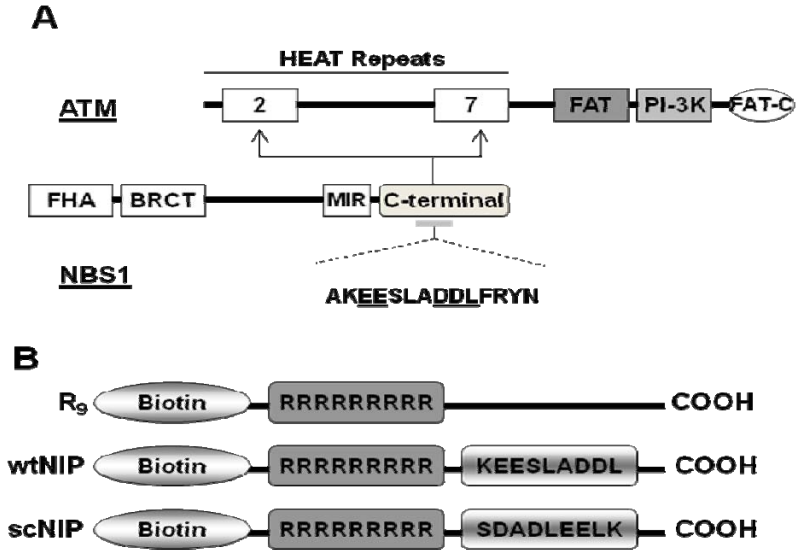

Figure 6. Development of the NBS1 Inhibitory Peptides (NIPs). *A.* Schematic illustration of functional domains of ATM an NBS1 and their interaction. The C-terminal of NBS1 is required for ATM activation and recruitment to sites of DNA damage. It consists of at least two sets of amino acid residues, 736-737 (EE) and 741-742 (DDL), that are evolutionarily conserved and necessary for ATM binding. NBS1 binds to two sets of Heat Repeats (Heat Repeat 2 (a.a. 248-522), and Heat Repeat 7 (a.a.1436-1770), in ATM. *B.* The amino acid sequences for the R9, wtNIP, and scNIP peptides developed.

associate with ATM *in vitro* (Cerosaletti and Concannon, 2003; Cerosaletti and Concannon, 2004; Cerosaletti et al., 2006). In addition, it has recently been shown that expression of an NBS1 transgene lacking the ATM binding domain in NBS cells leads to a dramatic reduction in ATM activation (Difilippantonio et al., 2005). Therefore NBS1-ATM interaction can be an appealing target for developing radiosensitizers, since inhibiting NBS1 association with ATM leads to suboptimal ATM activation after IR, thereby sensitizing cells to radiation. One approach to inhibiting NBS1-ATM interaction would be to use small peptides containing the conserved C- terminal sequence of NBS1 which can presumably compete with endogenous NBS1-ATM interaction. To test this hypothesis, we generated several fusion peptides using a polyarginine sequence (R_9) for internalization. These include the polyarginine (R_9) internalization sequence alone, and a wild-type NBS1 inhibitory peptide (wtNIP) corresponding to amino acids 734-748 of human NBS1. To serve as a negative control, we used a random sequence generator to produce a peptide in which a.a. 734-748 of human NBS1 were scrambled (scNIP) as shown in **Figure 6**. R_9, wtNIP and scNIP were synthesized and labeled with a biotin tag at their N-terminus for detection *in vitro*. Cells treated with the peptide were probed with a fluorescein-conjugated streptavidin antibody to determine the presence of the biotinylated peptides. Our data demonstrated that cells treated with R_9, wtNIP, and scNIP display significant cytoplasmic and nuclear localization (data not shown, see appendix).

We then set out to determine the length of time the peptides remain in cells as a way to ensure the peptides would be present throughout the DNA repair process after IR. As shown in **Figure 7**, immediately after incubation with the peptides, all sample groups show distinct presence of peptides. Within 2 hours of treatment cells continue to

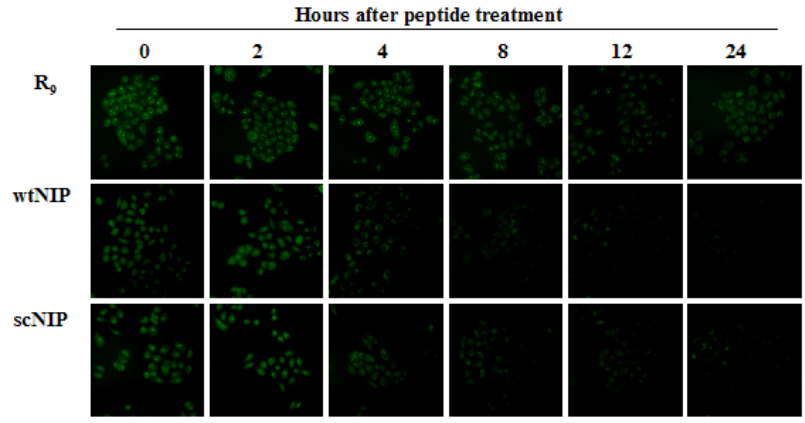

Figure 7. Degradation of the R9, wtNIP or scNIP peptides. HeLa cells were treated with 10μM R9, wtNIP, or scNIP for one hour and harvested at indicated time points before they were analyzed by immunofluorescence microscopy staining with an anti-streptavidin antibody.

display a strong distribution of R_9, wtNIP and scNIP but fluorescent intensity levels of wtNIP and scNIP begin to decrease within 4 hours with a substantial decline by 8 hrs. R_9 fluorescence remains slightly elevated at 8 hours while wtNIP and scNIP intensities are much weaker. This is in agreement with literature which suggests that R_9 sequences translocate easier and remain present longer than when they are not

coupled to a molecule or peptide (Jones et al., 2005). By 12 hours, cells treated with R_9 peptides still display prominent staining, while cells treated with wtNIP or scNIP show much weaker cytoplasmic staining with no observed nuclear staining. Within 24 hours, cells treated with R_9 show cytoplasmic staining, but the nuclear signal is no longer visible, cells treated with wtNIP or scNIP show no detectable presence of the peptides. These data suggest that the NIP peptides should be added to cells every 4-6 hours in the first 24 hours after treatment with IR in order to achieve maximal inhibitory effects.

F. Peptide Cytotoxicity

An ideal candidate for a radiosensitizer should possess little or no cytotoxicity by itself in order for investigations to purely assess the radiosensitizing effect on tumor cells. To test the cytotoxicity of the peptides, HeLa cells were treated with R_9, wtNIP or scNIP for 24h at ambient temperature. After incubation, media containing peptides was removed, fresh media added and the cultures incubated for additional 24 hours. At the end of the time course, the commercially available MTT cell viability assay (Promega Corp., Madison WI) was used to determine peptide cytotoxicity. A chemotherapeutic agent Taxol (from Sigma) was used as a positive control. Our data show that the NIP peptides possess minimal cytotoxicity to cells in doses below 10μM (**Figure 8**). Clonogenic surviving assays confirmed a minimal toxicity of the peptides (data not shown). A dose-response and time course experiments were also performed and repeated in other cell lines (data not shown), and the conclusion is firmed.

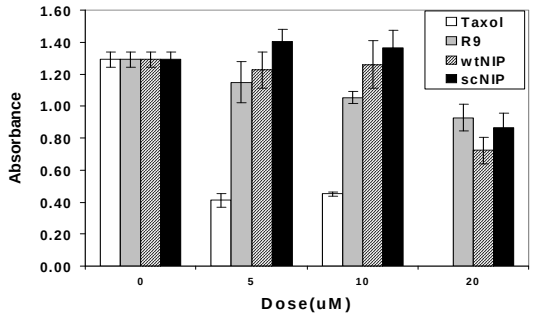

Figure 8. The cytotoxicity of the NIP peptides. Hela cells were treated Taxol and the NIP peptides at indicated doses. 24 hours after treatment, cell survival was quantified by a standard MTT assay.

G. wtNIP inhibits IR-induced γ-H2AX and NBS1 focus formation.

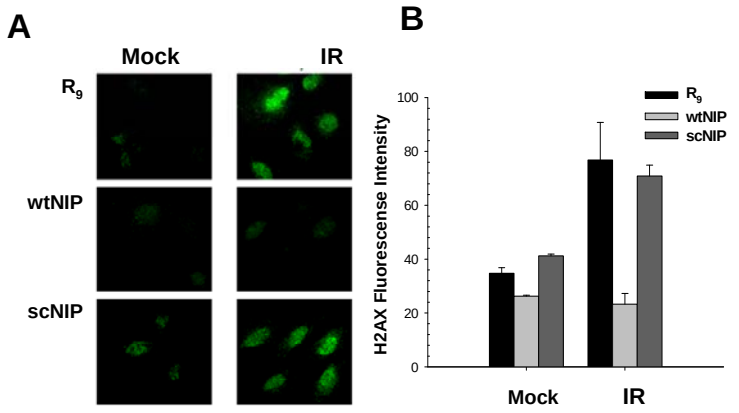

Figure 9. wtNIP can inhibit γ-H2AX foci formation. A. HeLa cells were treated with 10μM R9, wtNIP, or scNIP for 1hour, irradiated with 0 or 6Gy, and harvested 30 minutes later before immunofluorescence microscopy was employed to detect radiation induced-γ-H2AX foci. **B.** The mean γ-H2AX fluorescence intensity was determined for each image using Image Pro 5.1 software and is expressed in arbitrary units. Error bars represent +/-1 SD, graphed are the mean of three independent experiments

One of the earliest responses after DNA damage is the formation of γ-H2AX foci at sites of DSBs, an event that requires functional ATM (Burma et al., 2001; Furuta et al., 2003). Since the wtNIP peptide was designed to interfere with NBS1-ATM interaction and ATM activation, we investigated whether IR-induced γ-H2AX focus formation was inhibited by the peptide. Immunofluorescence microscopy was used to detect the presence of γ-H2AX foci in mock or irradiated cells in the presence of 10μM R_9, wtNIP or scNIP. We observed that while R_9 showed no inhibition on γ-H2AX focus formation, wtNIP significantly diminished IR-induced γ-H2AX foci (**Figure 9**). The scNIP peptide did not have any effects on IR-induced focus formation, suggesting that only wtNIP can display an inhibitory effect in the DNA damage response. These observations have been confirmed in other cell lines, including the prostate cancer cell line DU-145 (data not shown, see appendix)

To further support our conclusion, we investigated IR-induced NBS1 focus formation, an event considered to be an ATM-dependent process at the sites of DSBs (Lim et al., 2000). NBS1 foci are a result of ATM-mediated NBS1 phosphorylation on Serine 343. Using an anti-phospho-Ser343 NBS1 antibody, we observed that NBS1 phosphorylation was significantly inhibited in cells treated with wtNIP compared to those treated with R_9 or scNIP (data not shown, see appendix). The average number of foci in mock-irradiated cells was 5.96, 7.96, and 5.88 for 10μM R_9, wtNIP, and scNIP respectively. Cells treated with R_9 or scNIP displayed average foci of 24.52 and 30.84, while cells treated with wtNIP showed only 6 foci per nucleus 2hrs after treatment with 6Gy IR (data not shown, see appendix). These observations demonstrate that the wtNIP peptide can inhibit the ATM-mediated signaling pathway.

H. wtNIP increases radiation sensitivity.

The clonogenic survival assay was then used as a tool for determining *in vitro* radiosensitivity of cultured mammalian cells. To characterize the radiosensitizing

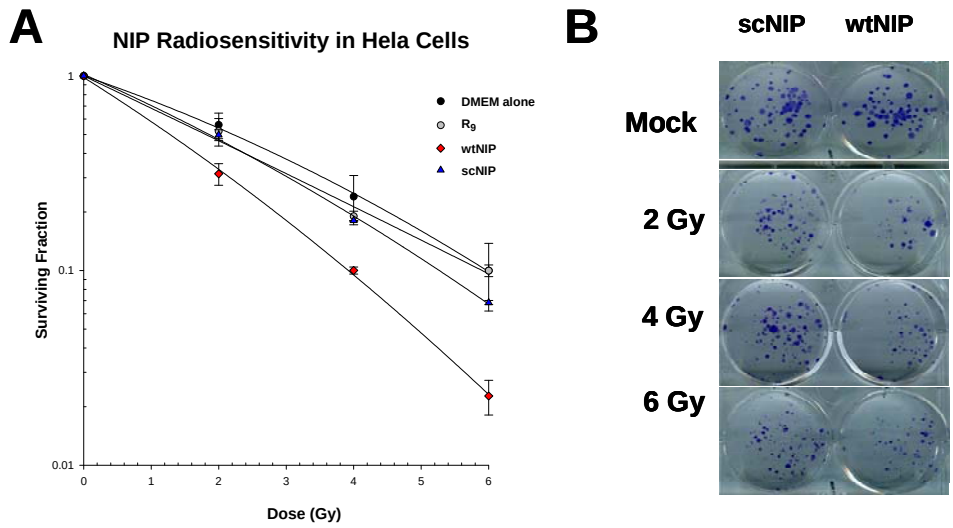

Figure 10. wtNIP increases cellular radiosensitivity. HeLa cells were seeded at limiting dilutions and treated with 10μM R9, wtNIP, or scNIP for one hour prior to irradiation, harvested 10-12 days later and stained with crystal violet. Shown in A is the surviving curve after indicated doses of radiation. Error bars represent +/- 1 SD, graphed are the mean of three independent experiments. ***B.*** Representative plates of the clonogenic assay for NIP mediated radiosensitivity.

potential of our peptides, Hela or DU-145 cells were plated in six-well plates at limiting dilutions, treated with either DMEM or 10μM R_9, wtNIP, or scNIP and mock irradiated or irradiated with 2-6Gy. At 6-8 hr intervals for 24 hrs following irradiation, fresh peptides were added, and colonies allowed to grow for 10-12 days. When HeLa or DU-145 cells were treated with 10μM wtNIP, both cell lines demonstrated a marked increase in radiosensitization with a significant difference observed in wtNIP and DMEM, R_9, or scNIP treated cells (Shown in **Figure 10A** is the surviving curve of HeLa cells, DU-145 data not shown). Radiation survival curves were then characterized based on D_0 to define the NIP's effect on radiosensitivity. D_0 represents the mean lethal dose which kills 63% of the cells and is a measure of the intrinsic radiosensitivity of the cell. The smaller the D_0 value, more radiosensitive the cell line. D_0 values for Hela and DU-145 treated with wtNIP were 1.9 and 2.4 compared to 3.0 and 3.4 for cells treated with scNIP. To better determine the radiosensitizing potential of the peptides in comparison to other small molecule inhibitors, we calculated the sensitizing enhancement ratio (SER) based on the following formula:

$$SER = \frac{\text{Surviving fraction at 2Gy of untreated group}}{\text{Surviving fraction at 2Gy of treated group}}$$

The SER for HeLa cells was 1.8 and for DU-145 was 1.5. These values are comparable to other tested radiosensitizers, including Gemcitabine, 5-Fluorouracil, Pentoxifylline, and Vinorelbine with SERs from 1.1-2.5 (Lawrence et al., 2001;

Robinson et al., 2003; Strunz et al., 2002; Araki et al., 2003). These are also comparable to ATM-specific radiosensitizers listed in Table 1.

To establish the statistical significance of wtNIP-induced radiosensitivity, Student's t-test (Paired 2 sample for means) was incorporated. The data were first fit to each experimental group over a dose range of 0-6Gy. Significant differences ($p<0.05$) in clonogenic survival were observed between cells treated with wtNIP, and those treated with DMEM, R_9, or scNIP. Collectively, these observations provide strong evidence for the radiosensitizing potential of the wtNIP peptide.

Task 2. To investigate the mechanism of peptide inhibition.

A. Mechanism of THM-SMC1 inhibition.

To study if the THM-SMC1 peptide directly inhibits ATM phosphorylation of SMC1, we performed western blot analysis in DU145 prostate cancer cells treated with mock or IR (6Gy) in the absence or presence of caffeine (a non-specific ATM inhibitor) or THM peptides (THM-only, THM-wtSMC1, and THM-mtSMC1). We found that, when cells were treated with THM-wtSMC1 peptides, IR-induced SMC1 phosphorylation was significantly reduced as compared to that of cells treated with THM-only or THM-wtSMC1 (**Figure 11**). This pattern is similar to that induced by caffeine treatment, suggesting that the THM-wtSMC1 can inhibit ATM-mediated SMC1 phosphorylation.

To further test whether the THM-wtSMC1 peptide can inhibit other ATM-dependent pathways, we tested NBS1 and Brca1 phosphorylation, which are also mediated by ATM in response to IR-induced DNA damage. We found that neither NBS1 nor Brca1

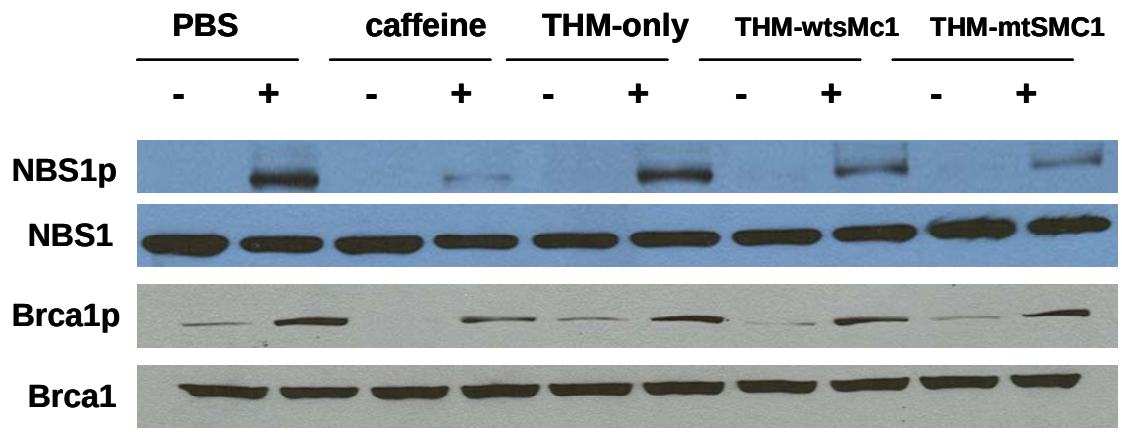

Figure 11. THM-wtSMC1 peptide does not affect IR-induced NBS1 and Brca1 phosphorylation. DU145 were treated either with PBS, Caffeine (10mM), or the THM-fusion peptides (10uM) for 1 hour before 6Gy radiation was delivered. Two hours after radiation, total cell lysates were immunoblotted with indicated antibodies.

phosphorylation was affected by THM-wtSMC1 peptides (**Figure 11**), demonstrating that the THM-wtSMC1 has a specific effect on ATM phosphorylation of SMC1. Since we have previously shown that the THM-SMC1 peptide can abrogate the IR-induced S-phase checkpoint, and that ATM-mediated SMC1, NBS1 and Brca1 phosphorylation is critical for activation of the checkpoint, our data indicated that SMC1 phosphorylation was a downstream event of the pathway.

B. Mechanisms of NBS1-inhibitory peptides.

Co-immnoprecipitation experiments were performed to determine whether R_9-conjugated can inhibit the NBS1-ATM interaction. When wtNIP is present in cells, ATM is no longer detectable in NBS1 immunoprecipitates (**Figure 12**). In contrast, MRE 11 is still present in the NBS1 complex. As a negative control, scNIP does not influence the NBS1-ATM interaction. These observations demonstrate that wtNIP can inhibit NBS1 binding with ATM, but does not disrupt the MRE11-NBS1 binding. Dose response experiments revealed that as low as 100nM wtNIP can inhibit the NBS1-ATM binding (data not shown).

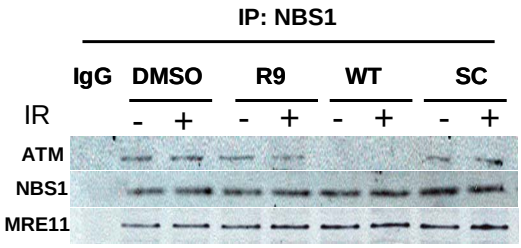

Figure 12. wtNIP inhibit NBS1-ATM binding. HeLa cells treated with the NIP peptides were irradiated (0 or 6Gy). Immune precipitation was performed with a rabbit NBS1 antibody. Immunization blotting was performed with monoclonal anti-ATM, NBS1& MRE11.

Task 3. To study radiosensitizing effects of the peptides on a prostate cancer xenograft model in nude mice.

1. Development of the THM-peptides for in vivo studies.

We first generated a set of fusion peptides containing the CNGRC tumor homing motif (THM) for the mouse studies. These include THM-only, THM-wtSMC1, THM-mtSMC1, THM-wtNIP, and THM-scNIP. The CNGRC sequence was linked to the N-terminus of the peptides. Biotin was labeled at the N-terminus of the fusion peptides in order for *in vivo* detection. Since it was possible that the THM sequence may have an inhibitory effect on NBS1-ATM interaction, we first tested the potential effect in PC-3 cells treated with the peptides/ irradiation (0 or 6Gy). 2 hours after IR, cells were harvested and co-immunoprecipitation experiments were performed using an anti-NBS1 antibody. The immunoprecipitates were then immunoblotted with anti-ATM or anti-NBS1 antibodies. Our data demonstrate that the THM sequence does not interfere with the NBS1-ATM binding (**Figure 13**). Therefore these peptides are suitable to further studies.

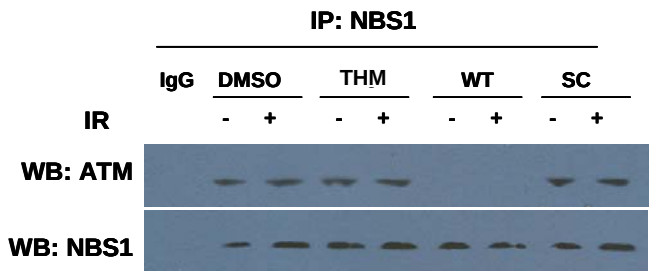

Figure 13. The tumor homing motif (THM) does not affect NBS1-ATM interactions. PC-3 cells treated with the THM fusion NIP peptides were irradiated (0 or 6Gy). Immune precipitation was performed with a rabbit anti-NBS1 antibody. Immunization blotting was performed with monoclonal anti-ATM and NBS1.

2. Establishment of PC-3 xenografts.

The *in vivo* studies were performed in the Department of Cancer Therapeutics at the Drug Development Division of Southern Research Institute. Specific pathogen-free, 4-6 week old male nu/nu (nude) mice were obtained from Harlan, Sprague, Dawley and housed in sterilized filter-topped cages kept in laminar flow isolators. PC-3 tumor cells (2×10^6 per mouse in PBS) were injected s.c. into the flanks of the mice. In all experiments, tumors were allowed to establish and grow before any treatment is initiated.

3. In vivo distribution of the peptides

When tumors reached approximately 100 mm^3, animals were randomized and treated with the THM-only, THM-wtNIP, THM-scNIP, THM-wtSMC1, or THM-mtSMC1 peptides at doses ranging from 0.5-2 mg/kg by one of two routes: intraperitoneal (*ip*) or

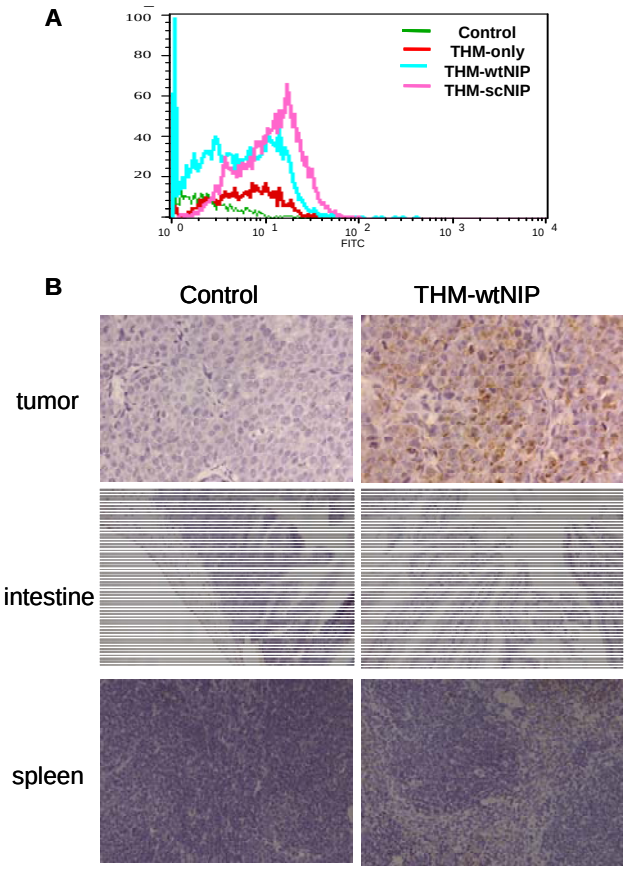

Figure 14. The THM-NIP fusion peptides home to the tumor tissue after *ip* injection. Nude mice bearing PC-3 xenograft were injected (via *ip*) with 200μg of the THM-fusion peptides. 2 hours after injection, the tumor tissue, intestine and spleen were harvested and processed for **(A)** flow cytometry analysis using FITC-conjugated streptavidin or **(B)** immunohistochemistry using the Vectastain ABC system with the DAB peroxidase substrate to visualize peptide localization *in vivo*.

intra-tumoral injection (*it*). 0, 6, 12, or 24 hours after injection, the mice were euthanized, and the tumor tissue and normal tissues surrounding the tumor tissue were obtained. The samples will be assessed by a flow-activated cell sorting (FACS) analysis when stained with an FITC-conjugated streptavidin antibody. These experiments provided information on how fast the peptide could reach the tumor tissue, how long they would remain in the tumors, and whether or not the peptides would also accumulate in normal tissues. Localization of the peptides within tumor tissues was analyzed by dissection of the tumor tissues up to 24 hours after peptide injection. The tumor tissue specimen was stained with FITC-conjugated streptavidin, and immunofluorescence microscopy

performed. We found that the tumor tissues injected with either route of the THM peptides showed at least of 80% of positive staining with an anti-streptavidin antibody, suggesting that the peptides can accumulate in tumor tissues (**Figure 14A**). Immuno-histochemistry experiments to investigate the localization of the THM peptides in tumor and normal tissues reveal that the THM-fusion peptides can specifically accumulated in tumor, but not in normal tissues (spleen and intestine) (**Figure 14B**).

4. Xenograft radiation.

We also performed preliminary experiments in order to obtain a radiation dose range for the xenograft studies. Mice with PC-3 xenografts reached 100 mm^3 were randomized and injected with peptides via *i.t*. Following a short interval to allow peptides to target tumors, mice were irradiated with a Precision X-RAD 320 Irradiation System. The dose rate for the irradiator at a distance of 50cm was 2.8Gy/min, while at a distance of 25 cm is 5.6 Gy/min. During the radiation procedure, mice were briefly (less than 5 minutes) restrained in a Plas Labs (Lansing, MI) clear plastic mouse-restraining device (tube) to allow the tumors to be targeted by radiation. Initial experiments have shown that 15Gy of local radiation was lethal to the majority of the mice, due to radiation damage to intestines. To over come this, we will 1) reduce the radiation dose to 10Gy, 2) shield the intestine area during radiation; and 3) reduce the area to be irradiated.

5. In vivo radiosensitization effect.

With modification of radiation doses and specific shielding techniques, we were able to complete the in vivo experiments testing the radiosensitization effect and normal tissue response using the PC-3 xenograft. Mice were intra-tumoral injected with series tumor homing peptides (THM-only, THM-wtSMC1. THM-mtSMC1, THM-wtNIP, and THM-scNIP) for 30 minutes before xenografts irradiated with 0 or 10 Gy of irradiation. Tumor growth and normal tissue responses were measured. As shown in **Figure 15**, we observed that, unlike in vitro experiments, there were no significant differences on radiation sensitivity in tumors treated with

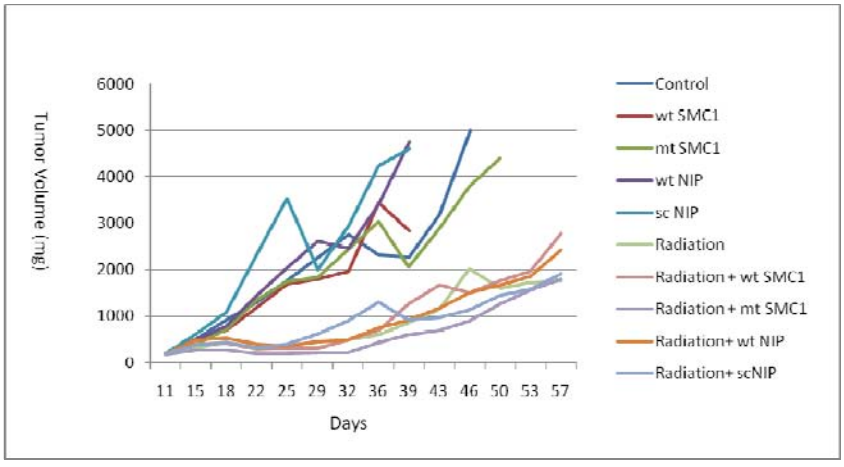

Figure 15. I*n vivo radiosensitization activity of small inhibitory peptides*. PC-3 human prostate cancer fragments were implanted subcutaneously in male athymic mice. Mice treated with indicated peptides clone or combined with radiotherapy at a signal dose of 0 or 10Gy. Tumor volume recorded twice weekly. Shown are medium tumor weights of each group as function of time after implantation

wtSMC1 or wtNIP peptides with that treated with control peptides (THM only, mtSMC1, or scNIP). Several reasons might contribute to the lack of radiosensitization effect: 1) peptide concentrations in xenografts might be critical for radiosensitization. Because of budgetary limitations, we were unable to perform peptide dose-response experiments combined with IR. Therefore it is possible that increasing peptides dosage might result in more robust radiosensitization effect. 2). it is also possible that the peptide half-life might influence radiosensitivity. Since we do not currently have a method to detect peptide half-life in nude mice, it is possible that the peptides might have been degraded when radiation-induced DNA response and DNA repair are occurring, therefore we could not obtain similar effects as shown in vitro studies. It is also noted that peptide treatment did not increase toxicity of radiation (data not shown).

KEY RESEARCH ACCOMPLISHMENTS

1. We have identified two molecular targets that can be explored for radiosensitization for prostate cancer radiotherapy;
2. We have characterized several fusion peptides that specifically target the ATM-mediated DNA damage response pathways;
3. We have elucidated molecular mechanisms of peptide inhibition;
4. An approach for identifying small molecules targeting DNA damage pathways have been enrolled in the NIH Roadmap Initiative for high throughput drug screening.

REPORTABLE OUTCOMES

A. Publications and meeting presentations:

1. Mickael Cariveau, Jessie Tang, Xiaoli Cui, and Bo Xu. Conserved NBS1 C-terminal small peptides can inhibit the ATM-mediated DNA damage response and enhance radiation sensitivity. *Molecular Pharmacology*, AUG 2007, 72 (2): 320-326.
2. Xi Tang, Zhou-guang Hui, Xiao-li Cui, Renu Garg, Michael B. Kastan, and Bo Xu. A novel ATM-dependent pathway regulates Protein Phosphatase 1 and Histone H3 phosphorylation in response to DNA damage. *Molecular Cellular Biology*. April 4, 2008, 2559-2566
3. Stephen L. Gasior, Timothy Wakeman, Bo Xu, Prescott L. Deininger. The human LINE-1 Retrotransposon Creates DNA DSBs*. J Mol Biol.* 2006 Apr 14; 357(5):1383-93.
4. Xi Tang and Bo Xu. Mechanisms governing radiation-induced PP1 activation. Short Talk at the FASEB Summer Research Meeting on Protein Phosphatases, July 13, 2008, Snowmass Village, CO
5. Xi Tang, Zhou-guang Hui, Xiao-li Cui, Renu Garg, Michael B. Kastan and Bo Xu. A novel ATM-dependent pathway regulates Protein Phosphatase 1 in response to DNA damage. Presented in Mini-Symposia, 2008 Annual meeting of American Association for Cancer Research, San Diego, CA, April 15, 2008—the AACR-WICR Brigid G. Leventhal Scholar Award winner.
6. Bo Xu. A novel pathway involving ATM, PP1 and I-2, presented in Gordon Research Conference Radiation Oncology, Feb 1, 2008, Ventura, CA
7. Xi Jessie Tang, Xiao-nan Sun, Renu Garg, and Bo Xu， Regulation of ATM Phosphorylation in the Absence of DNA Damage. Presented in the 98th Annual meeting of American Association for Cancer Research, Los Angeles, CA, April 2007
8. Mickael J. Cariveau, Xiao-Li Cui and Bo Xu， Interfering with NBS1-ATM Interaction to Inhibit the DNA Damage Response and Sensitize Tumor Cells to Radiation. Presented in the 98th Annual meeting of American Association for Cancer Research, Los Angeles, CA, April 2007
9. Stephen Gasior, Timothy Wakeman, Bo Xu, Prescott Deininger, The Human LINE-1 retrotransposon creates DNA double strand breaks. Presented in the

96th Annual meeting of American Association for Cancer Research, Anaheim, CA, April 2005

10. Shannon Callens and Bo Xu. Targeting ATM-SMC1 pathway: a novel approach to developing radiosensitizers for prostate cancer. Presented in the Prostate Cancer Symposium, February 17-19, Orlando, Florida 2005
11. Bo Xu. Development of fusion peptides that can interfere with ATM-mediated DNA damage pathways and increase tumor radiosensitivity. The 3rd International Symposium on Targeted Anticancer Therapies. March 3 to 5, 2005, Amsterdam, the Netherlands.

B. Awards/honors:

1. Bo Xu, The John R. Durant Award for Excellence in Cancer Research (Junior Faculty Category, Third Place), UAB Comprehensive Cancer Center Annual Retreat Competition, 2006
2. Xi Tang, postdoctoral fellow, AACR-WICR Brigid G. Leventhal Scholar Award for the 2008 AACR annual meeting

C. Patent Application:

Bo Xu and Mickael Cariveau: Targeting NBS1-ATM Interaction to Sensitize Cancer Cells to Radiotherapy and Chemotherapy, PCT/US1007/022886, pending, filed on October 30, 2007

D. Degrees obtained:

Timothy P Wakeman, PhD
Xi Tang, PhD
Anand Prakash, MS

E. Funding applied for based on the research:

1. 1R01ES016354-01A2 (Xu) NIEHS/NCI 07/01/09-06/30/13
   Project title: "A Novel Pathway Involving ATM, PP1 and I-2"
   Role on project: Principal Investigator
   Goal: Dissect the signaling pathways regulating PP1 after DNA damage.
   Status: Active

2. 1R21NS061748-01 (Xu), NIH/NINDS, 09/01/2007-08/30/2010
   Project title: "An HTS Assay for Inhibitors of NBS1-ATM Interactions"
   Role on project: Principal Investigator
   Goal: Develop a high throughput assay for identifying small molecules that can disrupt NBS1-ATM interactions.
   Status: Active.

3. 1R01CA133093-01A1 (Xu) NCI 04/01/09-03/31/14
   Project title: “Mechanisms of Mitotic Activation of the ATM Kinase”
   Role on project: Principal Investigator
   Goal: Study the molecular mechanisms of mitotic ATM activation and its role in spindle checkpoint.
   Status: Active

4. RSG-CDD-115003 (Xu) American Cancer Society 04/01/2009-03/31/2013
   Project title: “Targeting NBS1-ATM Interactions to Radiosensitize Tumor Cells”
   Role on project: Principal Investigator
   Goal: Develop novel sensitizers for cancer radiotherapy.
   Status: Pending.
   Note: the proposal was ranked No.1 in the ACS Cancer Drug Discovery study section. It has been approved for funding but placed in the Pay-If category.

F. Invited seminars/lectures:

1. “DNA damage response and human genetic diseases”, Tulane Human Genetics Program, September 28, 2004
2. “ The role of ATM in DNA damage response and aging”, The National Institute of Aging Workshop, September 30, 2004
3. “The mechanisms of the human G2/M Cell Cycle Checkpoints", LSU/Tulane Joint Cancer Center Seminar, December 16, 2004
4. “Sensing DNA double strand breaks”, Tianjin Medical University Cancer Institute, June 3, 2005
5. “Mechanisms of DNA damage-induced cell cycle checkpoints”, Cancer Hospital/Institute, Peking Union Medical College and Chinese Academy of Medical Sciences, June 12, 2005
6. “DNA double strand breaks”, Peking University Health Sciences Center, Cancer Hospital, July 4, 2005
7. “Sensing DNA double strand breaks”, Virginia Commonwealth University and the Massey Cancer Center, July 13, 2005
8. “Roles of ATM and ATR in chromium-induced cell cycle checkpoints”, Workshop on Chromium and Human Health, Portland, ME, August 19, 2005
9. “Regulation of DNA damage-induced cell cycle checkpoints”, Case Western Reserve University, August 25, 2005
10. “Sensing DNA double strand breaks”, Southern Research Institute, November 22, 2005
11. **“**DNA damage response mechanisms and new approaches to developing radiosensitizers” Methodist Hospital Residence Seminar Series, Methodist Hospital Houston, March 2006
12. “Mechanisms of DNA damage induced cell cycle checkpoints”, Nelson Institute of Environmental Sciences, NYU School of Medicine, March 21, 2006

13. “Advances in Modern Molecular Radiobiology” Chinese Association of Radiation Oncology, June 22, 2006, Beijing, China
14. “DNA damage response mechanisms, from fundamental biology to cancer drug discovery”, Georgia State University, Atlanta, GA, December 7, 2007,
15. “Recent Advances in Cancer Drug Discovery”, Samford University School of Pharmacy, March 12, 2008
16. “Modern Molecular Radiobiology: Molecular targeted Radiosensitization”, Department of Radiation Oncology, The Methodist Hospital, Houston, TX. March 19, 2008
17. “DNA damage response mechanisms, from fundamental biology to cancer drug discovery”, The Methodist Hospital Research Institute, Houston, TX. March 20, 2008
18. “Roles of ATM in DNA damage response”, The 2nd International Conference on Frontiers in Biomedical and Environmental Health Sciences: DNA Repair and Cancer Biology. April 16-20, 2008, Hangzhou, China
19. “ATM and Breast Cancer”, Tianjin Medical University Cancer Hospital, April 22, Tianjin , China
20. “DNA damage response and human diseases”, Institute of Biophysics, Chinese Academy of Sciences, Beijing, China, April 23, 2008
21. “Damage response mechanisms and genetic instability”, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China, April 24, 2008
22. “Molecular Radiation Biology”, Tianjin Medical University General Hospital, Tianjin, China, April 25, 2008
23. “Targeting DNA damage responses for cancer drug discovery”, Nantong Biomics, Nantong, China, May 14, 2008
24. “Molecular Cancer Biology and Molecular Targeted Cancer Therapeutics”, Nantong University Medical School, Nantong, China, May 15, 2008
25. “Targeting DNA damage pathways by small inhibitory peptides”, 1st Annual World Congress of ibio2008, Hangzhou, China, May 19, 2008 (also served as session co-chair)
26. “DNA damage response mechanisms: implications for cancer drug development” UAB Comprehensive Cancer Center Experimental Therapeutics Seminar Series, November 22, 2008
27. “Mitotic functions of ATM”, H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL, Dec 8, 2009
28. “ATM-mediated Signaling Pathways”, The Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA, Jan 6, 2009
29. “DNA damage responsive mechanisms: from fundamental biology to cancer drug discovery”, Temple University, Philadelphia, PA, Jan 20, 2009
30. “ATM-mediated pathways: implication in cancer biology and cancer drug discovery” University of Virginia, Charlottesville, VA, May 13, 2009

CONCLUSIONS

During the project performance period, we have generated and characterized several small inhibitory peptides that can interfere with DNA damage-induced signaling pathways mediated by the ATM kinase. We have shown *in vitro* radiosensitization effects of the peptides. We have also generated several fusion peptides that have the tumor-homing ability. However, as compared to *in vitro* data, we have not observed consistent in vivo radiosensitization activities of the fusion peptides. These discrepancies might be due to that peptide concentrations and half-lives *in vivo* are different from the *in vitro* studies. More experiments are required to establish optimal doses for *in vivo* studies. In order to overcome the disadvantages of the peptides, we have also proposed to use the approaches we have developed *in vitro* to screen for small molecules to target DNA damage response pathways.

The completion of this project will have significant impacts on the concept development of molecular targeted radiosensitization, which will significantly improve the prostate cancer treatment outcome. We have showed the proof-of-principal of the specificity and activity of targeting ATM phosphorylation of SMC1 and NBS1-ATM interactions. Future investigations and efforts focusing on identifying small inhibitory molecules should shed lights on developing novel radiosensitizers for prostate cancer radiotherapy.

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Appendix

1. **Publications**
2. **Curriculum Vitae**
3. **Patent application**

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Characterization of an NBS1 C-Terminal Peptide That Can Inhibit Ataxia Telangiectasia Mutated (ATM)-Mediated DNA Damage Responses and Enhance Radiosensitivity[S]

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ABSTRACT

ATM and NBS1, mutation of which lead to the human autosomal recessive diseases ataxia telangiectasia and Nijmegen breakage syndrome (NBS), respectively, are essential elements in the cellular response to DNA damage induced by ionizing radiation (IR). ATM is a member of the phosphatidylinositol 3-kinase family and is activated by IR in an NBS1-dependent manner. The extreme C terminus of NBS1 contains an evolutionarily conserved sequence motif that is critical for binding to and activation of ATM after IR. ATM phosphorylates a series of targets to initiate cell cycle arrest and promote cell survival in response to DNA damage. Therefore, targeting the NBS1-ATM interaction may lead to a novel approach for specific ATM inhibition and radiosensitization. We developed small peptides containing the conserved C-terminal sequence of NBS1 to investigate whether these peptides can interfere with the DNA damage pathway. We found that wild-type NBS1 inhibitory peptides (wtNIP) can abrogate NBS1-ATM association in the presence or absence of IR. We also found that cells exposed to wtNIP displayed a significant reduction in radiation-induced γ-H2AX and NBS1 focus formation compared with cells treated with control peptides, demonstrating that wtNIP possesses a strong inhibitory effect on ATM. The inhibitory effect of wtNIP also leads to a significant decrease in clonogenic survival in response to IR. Furthermore, wtNIP does not radiosensitize cells with defective ATM, suggesting a specific inhibition of ATM. Together , these data provide a proof of principle for the use of NBS1 C-terminal small peptides as specific ATM inhibitors and radiosensitizers.

The DNA damage response is controlled by a concise series of signaling events that result in activation of cell cycle checkpoints, DNA repair, and apoptosis. This network is composed of a number of gene products, which include sensors, transducers, and effectors. DNA double-strand breaks (DSBs) are detected by sensor molecules that trigger the activation of transducing kinases. Transducers then amplify the signals by phosphorylation of effector molecules to regulate the signaling cascades that initiate cell cycle checkpoints, influence DNA repair machinery, or trigger apoptotic pathways. One central element in the network is the *ATM* gene, mutation of which contributes to the human autosomal recessive disorder ataxia-telangiectasia (A-T) (Shiloh, 2003). A-T is characterized by progressive neurodegeneration, variable immunodeficiency, an extremely high predisposition to the development of lymphoid malignancies, and a hypersensitivity to IR. Cells derived from patients with A-T show a variety of abnormalities, including cell cycle checkpoint defects, chromosomal instability, and hypersensitivity in response to IR. *ATM* is remarkable for its large size and the existence of a sequence in its carboxyl terminus similar to phosphatidylinositol 3-kinases. A family of genes, including *Tel1*, *Mec1*, and *Rad3* in yeast, *Mei-41* in *Drosophila melanogaster*, and *ATR* and *DNA-PK* in vertebrates, are similar in size and presence of the carboxyl terminal kinase sequence

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doi:10.1124/mol.107.036681.
[S] The online version of this article (available at http://molpharm.aspetjournals.org) contains supplemental material.

ABBREVIATIONS: DSB, double-strand break; A-T, ataxia-telangiectasia; IR, ionizing radiation; HEAT, huntingtin/elongation factor 3/the 65 kDa α-regulatory subunit of protein phosphatase 2A/yeast PI-3K TOR1; MRN, Mre11-Rad50-NBS1 complex; NBS1, Nijmegan breakage syndrome; ATM, ataxia telangiectasia mutated; DMEM, Dulbecco's modified Eagle's media; FBS, fetal bovine serum; NIP, NBS1 inhibitory peptide; wtNIP, wild-type NBS1 inhibitory peptide; scNIP, scrambled NBS1 inhibitory peptide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; SER, sensitizing enhancement ratio; ATR, ataxia telangiectasia related; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; siRNA, small interfering RNA; LY294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; KU55933, 2-morpholin-4-yl-6 thianthren-1-yl-pyran-4-one.

and are all involved in controlling DNA damage response (Abraham, 2001). The functional domains of the ATM protein include several HEAT repeats that act as scaffolding for assembly of molecular components, a phosphatidylinositol 3-like kinase domain that can phosphorylate serine/threonine followed by glutamine (the S/T-Q consensus sequence), and a FAT carboxyl-terminal domain that may regulate protein activity and stability (Perry and Kleckner, 2003). ATM activation requires functional NBS1 (Cerosaletti and Concannon, 2004; Difilippantonio et al., 2005; Falck et al., 2005; Cerosaletti et al., 2006). Mutations in the *NBS1* gene are responsible for Nijmegen breakage syndrome (NBS), a hereditary disorder that imparts an increased predisposition to development of malignancy and a hypersensitivity to IR (Shiloh, 1997). NBS1 forms a complex with Mre11 and Rad50 to be called the MRN complex. MRN is highly conserved, and it influences each aspect of chromosome break metabolism (Varon et al., 1998). Studies have shown that the MRN complex can detect DNA double-strand breaks and recruit ATM to damaged DNA molecules (Lee and Paull, 2004, 2005). The C terminus motif of NBS1 contains a conserved sequence motif that binds to two of the HEAT repeats (2 and 7) of ATM. This interaction is essential to activate the kinase (Falck et al., 2005).

Because the binding of NBS1 is critical for ATM to be functioning in response to DNA damage, we hypothesized that interfering with the NBS1-ATM interaction may block ATM activation and confer radiosensitization. To test this hypothesis, we developed several small peptides containing the conserved C-terminal sequence motif of NBS1 and fused them to a polyarginine internalization sequence. Herein, we describe the characterization of the C-terminal NBS1 inhibitory peptide in terms of internalization, half-life, cellular cytotoxicity, effects on the DNA damage response, and radiosensitivity. Together, these data may lead to a better understanding of the mechanisms that could be used to increase the radiosensitivity of cancer and provide data that could be rapidly translated into the development of novel radiosensitizing drugs.

Materials and Methods

Cell Culture. Human tumor cell lines HeLa and DU-145 (American Type Culture Collection, Manassas, VA), and human simian virus-40 transformed fibroblast cell line GM9607 (Corriell Cell Repositories, Camden, NJ) were maintained in exponential growth in DMEM/10% FBS, in a 5% CO_2 humidified atmosphere. The glioma cell line M059J (Corriell Cell Repositories) were maintained in exponential growth in RPMI 1640 medium/15% FBS in a 5% CO_2 humidified atmosphere.

Peptide Synthesis. All peptides were synthesized by Abgent (San Diego, CA) and labeled with a biotin tag at their N termini for detection in vitro. Three peptides were produced: 1) one containing the polyarginine (R_9) internalization sequence alone, 2) a wild-type NBS1 inhibitory peptide (wtNIP) corresponding to amino acids 735 to 744 of human NBS1, and 3) a random sequence peptide in which amino acids 735 to 744 of human NBS1 were scrambled (scNIP). The peptides were dissolved in dimethyl sulfoxide, stored at −20°C, and reconstituted in DMEM/10% FBS before use.

Irradiation. An X-RAD 320 Irradiation Cabinet (Precision X-Ray, East Haven, CT) was employed at 320 kV and 160 mA, with a 0.8-mm Sn + 0.25-mm Cu + 1.5-mm Al (half-value layer ≅ 3.7 Cu) filter at a target-to-source distance of 20 cm and a dose rate of 3.4 Gy/min. All irradiations were conducted under normal atmospheric pressure and temperature.

Immunoprecipitation and Western Blotting. For coimmunoprecipitation of ATM, NBS1, and MRE11, cells were lysed for 1 h in ice-cold lysis buffer, which consisted of 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 5 mM Na_3VO_4, 1 mM NaF, and 1 mM phenylmethylsulfonyl fluoride. After centrifugation, supernatants were incubated with antibodies. After extensive washing with the lysis buffer, immunoprecipitates were analyzed by immunoblot using specific antibodies. For Western blotting analysis, samples (cell lysates or immunoprecipitates) were separated on to 2% SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and probed with various antibodies.

Immunofluorescence Microscopy. Exponentially growing cultures of cells were plated on sterile 22-cm^2 coverslips and incubated for 24 h at 37°C in 5% CO_2 humidified air before they were treated with the NIP peptides at room temperature. Coverslips were washed with phosphate-buffered saline and fixed with 4% paraformaldehyde and 0.25% Triton X-100 for 15 min at room temperature, blocked for 30 min at room temperature, and incubated with fluorescein isothiocyanate-conjugated streptavidin or anti-γ H2AX and phospho-NBS1 antibodies (Rockland Immunochemicals, Gilbertsville, PA) for 1 h at room temperature. Coverslips were then mounted with Vectashield Elite (Vector Labs, Burlingame, CA) and observed with a Leica fluorescence microscope. Images were captured at 40× magnification using a Retiga EXi digital camera (QImaging, Surrey, BC, Canada) and analyzed with Image-Pro Plus software (ver. 4.1; Media Cybernetics, Inc., Bethesda, MD).

MTT Assay. For cytotoxicity studies, exponentially growing cultures of HeLa or DU-145 cells were harvested, plated in 96-well plates (5000 cells/well) in complete media, and incubated overnight. On the following day, cells were treated with the NIP peptides (0, 5, 10, 20, 50, or 100 μM) or paclitaxel (Taxol; 0, 10, 50 or 100 μM) as a positive control. At the end of the time course, an MTT cell viability assay (Promega Corp., Madison, WI) was used according to the manufacturer's guidelines to determine peptide cytotoxicity.

Colony Formation Assays. To determine radiosensitivity, the colony-forming assay was incorporated. Cells were harvested with 0.125% trypsin/0.05% EDTA, pelleted, and resuspended in 1 ml of fresh media with a 22-gauge needle to disperse clumps before hemocytometer counting in trypan blue. Cells were then plated at limiting dilutions in six-well plates and allowed to adhere overnight. Cultures were treated with phosphate-buffered saline, R_9, wtNIP, or scNIP for 1 h and irradiated (0–6Gy). Fresh peptides were added every 4 h until 24 h after IR, when the medium was replaced with peptide-free medium. Cultures were incubated for 1 to 2 days, harvested, and stained with 0.5% crystal violet in methanol. Colony number was determined with a dissecting microscope. A population of >50 cells was counted as one colony, and the number of colonies was expressed as a percentage of the value for untreated mock-irradiated control cells. The surviving curves were plotted by linear regression analyses, and the D_0 value represents the radiation dose that leads to 37% of survival. To determine the radiosensitizing potential of the peptides compared with other small-molecule inhibitors, we calculated the sensitizing enhancement ratio (SER) based on the dose of radiation required to reduce survival to 37% in the presence of scNIP or wtNIP. The following formula was used:

$$\text{SER} = \frac{D_0 \text{ for scNIP-treated cells}}{D_0 \text{ for wtNIP-treated cells}}$$

Statistics. To establish statistical significance, Student's t test was incorporated. The data were first fit to each experimental group over a dose range of 0 to 6 Gy. Significant differences were established at $p < 0.05$.

Results

Internalization and Cytotoxicity of the C-Terminal NBS1 Inhibitory Peptides. Previous studies have revealed that the C-terminal NBS1 domain is critical for its binding to ATM, and an NBS1 truncated derivative lacking the C-terminal 20 residues does not associate with ATM in vitro (Cerosaletti and Concannon, 2003, 2004; Falck et al., 2005; Cerosaletti et al., 2006). In addition, it has been shown that expression of an NBS1 transgene lacking the ATM binding domain in NBS cells leads to a dramatic reduction in ATM activation (Difilippantonio et al., 2005). Because inhibiting NBS1 association with ATM leads to suboptimal ATM activation after IR, the NBS1-ATM interaction can be a novel target for developing radiosensitizers. One approach to inhibiting NBS1-ATM interaction would be to use small peptides containing the conserved C-terminal sequence,which will presumably compete with endogenous NBS1-ATM interactions (Fig. 1A). Therefore, we designed peptides containing two functional domains: one an interfering domain that will inhibit the NBS1-ATM association, and the other an internalization domain that will transport the interfering peptides into cells. For the interfering domain, we used the amino acid sequences containing the conserved C-terminal motif of NBS1 as shown in Fig. 1B. This sequence contains the shortest ATM binding motif based on in vitro data (data not shown). For the internalization domain, we used a polyarginine sequence, which has been shown to have a significant efficiency of transporting small peptides and proteins across the plasma membrane (Fuchs and Raines, 2004; Deshayes et al., 2005) Three peptides were generated, including the R_9-alone, and a wtNIP corresponding to amino acids 73to 44 of human NBS1. The third peptide was designed as a negative control, using a random sequence generator to produce a peptide in which amino acids 735 to 744 of NBS1 were scrambled (scNIP). These peptides were labeled with a biotin tag at their N termini for detection in vitro.

We first evaluated the internalization of the fusion peptides. Treatment of HeLa cells with R_9, wtNIP, or scNIP at a concentration of 10 μM for 1 h led to a significant cellular uptake of peptide (Fig. 2). R_9, wtNIP, and scNIP internalization was localized to the cytoplasmic and nuclear compartments, whereas the control group, treated with DMEM alone, shows no fluorescent signal. Because the peptides would be used in radiation studies, we then determined the length of time the peptides remain in cells to ensure that the peptides would be present throughout the DNA repair process after IR. Cells treated with wtNIP or scNIP have significantly decreased fluorescence 8 h after treatment (Supplemental

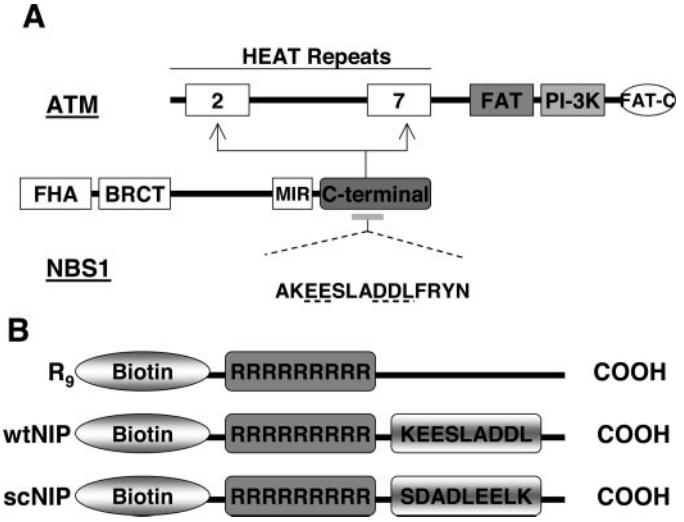

Fig. 1. Development of the NBS1 inhibitory peptides. A, schematic illustration of functional domains of ATM and NBS1 and their interaction. The C terminus of NBS1 is required for ATM activation and recruitment to sites of DNA damage. It consists of at least two sets of amino acid residues, 736 to 737 (EE) and 741 to 742 (DDL), that are evolutionarily conserved and necessary for ATM binding. NBS1 binds to two sets of the HEAT repeats [HEAT repeat 2 (amino acids 248–522) and HEAT repeat 7 (amino acids 1436–1770)] in ATM. B, the amino acid sequences for the R_9, wtNIP, and scNIP peptides developed.

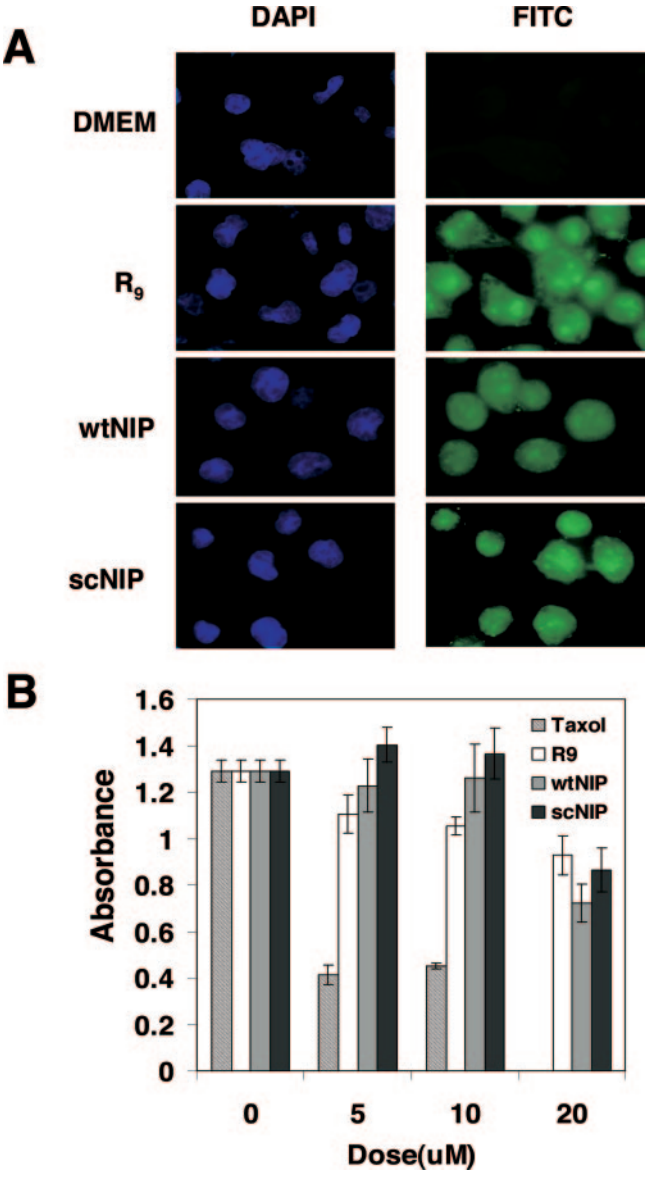

Fig. 2. Peptide internalization and cytotoxicity. A, HeLa cells were treated with 10 μM R_9, wtNIP, or scNIP for 1 h and analyzed by immunofluorescence microscopy after staining with fluorescein-conjugated streptavidin. B, HeLa cells were treated with paclitaxel and the NIP peptides at indicated doses. Twenty-four hours after treatment, cell survival was quantified by a standard MTT assay.

Fig. 1), suggesting that the NIP peptides should be added to cells every 4 to 6 h in the first 24 h after treatment with IR to achieve maximum inhibitory effects.

We then determined in vitro cytotoxicity of R_9, wtNIP and scNIP. HeLa cells grown in 96-well plates were treated with the peptides (0, 5, 10, 20, 50, or 100 μM) or paclitaxel (0, 10, 20, 50, or 100 μM) for 24 h. After treatment, the MTT assay was used to measure the production of solubilized formazan, a metabolic indicator of cell proliferation. The peptides demonstrated no growth inhibitory or cytotoxic effects up to 72 h after treatment (Fig. 2B), when the peptide doses were lower than 20 μM. Based on the cytotoxicity observed in the MTT assay, we chose 10 μM as the working concentration for all subsequent experiments. The effect of 10 μM R_9, wtNIP, and scNIP on clonogenic survival displayed no significant difference between treatment groups ($p < 0.05$) (Data not shown). It is noteworthy that dose and time course experiments have been preformed in several other cell lines, and our data confirmed rapid internalization and minimal cytotoxicity of these peptides (data not shown).

wtNIP Abrogated the NBS1-ATM Interaction. To investigate whether R_9-conjugated NIP peptides could inhibit NBS1-ATM interactions, we performed coimmunoprecipitation experiments in cells treated with the NIP peptides. Four hours after peptide treatment, HeLa cells were harvested and subjected to immunoprecipitation using an anti-NBS1 antibody. The immunoprecipitates were then blotted with anti-ATM, NBS1, and MRE11 antibodies. We observed a normal level of ATM-NBS1 association in R_9-treated cells compared with control cells. However, in wtNIP-treated cells, NBS1 was no longer able to bring down ATM (Fig. 3). Furthermore, the wtNIP affected only the NBS1-ATM interaction and did not interfere with NBS1 binding to MRE11. In contrast, scNIP did not affect the NBS1-ATM interaction. In cells treated with IR, wtNIP showed an effect similar to that in unirradiated cells. These observations demonstrate that wtNIP can abrogate the NBS1-ATM interaction in the absence or the presence of DNA damage.

wtNIP Inhibits IR-Induced γ-H2AX and NBS1 pSer343 Focus Formation. One of the earliest responses to IR-induced DNA damage is the formation of γ-H2AX foci, which requires functional ATM (Burma et al., 2001; Furuta et al., 2003). Because wtNIP showed an inhibitory effect on the NBS1-ATM interaction, we investigated whether IR-induced γ-H2AX focus formation was inhibited by the peptide. Immunofluorescence microscopy was used to detect the presence of γ-H2AX foci in mock-irradiated or irradiated cells in the presence of R_9, wtNIP or scNIP. The average number of γ-H2AX foci/nucleus in HeLa cells significantly increased after IR in cells treated with R_9 (42 foci/nucleus) or scNIP (41 foci/nucleus), whereas cells treated with wtNIP displayed only an average of 6.9 γ-H2AX foci/nucleus, similar to that of mock-irradiated cells (Fig. 4). Similar results were observed in DU-145 cells, whereas R_9 or scNIP exposure did not affect IR-induced focus formation, and wtNIP showed significantly reduced H2AX foci/nucleus (Supplemental Fig. 2). Therefore, IR-induced γ-H2AX focus formation can be inhibited by wtNIP.

To further support the idea that wtNIP can inhibit ATM-mediated DNA damage pathways, we investigated IR-induced NBS1 focus formation, an event considered to be an ATM-dependent process at the sites of DSBs (Lim et al., 2000). NBS1 foci are a result of ATM-mediated NBS1 phosphorylation on serine 343. Using an anti-phospho-Ser343 NBS1 antibody, we observed that NBS1 phosphorylation was significantly inhibited in cells treated with wtNIP compared with those treated with R_9 or scNIP (Fig. 5A and supplemen-

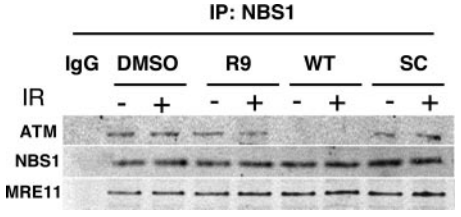

Fig. 3. wtNIP inhibits NBS1-ATM binding. HeLa cells treated with the NIP peptides were irradiated (0 or 6 Gy). Immunoprecipitation was performed with a rabbit NBS1 antibody, and Western blotting was performed with monoclonal antibodies against ATM, NBS1, or MRE11.

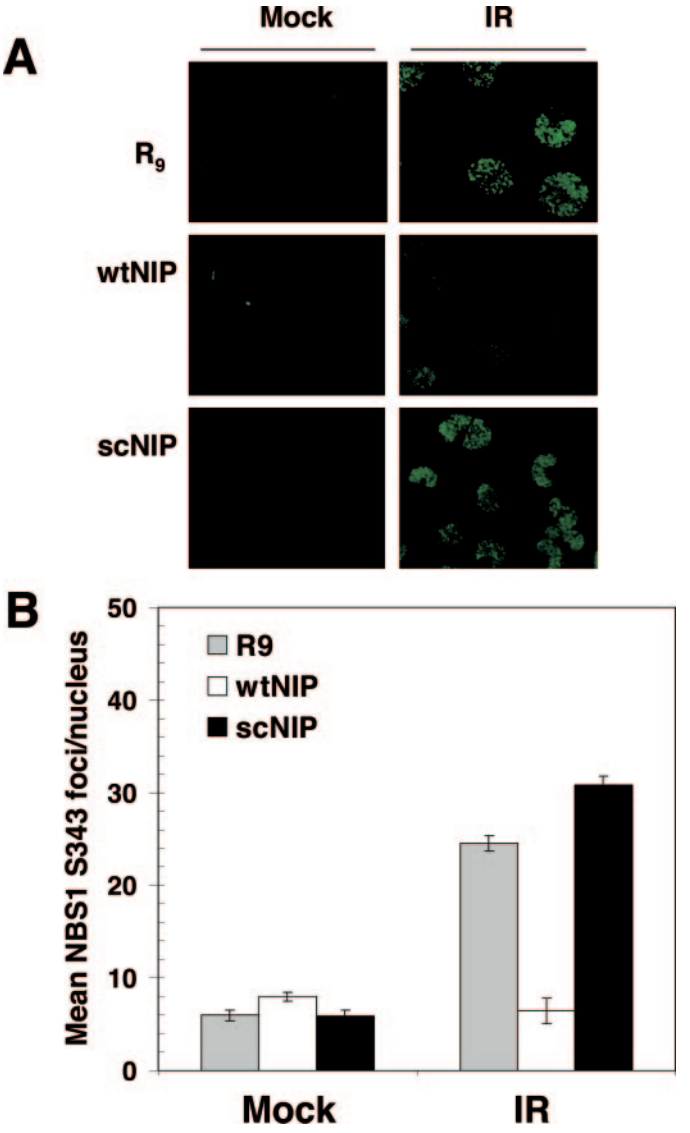

Fig. 4. WtNIP can inhibit γ-H2AX focus formation. A, HeLa cells were treated with 10 μM R_9, wtNIP, or scNIP for 1 h, irradiated with 0 or 6Gy, and harvested 30 min later before immunofluorescence microscopy was employed to detect radiation induced-γ -H2AX foci. B, the mean γ-H2AX nuclear foci per nucleus were determined for each image using Image-Pro Plus 5.1 software and is expressed in arbitrary units. Error bars represent ± 1 S.D.; graphed are the mean of three independent experiments.

tal Fig. 3A). The average number of foci in mock-irradiated HeLa cells was 6, 8, and 6 for R_9, wtNIP, and scNIP, respectively. Cells treated with R_9 or scNIP displayed 25 and 31 foci per nucleus, whereas cells treated with wtNIP showed only 6 foci per nucleus after treatment with 6-Gy IR (Fig. 5B).

It is important to note that there was a low level of background focus formation for both NBS1 and γ-H2AX phosphorylation, which has been correlated to mitosis in normally growing mammalian cell cultures (McManus and Hendzel, 2005).

wtNIP Increases Radiation Sensitivity. We then tested whether exposure to the NIP peptides will increase cellular radiosensitivity using the colony forming assay. Figure 6A depicts the survival curves for HeLa cells treated with R_9, wtNIP, or scNIP over a dose range of 0 to 6 Gy. We found that neither R_9 nor scNIP affects radiosensitivity, whereas wtNIP can significantly decrease IR-induced survival. Radiation survival curves were characterized based on D_0 to define the effect of NIP effect on radiosensitivity. D_0 represents the mean lethal dose required for 37% survival and is a measure of the intrinsic radiosensitivity of the cell. D_0 values for HeLa treated with wtNIP were 1.9 compared with 3.0 for cells treated with scNIP. To establish the statistical significance of wtNIP-induced radiosensitivity, Student's *t* test (paired two-sample for means) was incorporated. The data were first fit to each experimental group over a dose range of 0 to 6 Gy. Significant differences ($p < 0.05$) in clonogenic survival were observed between cells treated with wtNIP and those treated with DMEM, R_9, or scNIP. The SER was 1.58. This is comparable with other tested radiosensitizers, including gemcitabine, 5-fluorouracil, pentoxifylline, vinorelbine, and some ATM-specific radiosensitizers with SERs from 1.1 to 2.5 (Zhang et al., 1998; Lawrence et al., 2001; Robinson and Shewach, 2001; Strunz et al., 2002; Collis et al., 2003; Zhang et al., 2004). These observations have been confirmed in the prostate cancer cell line DU-145 (data not shown) with an SER of 1.46. Taken as a whole , they provide strong evidence for the radiosensitizing potential of the wtNIP peptide.

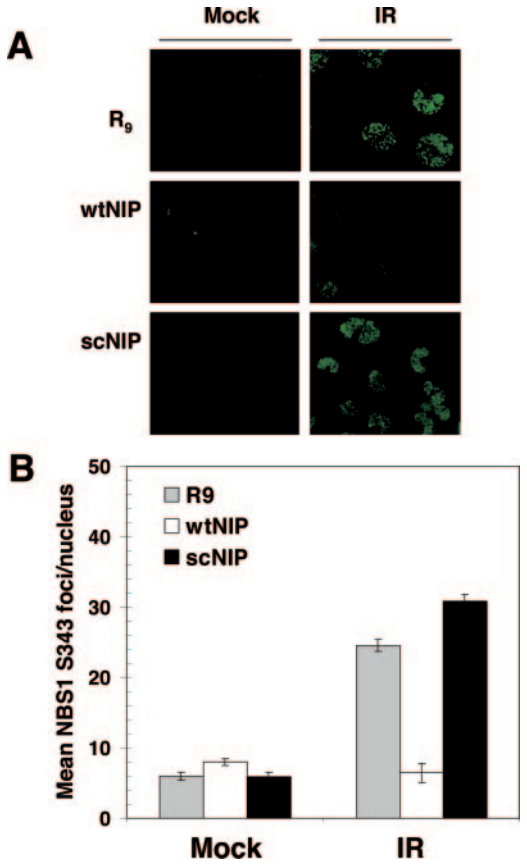

Fig. 5. Exposure to the wtNIP peptide abrogates IR-induced NBS1 phosphorylation. A, HeLa cells were treated with 10 μM R_9, wtNIP, or scNIP for 1 h, irradiated with 0 or 6Gy, and harvested 120 min later before immunofluorescence microscopy was employed to detect radiation induced-NBS1 focus formation using an anti-Ser343 NBS1 antibody. B, the mean number of NBS1 foci per nucleus was determined from a population of at least 25 cells in three independent experiments. Error bars represent ± 1 S.D.; graphed are the mean of three independent experiments.

Because wtNIP contains the conserved ATM binding sequence of NBS1, and this sequence is also conserved in the C terminus of ATR-interacting protein and KU80, the interacting proteins of ATR and DNA-PKcs, respectively, it was possible that it might also inhibit ATR or DNA-PKcs (Abraham, 2001). To test this possibility, we performed colony-forming assays in cell lines with defective ATM (GM9607) or DNA-PKcs (M059J). Although treatment with wtNIP led to an increase in radiosensitivity in M059J cells (Fig. 6C) with an SER of 1.83, GM9607 (Fig. 6D) displayed no change in radiosensitivity. Because GM9607 cells are ATM-deficient and have functional ATR and DNA-PKcs, our observations strongly suggest that wtNIP can specifically target ATM, but not ATR or DNA-PKcs, to achieve radiosensitization.

Discussion

Because ATM is central to cellular responses to irradiation, blocking its activation or activity could make any type of tumor much more sensitive to radiation. Since cloning the gene in 1995, investigators have employed several methods to develop specific ATM inhibitors. These methods include antisense RNA, small interfering RNA (siRNA), and screening of small molecule inhibitors of ATM. Subcloning a full-length cDNA of ATM in the opposite orientation into CB3AR cells significantly increased radiosensitivity (Zhang et al., 1998). The development of siRNA also led to the generation of an siRNA that could inhibit ATM function in prostate cancer cells. Both DU-145 and PC-3 cells, when transfected with these plasmids, exhibited an increase in radiosensitivity at clinically relevant radiation doses (Collis et al., 2003). More recently, the use of high-throughput screening has provided a new generation of ATM inhibitors that can be quickly translated to clinical studies. By screening a combinatorial library of compounds around the DNA-PKcs inhibitor LY294002, Hickson et al. (2004) reported a compound (KU55933) to selectively inhibit the ATM kinase. Their studies have shown a significant increase in radiosensitivity in HeLa cells. However, the in vivo radiosensitization effect and the toxicity of the compounds have not been reported.

Despite these promising findings, one of the major concerns of developing ATM inhibitors is the uncertainty of pleiotropic effects of such inhibitors. Due to the complex effects associated with malfunction of the protein kinase, the outcome of directly targeting ATM kinase activity can be complicated, in that it is unclear whether the only effect of these reagents will be to confer radiosensitization.

Instead of directly inhibiting the ATM kinase activity to

increase radiosensitivity, an alternative approach is to target IR-induced ATM activation, because this will directly lead to an increase in radiosensitivity without interfering with other important functions of ATM in the absence of DNA damage. Because the NBS1-ATM interaction is important for IR-induced activation of ATM, selectively disrupting the signaling pathway would be a novel approach for developing radiosensitizers. Furthermore, because the C-terminal of NBS1 association with ATM is necessary for ATM activation, we reasoned that a small peptide containing a portion of this conserved C-terminal domain (i.e., KEESLADDL) would compete with the NBS1-ATM association in vivo and sensitize tumor cells to radiation. Our data demonstrate that the wild-type NBS1 peptide can be used to inhibit ATM activation and induce radiosensitization.

Because the wtNIP peptide contains the conserved sequence among the phosphatidylinositol 3-kinase interacting proteins, such as ATR-interacting protein and Ku80 (Falck et al., 2005), we further reasoned that wtNIP could possibly interfere with ATR and DNA-PKcs activation. We tested the radiosensitizing effect of the peptides in cells with deficient ATM or DNA-PKcs. If the wtNIP could inhibit ATR or DNA-PKcs, then the ATM-deficient cells should be sensitized. However, the radiosensitivity of GM9607, which lacks ATM but has functional ATR and DNA-PKcs, was not affected by the peptide. In contrast, the DNA-PKcs mutant cells showed an increased radiosensitivity similar to that of HeLa and DU-145 cells treated with wtNIP. These observations therefore demonstrate specific ATM inhibition by the wtNIP peptide.

In summary, we have established a proof of principle in vitro, with results that may lend insight into a novel approach to the development of powerful radiosensitizers for clinical cancer therapy and use the peptides as specific ATM

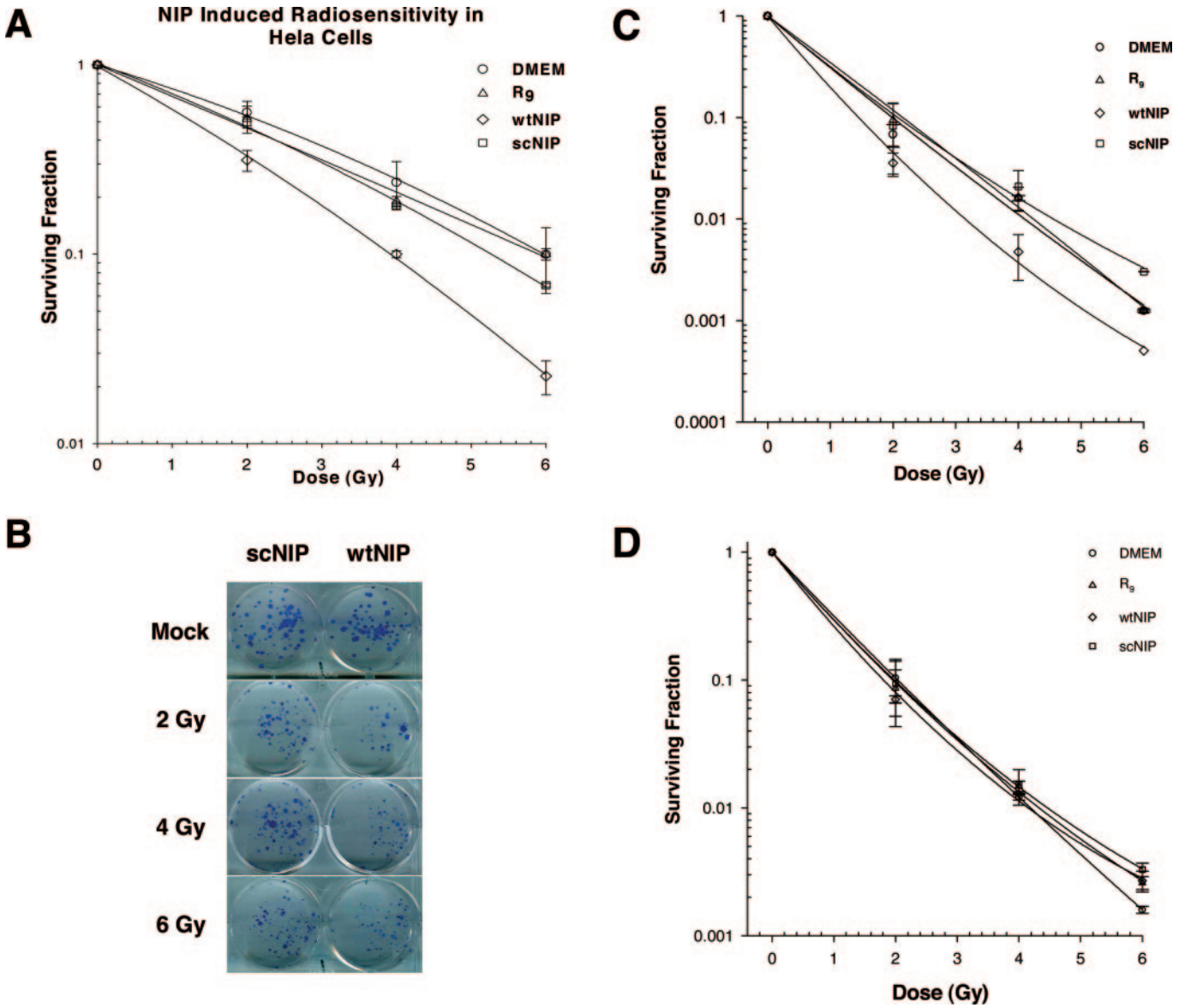

Fig. 6. WtNIP increases cellular radiosensitivity. Cells were seeded at limiting dilutions and treated with 10 μM R_9, wtNIP, or scNIP for 1 h before irradiation, continuously exposed to the peptides for 24 h, harvested 10 to 12 days later, and stained with crystal violet. Shown in A (HeLa), C (MO59J), and D (GM9607) are the surviving curves after indicated doses of radiation. Error bars represent ± 1 S.E.M.; graphed are the mean of three independent experiments. B, representative plates of the clonogenic assay for NIP-mediated radiosensitivity in HeLa cells.

inhibitors for further elucidation of signaling pathways involved in the DNA damage response. However, the use of the polyarginine-mediated NBS1 peptide as a therapeutic agent still faces challenges such as peptide stability, toxicity, tumor specific targeting, and immunogenic effects, etc. Using the current concept to establish an assay for high-throughput screening to identify small molecules that can target the NBS1-ATM interaction will eventually lead to novel radiosensitizers usable for clinical settings. Future studies are also necessary to determine the structure of the NBS1-ATM interaction complex and how wtNIP competes with the interaction.

Acknowledgments

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A Novel ATM-Dependent Pathway Regulates Protein Phosphatase 1 in Response to DNA Damage▽†

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Protein phosphatase 1 (PP1), a major protein phosphatase important for a variety of cellular responses, is activated in response to ionizing irradiation (IR)-induced DNA damage. Here, we report that IR induces the rapid dissociation of PP1 from its regulatory subunit inhibitor-2 (I-2) and that the process requires ataxia-telangiectasia mutated (ATM), a protein kinase central to DNA damage responses. In response to IR, ATM phosphorylates I-2 on serine 43, leading to the dissociation of the PP1–I-2 complex and the activation of PP1. Furthermore, ATM-mediated I-2 phosphorylation results in the inhibition of the Aurora-B kinase, the down-regulation of histone H3 serine 10 phosphorylation, and the activation of the G_2/M checkpoint. Collectively, the results of these studies demonstrate a novel pathway that links ATM, PP1, and I-2 in the cellular response to DNA damage.

Optimal cellular responses to DNA damage are mediated by protein kinases and phosphatases in order to promote survival and limit genetic instability. The *a*taxia-*t*elangiectasia *m*utated kinase, ATM, plays a crucial role in the cellular response to DNA damage. The loss of ATM functions in humans results in progressive neurodegeneration, immunodeficiency, glucose intolerance, sterility, predisposition to lymphoblastoid malignancies, and radiation sensitivity (25). Cellular phenotypes of ATM deficiency include suboptimal activation of radiation-induced cell cycle checkpoints, increased spontaneous and DNA damage-induced chromosomal breakage and gaps, and hypersensitivity to radiation, etc. Human ATM is a 3,056-amino-acid polypeptide which shares several features with other members of the phosphatidylinositol 3-kinase-like kinase family, including a FAT (FRAP, ATR, and TRRAP) domain, a phosphatidylinositol 3-kinase domain, and a FAT carboxyl-terminal domain (14). ATM, like other members of this protein kinase family, phosphorylates its substrates on serines or threonines that are followed by glutamine (the SQ or TQ motif) (12, 22). A number of downstream targets have been identified. These substrates include many tumor suppressors, such as p53, Brca1, and Chk2, and the functional significance of ATM phosphorylation has been studied previously. For example, in response to ionizing radiation (IR), ATM phosphorylates BRCA1 (at Ser 1387) (32), CHK2 (at Thr 68) (7), FANCD2 (at Ser 222) (27), NBS1 (at Ser 278 and Ser 343), and SMC1 (at Ser 957 and Ser 966) (13, 33) to facilitate the S-phase checkpoint. Large-scale proteomic analyses of proteins phosphorylated on the ATM and ATR consensus sites in response to DNA damage have identified more than 900 regulated phosphorylation sites encompassing over 700 proteins. Functional analyses of a subset of this data set have indicated that this list is highly enriched with proteins involved in the DNA damage response (17).

Protein serine/threonine phosphatases, which in humans include protein phosphatase 1 (PP1), PP2A, PP2B, PP4, PP5, PP6, and PP7, function by reversing the phosphorylation of key structural and regulatory proteins (3, 4). In this family, PP2A and PP5 have been reported previously to regulate ATM serine 1981 autophosphorylation after DNA damage (2, 8, 34). It has also been reported previously that PP1 is activated in an ATM-dependent manner in response to DNA damage (9). However, how ATM activates PP1, as well as the physiological function of ATM-mediated PP1 activation in the DNA damage response, remains unknown.

PP1 interacts with its regulatory subunits in controlling the specificity and diversity of the phosphatase function (3). Inhibitor-2 (I-2), a 23-kDa phosphoprotein, is a well-documented PP1 regulatory subunit. I-2 was originally isolated as a heat-stable protein from skeletal muscle extracts that could specifically inhibit PP1 activity (11). PP1 forms a stable and inactive complex with unphosphorylated I-2, and the activation of the complex is accompanied by the phosphorylation of I-2 (21). One established model is that glycogen synthase kinase 3 (GSK-3)-mediated threonine 72 phosphorylation of I-2 promotes a conformational change in the PP1–I-2 complex (1, 16). I-2 can be phosphorylated on other serine sites by casein kinase I (CKI) and CKII, cdc2, and mitogen-activated protein kinases (15, 29). Though phosphorylation by CKII does not alter I-2 activity, it greatly facilitates the subsequent phosphorylation by GSK-3 (3). Previous deletion and mutagenesis studies have demonstrated that the N-terminal domain of I-2 interacts with

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PP1 (23) and can be dephosphorylated by PP1. Despite these findings, it is not known whether the PP1–I-2 complex is involved in DNA damage responses.

To study the mechanism of ATM-mediated PP1 activation, we investigated the IR-induced dissociation of the PP1–I-2 complex. We report here that the activation of PP1 is governed by ATM phosphorylation of I-2 at serine 43 in response to DNA damage and that ATM-mediated PP1 activation leads to the activation of the G_2/M checkpoint through the inhibition of the Aurora-B kinase.

MATERIALS AND METHODS

Cell culture. Human cells lines 293T and HeLa (from the American Type Culture Collection, Manassas, VA), simian virus 40 (SV40)-transformed human fibroblast cell lines GM0637 and GM9607 (from the NIGMS Human Mutant Cell Repository, Camden, NJ), and SV40-transformed fibroblast cell lines PEB-vector and PEB-YZ5 (32) were grown as monolayers in Dulbecco's modified Eagle medium with high glucose levels. The cell culture medium was supplemented with 10% fetal bovine serum and 1% penicillin and 1% streptomycin. Epstein-Barr virus (EBV)-immortalized lymphoblastoid cell lines from healthy persons (GM0536; NIGMS) and from persons who were homozygous for the ATM mutation (GM1526) were cultured in RPMI 1640 supplemented with 15% fetal bovine serum. All cells were maintained in a humid incubator at 5% CO_2 and 37°C.

Irradiation. For irradiation treatments, an X-RAD 320 irradiation cabinet (Precision X-Ray, East Haven, CT) was utilized at 320 kV and 160 mA, with a 0.8-mm Sn, 0.25-mm Cu, 1.5-mm Al (half-value layer at 3.7 mm Cu) filter at a target-source distance of 20 cm and a dose rate of 3.4 Gy/min. All irradiation treatments were conducted under normal atmospheric pressure and at room temperatures.

Antibodies. Rabbit polyclonal antibodies against PP1 and phospho-histone H3 phosphorylated at serine 10 were purchased from Upstate Biotechnology (Temecula, CA). A rabbit polyclonal phospho-histone H1 antibody was obtained from Abcam (Cambridge, MA). A rabbit polyclonal antibody against I-2 was purchased from Calbiochem (San Diego, CA). Mouse anti-Flag antibodies M2 and M5 were obtained from Sigma-Aldrich (St. Louis, MO). The mouse anti-Xpress antibody was purchased from Invitrogen (Carlsbad, CA). The rabbit polyclonal antibody against phospho-Ser 43 of I-2 was generated through Alpha Diagnostic International (San Antonio, TX). Synthetic peptides representing the sequence surrounding serine 43 of I-2 and containing a phosphorylated serine linked with keyhole limpet hemocyanin at the site corresponding to serine 43 were generated. The immunogens were then injected into rabbits, and a polyclonal antibody was generated and purified.

Plasmids. Vectors that expressed glutathione *S*-transferase (GST)-conjugated I-2 and PP1 peptides were made by cloning complementary oligonucleotides that encoded the desired peptides (14 amino acids) into the BamHI-SmaI sites of pGEX-2T (Amersham Pharmacia Biotech, Piscataway, NJ). The QuikChange site-directed mutagenesis kit (Stratagene, Cedar, TX) was used to generate the serine-to-alanine mutant peptide. To construct Xpress-tagged I-2 expression vectors, we amplified the entire I-2 coding region by PCR with the following primers: 5′-CTGCGAGTCTCTGCTGTGCC-3′ and 5′-TGTGAAGAACAAG AAGCAACGTAC-3′. The PCR products were cloned into an Xpress-tagged pCDNA6 vector (Invitrogen, Carlsbad, CA) with the EcoRV restriction site. We then utilized the QuikChange site-directed mutagenesis kit to generate the serine-to-alanine mutant form. The oligonucleotides used for mutation were as follows: 5′-GAGCAAAAAAGCCCAGAAGTGG-3′ and 5′-CCACTTCTGGG CTTTTTTGCTC-3′.

Nuclear and cytoplasmic fractionation. Nuclear and cytoplasmic fractionation was carried out with a nuclear extraction kit (Chemicon, Temecula, CA), which was modified according to our experiments. Cells were collected with trypsinization and rinsed with ice-cold 1× phosphate-buffered saline (PBS) or 1× Tris-buffered saline. Then the sample was centrifuged at 250 × *g* for 5 min at 4°C. Cell pellets were resuspended with 10 cell pellet volumes of ice-cold 1× cytoplasmic lysis buffer containing 0.5 mM dithiothreitol and diluted protease inhibitor. The cell suspension was then centrifuged, and cell pellets were kept for resuspension with 5 volumes of ice-cold 1× cytoplasmic lysis buffer. The resuspended cells were disrupted using a syringe with a small-gauge needle (27 gauge), and the disrupted cell suspension was centrifuged at 8,000 × *g* for 20 min at 4°C. The supernatant contained the cytosolic portion of the cell lysates, while the remaining pellet contained the nuclear portion. The nuclear pellet was resuspended in a volume of ice-cold nuclear extraction buffer corresponding to two original cell pellet volumes and containing 0.5 mM dithiothreitol and diluted protease inhibitor. The nuclei were disrupted using a fresh syringe with a 27-gauge needle, and the nuclear suspension was gently agitated with an orbital shaker at 4°C for 1 h. The nuclear suspension was then centrifuged at 8,000 × *g* for 5 min at 4°C. The supernatant contained the nuclear portion of the cell lysates.

Immunoprecipitation. Cells were irradiated with 0 or 6 Gy, and the cell lysates were prepared as described in the previous section. The supernatants were incubated with anti-Flag M2, anti-Aurora-B, anti-PP1, or anti-Xpress antibodies. After extensive washing with the lysis buffer, immunoprecipitates were used for in vitro kinase assays or in vitro phosphatase assays or were analyzed by immunoblotting.

In vitro kinase assays. In vitro kinase assays for Flag-tagged ATM and Aurora-B were performed as described previously (12, 18). The immunoprecipitates were suspended in 50 μl of kinase buffer containing 10 μCi of [λ-^{32}P]ATP, 1 mM unlabeled ATP, and 1 μg of substrates (GST-conjugated peptides, recombinant I-2, or histone H3). The kinase reaction was conducted at 30°C for 30 min and stopped by the addition of sodium dodecyl sulfate-polyacrylamide gel electrophoresis loading buffer. The kinase assay products were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. The phosphorylation signal was analyzed by autoradiography and quantified by a phosphorimager.

In vitro phosphatase assays. The in vitro phosphatase assays were performed using a Ser/Thr phosphatase assay kit (Upstate). Cytoplasmic, nuclear, or exogenous PP1 was immunoprecipitated, and the PP1 immune complex beads were incubated with a phosphopeptide (KRpTIRR, where p indicates the site of phosphorylation) at room temperature for 30 min. The beads were pelleted, and a 25-μl sample of the supernatant was analyzed for free phosphate in the malachite green assay by dilution with 100 μl of a developing solution (malachite green). After incubation for 15 min, the release of phosphate was quantified by measuring the absorbance at 650 nm in a microtiter plate reader.

Histone H1 and H3 phosphorylation staining. The histone H1 and H3 phosphorylation assay results were assessed as described previously (27, 29). Cells were harvested 90 min after IR, washed with PBS, and fixed in a suspension with 2 ml of 70% ethanol. After fixation, cells were washed twice with PBS, suspended in 1 ml of 0.15% Triton X-100 in PBS, and incubated on ice for 5 min. After centrifugation, the cell pellet was suspended in 100 μl of PBS containing 1% bovine serum albumin (BSA) and 0.75 μg of a polyclonal antibody that specifically recognized the phosphorylated form of histone H3 or H1 (Upstate or Abcam, Cambridge, MA, respectively) and the suspension was incubated for 3 h at room temperature. Then the cells were rinsed with PBS containing 1% BSA and incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted at a ratio of 1:30 in PBS containing 1% BSA. After a 30-min incubation at room temperature in the dark, the cells were washed again, resuspended in a mixture of 25 μg of propidium iodide/ml and 0.1 mg of RNase A (Sigma)/ml in PBS, and incubated at room temperature for 30 min before the fluorescence was measured. Cellular fluorescence was measured by using a Becton Dickinson FACSCalibur flow cytometer-cell sorter.

RESULTS

IR induces ATM-dependent dissociation of the PP1–I-2 complex. In this study, we first confirmed that PP1 was activated in response to IR-induced DNA damage in an ATM-dependent manner (see Fig. S1A, C, and D in the supplemental material). We also found that only the nuclear fraction of PP1 was activated after IR and that the cytoplasmic fraction did not display IR-induced activation (see Fig. S1B in the supplemental material). Since PP1 is negatively regulated by I-2 in a variety of cellular events, it is possible that PP1 activation involves I-2. PP1 and I-2 form a complex to regulate PP1 activity; therefore, we explored the possibility of an alteration of the PP1–I-2 complex in response to DNA damage. The immunoprecipitation of PP1 from unirradiated cells brought down I-2, but following IR, I-2 was no longer detectable in the PP1 immunoprecipitates (Fig. 1A). We then tested the ATM dependency of this dissociation. While GM0637 cells (with functional ATM) had a noticeable dissociation of PP1–I-2 after

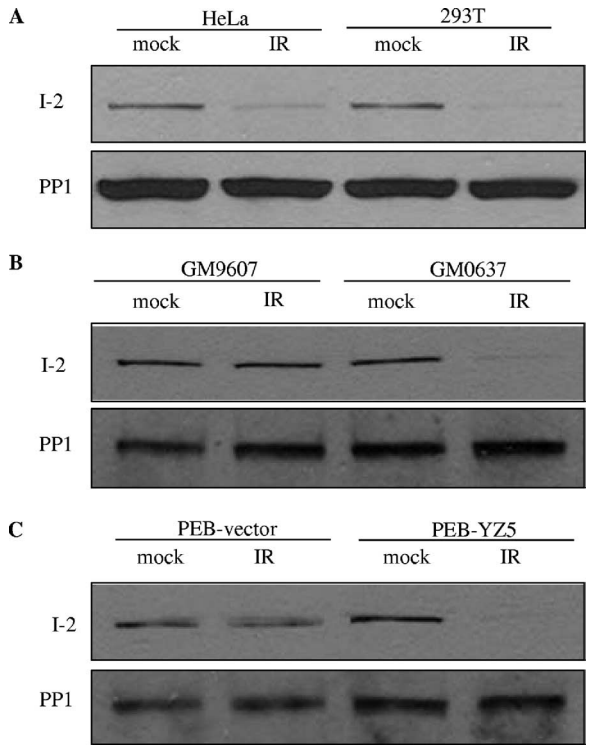

FIG. 1. The IR-induced dissociation of PP1 and I-2 requires functional ATM. Two hours after IR, immunoprecipitation from nuclear extracts was performed using an anti-PP1 antibody and immunoblot analyses were conducted using antibodies against I-2 or PP1 from HeLa and 293T cells (A), fibroblasts proficient (GM0637) or deficient (GM9607) in ATM (B), and isogenic fibroblasts deficient (PEB-vector) or proficient (PEB-YZ5) in ATM (C).

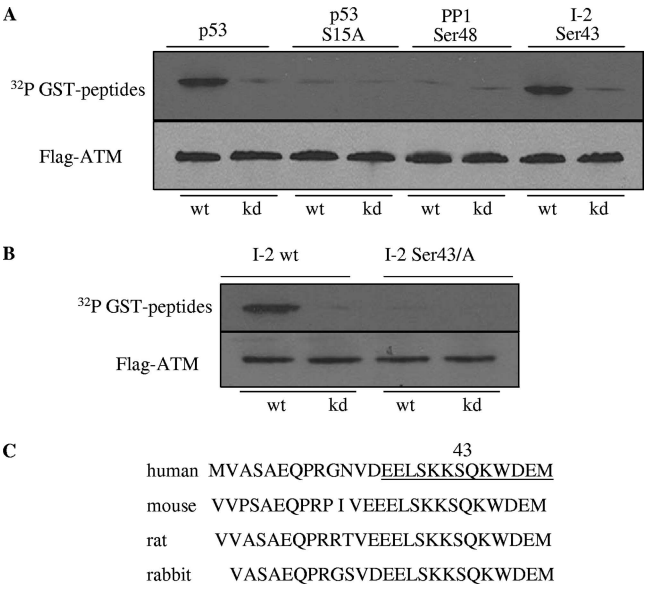

FIG. 2. ATM phosphorylates I-2 at serine 43 in vitro. (A) Immunoprecipitated Flag-tagged wild-type (wt) or kinase-dead (kd) ATM was incubated with recombinant proteins consisting of fusions between GST and peptides derived from various regions of human PP1 or I-2. The positions of the amino acids corresponding to each peptide are indicated at the top. p53 peptides (amino acids 1 to 101 for either the wild-type or the serine 15-to-alanine mutant form) were used as controls. (B) The full-length wild-type or serine 43-to-alanine mutant form of I-2 was used as the substrate for wild-type or kinase-dead ATM for the in vitro kinase assay. (C) Sequence homology of I-2 around serine 43 in different species. Underlining in the sequence from human I-2 indicates a sequence highly conserved among mammalian species.

IR, no detectable IR-induced PP1–I-2 dissociation occurred in GM9607 (ATM-deficient) cells (Fig. 1B). This phenotype was also observed in isogenic cell lines with an ATM deficiency (PEB-vector cells) and with reconstituted ATM (PEB-YZ5 cells) (Fig. 1C), demonstrating that ATM is required for the IR-induced PP1–I-2 dissociation.

ATM phosphorylates I-2 at serine 43 in vitro. Since the phosphorylation of the inhibitory subunit I-2 can activate PP1 (3), we wondered whether the direct phosphorylation of I-2 by ATM might lead to the activation of the phosphatase after IR. It was also possible that ATM could phosphorylate PP1 to activate it directly. To test these possibilities, we examined the ability of ATM to phosphorylate these substrates in vitro. A sequence search found that there was only one putative ATM target site (SQ or TQ) in PP1 (serine 48 and the adjacent glutamine) and one in I-2 (serine 43 and the adjacent glutamine). Recombinant GST fusion peptides containing amino acid sequences with these SQ sites were used as substrates for ATM in an in vitro kinase assay. GST-p53 (amino acids 1 to 101) and GST-p53 (amino acids 1 to 101 of the S15A mutant form) served as positive and negative substrate controls, respectively. A peptide containing serine 43 of I-2 exhibited a strong phosphorylation signal, while the phosphorylation of the PP1 serine 48 peptide was not above the background level (Fig. 2A). These observations suggested that serine 48 of PP1 was not likely to be a direct target of ATM but that serine 43 of I-2 was promising as a target. Progressing on to full-length I-2 protein as a substrate, we found that ATM phosphorylated wild-type I-2 but not I-2 with a serine 43-to-alanine mutation (Fig. 2B). Thus, serine 43 of I-2 appears to be an ATM target in vitro. It is noted that serine 43 and its surrounding residues are highly conserved among mammalian species (Fig. 2C).

ATM phosphorylates I-2 at serine 43 in vivo after IR. To better study the potential phosphorylation of I-2 in vivo, we generated an antibody that specifically recognizes I-2 serine 43 phosphorylation. The specificity of the phosphospecific antibody was demonstrated by its ability to recognize the phosphorylated, but not the unphosphorylated, peptide sequence (see Fig. S2 in the supplemental material). This antibody was used to probe for I-2 serine 43 phosphorylation in cells before and after IR. Several cell lines were tested, including HeLa and 293T cell lines, EBV-transformed lymphoblast cell lines with (GM 0536) and without (GM1526) ATM, and the isogenic cell lines PEB-vector (ATM null) and PEB-YZ5 (ATM complemented). Though total I-2 protein was easily detectable by Western blot analyses, with no notable changes before and after IR, no reactivity with the phosphoserine-specific antibody in unirradiated cells was seen. However, significant reactivity with the anti-phospho-serine 43 antibody in extracts from irradiated cells with functional ATM was observed (Fig. 3). No reactivity with the phosphoserine-specific antibody in ATM-deficient cells after IR was detected. Thus, ATM directly phos-

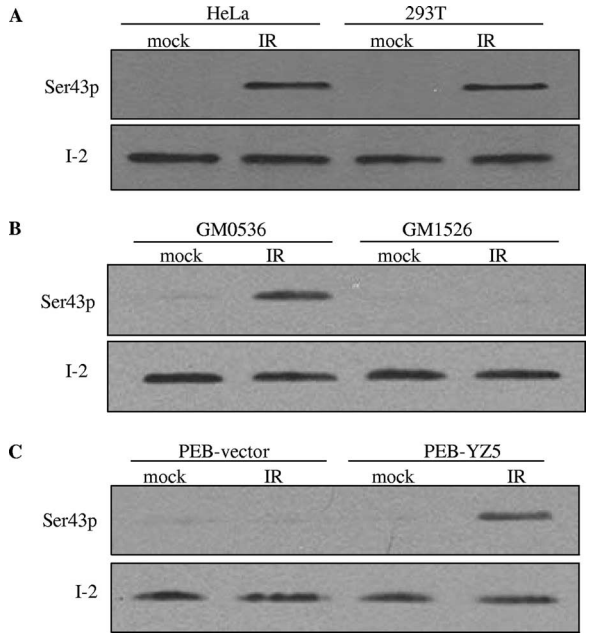

FIG. 3. ATM is required for IR-induced I-2 serine 43 phosphorylation. Shown are Western blot analyses of nuclear extracts from HeLa cells, 293T cells, EBV-transformed lymphoblast cell lines proficient (GM0536) and deficient (GM1526) in ATM, and SV40-transformed human fibroblast isogenic cell lines PEB-vector (ATM deficient) and PEB-YZ5 (expressing reconstituted ATM) with the phospho-serine 43 antibody (Ser43p).

phorylates I-2 at serine 43 in vitro and there is an ATM-dependent phosphorylation of this site in vivo after IR.

ATM phosphorylation of I-2 at serine 43 is required for IR-induced PP1–I-2 dissociation and PP1 activation. To assess the functional significance of the ATM phosphorylation of I-2, we explored the effects of ATM-dependent I-2 phosphorylation on the PP1–I-2 complex. 293T cells were transiently transfected with Xpress-tagged constructs expressing either a wild-type or a serine 43-to-alanine (S43A) mutant form of I-2, with or without IR. The exogenous I-2 was immunoprecipitated, and the immunoprecipitates were probed with the anti-Xpress or anti-PP1 antibodies. While wild-type I-2 brought down PP1 in the absence of IR, Xpress-tagged wild-type I-2 no longer bound to PP1 after DNA damage. In contrast, the S43A mutant form of I-2 remained bound to endogenous PP1 after IR (Fig. 4A). A reciprocal experiment showed that PP1 still associated with S43A mutant I-2 after IR (Fig. 4B). These observations demonstrate that ATM-mediated I-2 phosphorylation on serine 43 is required for the dissociation process of the PP1–I-2 complex in response to IR-induced DNA damage.

We then investigated whether serine 43 phosphorylation of I-2 was required for IR-induced PP1 activation. We transfected 293T cells with wild-type or S43A mutant I-2 and assessed potential dominant-inhibitory effects on PP1 activity. 293T cells transiently transfected with either vector only or vectors expressing wild-type I-2 exhibited normal IR-induced PP1 activation. In contrast, cells transfected with vectors expressing the S43A form of I-2 exhibited substantially impaired PP1 activation after IR (Fig. 4C). Thus, S43A mutant I-2 has dominant-inhibitory effects on endogenous PP1 activation after IR, demonstrating that ATM-mediated I-2 phosphorylation is required for IR-induced PP1 activation.

To further study in vivo activation of PP1 toward its substrates, we assessed the dephosphorylation of histone H1, a process mediated by PP1 (24). A flow cytometry-based assay was employed to measure H1 phosphorylation in the absence or presence of DNA damage. We found that H1 phosphorylation was significantly reduced after IR. Expressing vector only or wild-type I-2 did not alter the IR-induced inhibition of H1 phosphorylation, while expressing S43A mutant I-2 abolished the process (see Fig. S3 in the supplemental material). Therefore, our data demonstrate that in vivo PP1 activity is enhanced by ATM-mediated phosphorylation of I-2 after DNA damage.

ATM phosphorylation of I-2 is critical for IR-induced PP1 threonine 320 dephosphorylation. It was shown previously that nuclear PP1 contains the consensus sequence for phosphorylation by Cdk2 (9). PP1 threonine 320 phosphorylation inactivates PP1, and phosphorylation is attenuated after DNA damage in an ATM-dependent manner. Whether ATM-mediated I-2 phosphorylation interacts with the ATM-dependent inhibition of PP1 threonine 320 phosphorylation is not clear. To test this possibility, we performed experiments with 293T cells expressing vector only or wild-type or S43A mutant I-2 to investigate the change in threonine 320 phosphorylation after IR. We found that the S43A mutation can abolish IR-induced threonine 320 dephosphorylation (Fig. 5), suggesting that ATM-mediated I-2 serine 43 phosphorylation may function as an upstream cascade of the signaling pathway.

ATM phosphorylation of I-2 at serine 43 is required for the IR-induced G_2/M checkpoint. Previously, we reported that the ATM-dependent G_2/M checkpoint is correlated with the down-regulation of histone H3 serine 10 phosphorylation (30, 31). However, how ATM links to H3 serine 10 phosphorylation was not known. Since PP1 is considered to be the major phosphatase required for the down-regulation of serine 10 phosphorylation (10) and the activation of PP1 is associated with the down-regulation of H3 serine 10 phosphorylation (see Fig. S4 in the supplemental material), it seemed likely that ATM-mediated I-2 serine 43 phosphorylation after IR would be important for the inhibition of H3 serine 10 phosphorylation in response to IR. To test this hypothesis, we transfected HeLa cells with the wild-type or the S43A mutant form of I-2 and performed a flow cytometry analysis using an anti-phospho-H3 serine 10 antibody. We found that cells expressing vector only or wild-type I-2 showed robust down-regulation of H3 phosphorylation and activation of the G_2/M checkpoint after IR. However, cells expressing the dominant negative form of I-2 (the S43A mutant form) displayed no significant reduction in histone H3 phosphorylation in response to IR (Fig. 6A). These observations demonstrate a role for ATM-dependent I-2 serine 43 phosphorylation in the regulation of histone H3 serine 10 phosphorylation and the G_2/M checkpoint in response to DNA damage.

We also explored whether PP1 activation regulated the Aurora-B kinase, the kinase essential for H3 serine 10 phosphorylation. It was shown previously that Aurora-B was inhibited after IR-induced DNA damage (18). We found that IR-induced Aurora-B inhibition required functional ATM (Fig.

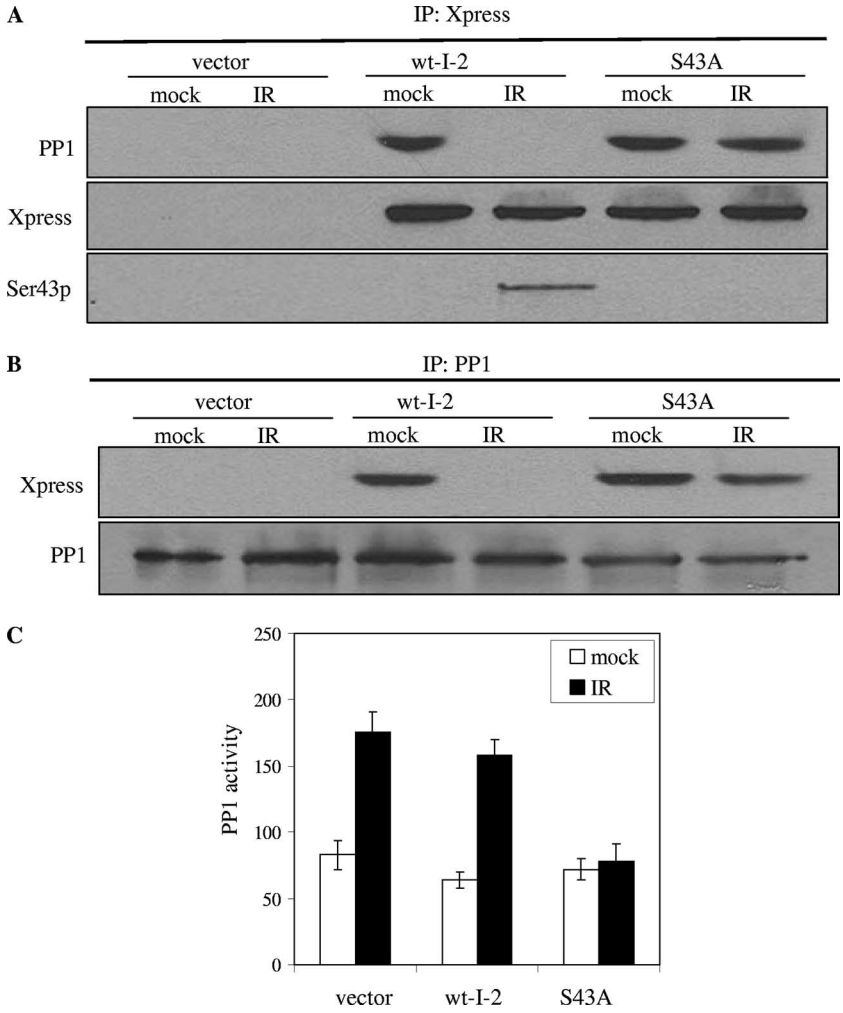

FIG. 4. ATM phosphorylation of I-2 at serine 43 is required for the dissociation of the PP1–I-2 complex and the activation of PP1. 293T cells were transfected with an empty vector or the Xpress-tagged wild-type or S43A mutant form of I-2 and mock treated (0 Gy) or treated with IR (6 Gy). (A) The exogenous I-2 was immunoprecipitated with an anti-Xpress antibody, and the immunoprecipitates (IP) were probed with an anti-PP1 or anti-Xpress antibody. wt-I-2, wild-type I-2; Ser43p, phospho-serine 43 antibody. (B) Endogenous PP1 was immunoprecipitated with anti-PP1, and the immunoprecipitates were probed with anti-PP1 or anti-Xpress antibodies. (C) Endogenous PP1 was immunoprecipitated and subjected to in vitro phosphatase assays. Error bars represent ±1 standard deviation, and the means of results from three independent experiments are graphed.

6B). Further, cells expressing the S43A mutant form of I-2 did not show the inhibition of Aurora-B compared to that in the appropriate controls (Fig. 6C). These observations demonstrate that ATM-mediated I-2 phosphorylation and PP1 activity can inhibit Aurora-B kinase activity, thereby causing reduced H3 phosphorylation and activation of the G_2/M checkpoint.

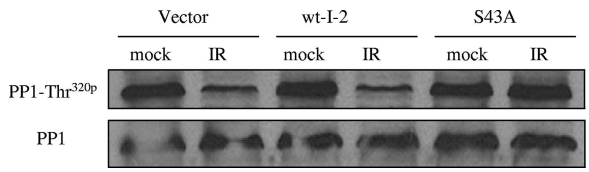

FIG. 5. ATM phosphorylation of I-2 at serine 43 is required for the dephosphorylation of PP1 threonine 320 in response to DNA damage. 293T cells were transiently transfected with an empty vector, Xpress-tagged wild-type I-2 (wt-I-2), or S43A mutant I-2 and treated without or with IR (6 Gy). Nuclear extracts were subjected to immunoblotting using anti-phospho-threonine 320 (PP1-Thr^{320p}) or anti-PP1 antibody.

DISCUSSION

Dissecting ATM-mediated signaling pathways in the cellular response to DNA damage can provide important insights into how the loss of ATM function causes such a devastating disease, ataxia-telangiectasia (A-T), in humans. Upon DNA damage, ATM binds strongly to damaged sites and its kinase activity is enhanced. Activated ATM in turn phosphorylates a list of substrates in pathways that together ensure cellular survival and recovery. A number of ATM-mediated signaling pathways have been revealed, and the functional significance of these pathways has been studied extensively. However, due to the complexity of the A-T phenotypes, detailed mechanisms on

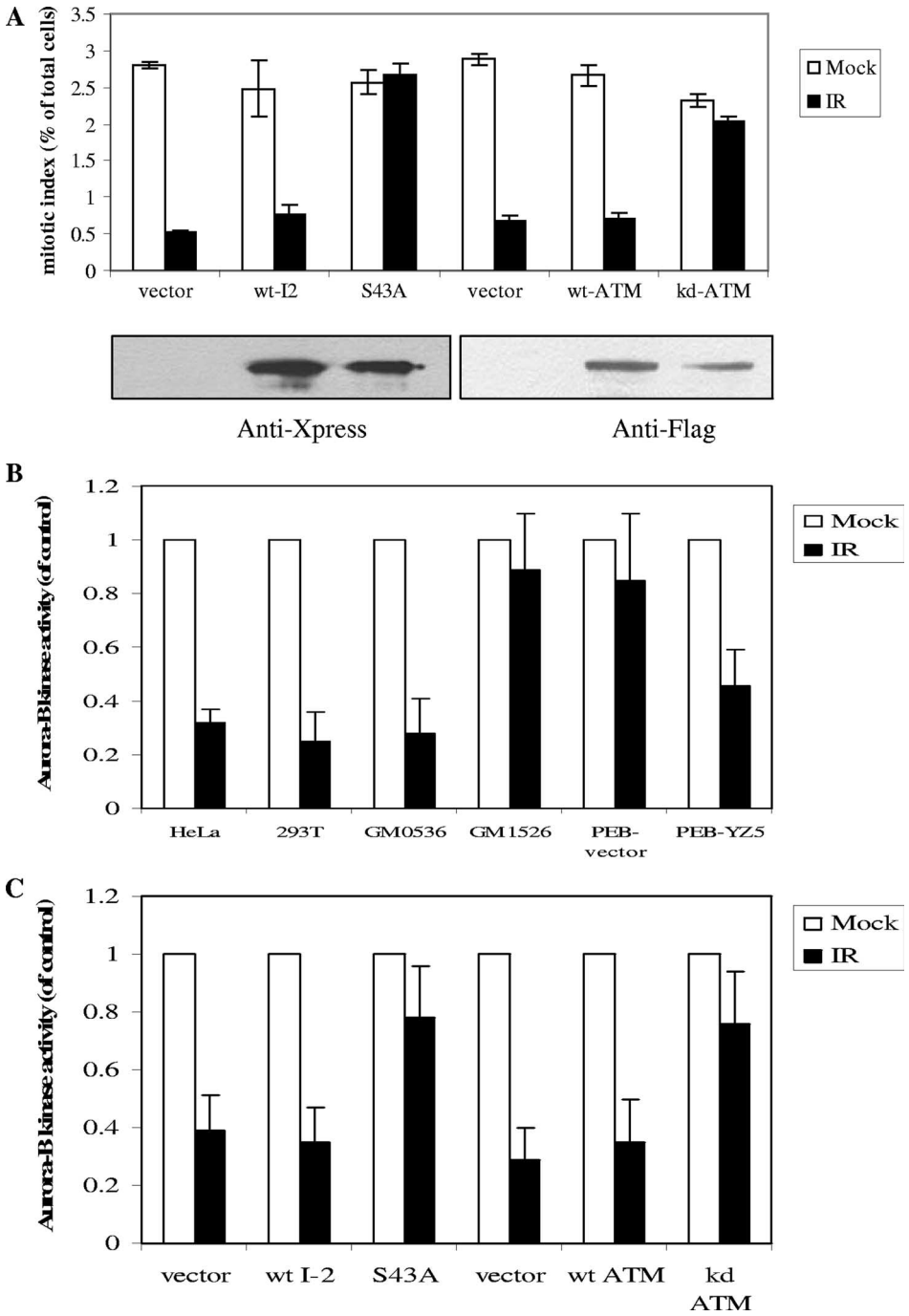

FIG. 6. ATM phosphorylation of I-2 is required for the activation of the G_2/M checkpoint and the inhibition of Aurora-B in response to IR-induced DNA damage. (A) HeLa cells were transfected with an empty vector, Xpress-tagged wild-type I-2 (wt-I2), the S43A mutant form of I-2 (S43A), wild-type ATM (wt-ATM), or kinase-dead ATM (kd-ATM) and treated without IR or with IR (6 Gy). Ninety minutes after IR, cells were harvested and subjected to the flow cytometry-based phospho-histone H3 staining assay. Error bars represent ±1 standard deviation, and the means of results from three independent experiments are graphed. Shown under the bar graph are the Western blot results demonstrating the expression patterns of the exogenous proteins. (B) Cells were treated without or with IR (6 Gy), and Aurora-B was immunoprecipitated and subjected to in vitro kinase assays using histone H3 as the substrate. Phosphorylation signals were quantified by a phosphorimager. (C) HeLa cells were transfected with an empty vector, the Xpress-tagged wild-type or S43A mutant form of I-2, or wild-type or kinase-dead ATM and treated without IR or with IR (6 Gy). The Aurora-B kinase assays were performed 90 min after IR. In panels B and C, values for activity levels are shown relative to the activity level of the control, which was set at 1.

how the loss of ATM leads to a variety of A-T phenotypes remains to be further explored. In this report, we highlight a novel signaling pathway that involves ATM, PP1, and I-2. We demonstrate that I-2 is a substrate of ATM and that ATM phosphorylation of I-2 at serine 43 is required for the activation of PP1 in response to DNA damage.

Previously, we reported a rapid and ATM-dependent G_2/M checkpoint that correlates with the down-regulation of histone

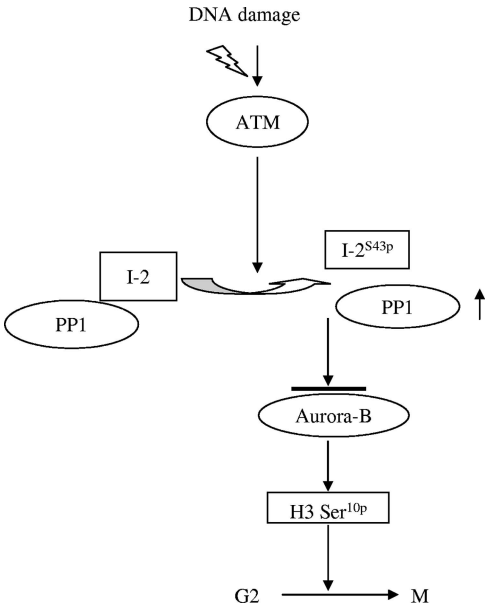

FIG. 7. Proposed role of ATM-mediated I-2 phosphorylation in the activation of PP1 and the signaling cascade in G_2/M checkpoint regulation in response to IR-induced DNA damage. I-2^{S43p}, I-2 phosphorylated at serine 43; H3 Ser^{10p}, H3 serine 10 phosphorylation.

H3 serine 10 phosphorylation in response to IR (30). However, how ATM links to regulators of histone H3 serine 10 phosphorylation was not known. It was reported previously that the enzymatic activities of PP1 are activated in response to IR in an ATM-dependent manner (9) and that Cdk2-mediated PP1 threonine 320 phosphorylation is attenuated after DNA damage. However, a detailed mechanism of ATM-mediated PP1 activation in response to DNA damage remained unknown. Starting with investigations of IR-induced PP1 activity, we found that IR induced an ATM-dependent dissociation of the PP1–I-2 complex. Further studies showed that ATM phosphorylated I-2 on serine 43 and that this phosphorylation led to the dissociation of the complex and the activation of PP1. This effect, in turn, resulted in the inhibition of Aurora-B, the down-regulation of histone H3 serine 10 phosphorylation, and the activation of the G_2/M checkpoint (Fig. 7).

Our data also demonstrate that ATM-mediated I-2 phosphorylation is an essential step for the attenuation of IR-induced threonine 320 phosphorylation. One possible explanation is that, after the dissociation of the PP1–I-2 complex, PP1 initiates autodephosphorylation which eventually activates the phosphatase. More-detailed investigations are required to determine whether threonine 320 dephosphorylation may also play a role in ATM-mediated I-2 phosphorylation and PP1–I-2 dissociation.

PP1 activity is also controlled by other regulatory subunits, such as I-1, NIPP1, and DARPP32 (21). Whether these regulators are involved in the DNA damage response and whether they dissociate from PP1 are not known. It is reasonable to suspect that some inhibitors are also involved in regulating PP1 activity in response to DNA damage. For example, I-1 has been shown previously to regulate cell growth and has been linked to PP1 in the G_1 cell cycle control (21).

Histone H3 serine 10 phosphorylation is critical for chromosome condensation and segregation, and it has been used previously as a mitotic marker for studying the activation of the G_2/M checkpoint. Our data demonstrate that the activation of PP1 governed by ATM phosphorylation of I-2 leads to the down-regulation of H3 serine 10 phosphorylation. We also found that activated PP1 leads to the inhibition of the Aurora-B kinase. Therefore, PP1 may prevent H3 phosphorylation to delay the transition from G_2 to M, thereby activating the G_2/M checkpoint. However, it is also possible that activated PP1 may directly dephosphorylate the phosphorylated H3 when cells are already in the M phase. Therefore, PP1 and I-2 serine 43 phosphorylation may also have a role to facilitate the mitotic exit. The latter scenario is supported by the evidence that yeast PP1 homolog Dis2 can down-regulate Chk1 activity for a checkpoint release (6). Therefore, the detailed mechanisms of ATM-mediated PP1 activation in the regulation of histone H3 serine 10 phosphorylation remain to be further investigated.

The functional significance of ATM-mediated phosphorylation of I-2 and activation of PP1 activity may extend beyond the roles of these processes in histone H3 modification and cell cycle checkpoint regulation. Since PP1 is a major eukaryotic protein serine/threonine phosphatase that regulates a variety of cellular functions, the regulation of PP1 through ATM phosphorylation of I-2 may have a significant impact on many cellular responses to DNA damage. Dephosphorylation by phosphatases can turn signals off or regulate the degradation of phosphorylated substrates, thus balancing the physiological effects of kinases (19).

One of the known physiological roles of I-2 is to control sperm motility (28). A testis-specific isoform of PP1 forms an inactive complex with I-2, and GSK-3-mediated I-2 phosphorylation which activates the PP1–I-2 complex results in an increase in the PP1 activity seen in nonmotile immature sperm. The exposure of the immature sperm to phosphatase inhibitors, such as okadaic acid and calyculin A, induces motility, suggesting that I-2 inhibits PP1 activity in mature mammalian sperm cells to facilitate their motility. The PP1–I-2 complex is also involved in insulin signaling (5, 20). These observations are particularly interesting since both A-T patients and A-T mice are sterile and have glucose intolerance and insulin resistance (26), suggesting a physiologically important link between ATM and PP1–I-2. Indeed, we have observed that ATM phosphorylates I-2 at serine 43 in response to insulin stimulation (unpublished data). The establishment of a serine 43 phosphorylation mutant knock-in mouse model to study the physiological significance of ATM-mediated I-2 phosphorylation is under way.

In summary, our data provide mechanistic insights into the activation process of PP1 in DNA damage response pathways in mammalian cells. The results of these studies also provide a foundation for future studies of the ATM–PP1–I-2 pathway in regulating cellular responses to stress.

ACKNOWLEDGMENTS

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14. **Kurz, E. U., and S. P. Lees-Miller.** 2004. DNA damage-induced activation of ATM and ATM-dependent signaling pathways. DNA Repair (Amsterdam) **3:**889–900.
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16. **MacKintosh, C., A. J. Garton, A. McDonnell, D. Barford, P. T. Cohen, N. K. Tonks, and P. Cohen.** 1996. Further evidence that inhibitor-2 acts like a chaperone to fold PP1 into its native conformation. FEBS Lett. **397:**235–238.
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19. **Moorhead, G. B., L. Trinkle-Mulcahy, and A. Ulke-Lemee.** 2007. Emerging roles of nuclear protein phosphatases. Nat. Rev. Mol. Cell Biol. **8:**234–244.
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24. **Paulson, J. R., J. S. Patzlaff, and A. J. Vallis.** 1996. Evidence that the endogenous histone H1 phosphatase in HeLa mitotic chromosomes is protein phosphatase 1, not protein phosphatase 2A. J. Cell Sci. **109:**1437–1447.
25. **Shiloh, Y., and M. B. Kastan.** 2001. ATM: genome stability, neuronal development, and cancer cross paths. Adv. Cancer Res. **83:**209–254.
26. **Shiloh, Y.** 2006. The ATM-mediated DNA-damage response: taking shape. Trends Biochem. Sci. **31:**402–410.
27. **Taniguchi, T., I. Garcia-Higuera, B. Xu, P. R. Andreassen, R. C. Gregory, S. T. Kim, W. S. Lane, M. B. Kastan, and A. D. D'Andrea.** 2002. Convergence of the fanconi anemia and ataxia telangiectasia signaling pathways. Cell **109:**459–472.
28. **Vijayaraghavan, S., D. T. Stephens, K. Trautman, G. D. Smith, B. Khatra, E. F. da Cruz e Silva, and P. Greengard.** 1996. Sperm motility development in the epididymis is associated with decreased glycogen synthase kinase-3 and protein phosphatase 1 activity. Biol. Reprod. **54:**709–718.
29. **Wang, H., and D. L. Brautigan.** 2002. A novel transmembrane Ser/Thr kinase complexes with protein phosphatase-1 and inhibitor-2. J. Biol. Chem. **277:**49605–49612.
30. **Xu, B., S.-T. Kim, and M. B. Kastan.** 2001. Involvement of Brca1 in S-phase and G_2-phase checkpoints after ionizing irradiation. Mol. Cell. Biol. **21:**3445–3450.
31. **Xu, B., S. T. Kim, D. S. Lim, and M. B. Kastan.** 2002. Two molecularly distinct G(2)/M checkpoints are induced by ionizing irradiation. Mol. Cell. Biol. **22:**1049–1059.
32. **Xu, B., A. H. O'Donnell, S. T. Kim, and M. B. Kastan.** 2002. Phosphorylation of serine 1387 in Brca1 is specifically required for the Atm-mediated S-phase checkpoint after ionizing irradiation. Cancer Res. **62:**4588–4591.
33. **Yazdi, P. T., Y. Wang, S. Zhao, N. Patel, E. Y. Lee, and J. Qin.** 2002. SMC1 is a downstream effector in the ATM/NBS1 branch of the human S-phase checkpoint. Genes Dev. **16:**571–582.
34. **Zhang, J., S. Bao, R. Furumai, K. S. Kucera, A. Ali, N. M. Dean, and X. F. Wang.** 2005. Protein phosphatase 5 is required for ATR-mediated checkpoint activation. Mol. Cell. Biol. **25:**9910–9919.

Bo Xu, M.D., Ph.D.
Curriculum Vitae

---CURRENT POSITION:

Principal Investigator and Head,
Laboratory of Molecular Radiobiology
Department of Biochemistry and Molecular Biology
Southern Research Institute
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---EDUCATION:

M.D. (M.B)	1985-1990	Norman Bethune University of Medical Sciences, Changchun, China, (Major: Medicine)
Ph.D.	1995-1998	Peking Union Medical College, Beijing, China, (Major: Radiation Oncology) Advisor: Prof. Weibo Yin
Postdoctoral Training	1998-2002	St Jude Children's Research Hospital, Memphis, TN Advisor: Michael B. Kastan MD, PhD

---PROFESSIONAL APPOINTMENTS:

1990-1995 Radiation Oncology Resident and Chief Resident, Department of Radiation Oncology, Tianjin Medical University General Hospital, Tianjin 300052, China

1995-1998 Chief Resident, Department of Radiation Oncology, Cancer Hospital/Institute, Chinese Academy of Sciences and Peking Union Medical College, Beijing, China

Oct. 1998- Aug. 2002: Postdoctoral Research Associate, Department of Hematology and Oncology, St. Jude Children's Research Hospital, Memphis, TN 38105

Aug. 2002- May 2006: Assistant Professor (tenure-track), Department of Genetics, Louisiana State University Health Sciences Center, New Orleans, LA 70112

May 2006-present: Principal Investigator and Head, Laboratory of Molecular Radiobiology, Southern Research Institute, Birmingham, AL 35205

Adjunct Faculty (Associate Professor), Department of Biochemistry and Molecular Genetics, and Department of Pharmacology and Toxicology, University of Alabama at Birmingham, Birmingham, AL 35205

February 2007- present: Member, UAB Comprehensive Cancer Center, Birmingham, AL 35205

---PROFESSIONAL SOCIETY MEMBERSHIPS:

Active member of the *American Association for Cancer Research* (AACR)
Member of the *American Society of Cell Biology (ASCB)*
Member of the *American Association for the Advancement of Science (AAAS)*

---HONORS/AWARDS:

1. The John R. Durant Award for Excellence in Cancer Research (Junior Faculty Category, Third Place), UAB Comprehensive Cancer Center Annual Retreat Competition, 2006
2. New Investigator award, Department of Defense, Prostate Cancer Research Group, 2004
3. The AACR-AFLAC Scholar-In-Training Award, American Association for Cancer Research, San Francisco, April, 2002
4. Aventis-Young Investigator Award, American Association for Cancer Research; San Francisco, April , 2000
5. Student of the Year. Graduate School of Peking Union Medical College, Beijing, China, 1997
6. Best Scientific Article Award. Chinese Cancer Research Foundation(CCRF); Xi An, China, April, 1994

---PUBLICATIONS:

(Selected from 49 peer-reviewed publications, citation updated on May 8, 2008 from ISI Web of Knowledge)

1. Lim DS, Kim ST, **Xu B**, Maser RS, Lin J, Petrini JH, Kastan MB. Atm phosphorylates p95/nbs1 in an S-phase checkpoint pathway. ***Nature*** 404:613-617; 2000, **Times Cited:** 477

2. **Xu B,** Kim ST and Kastan MB. Involvement of Brca1 in S-phase and G2-phase checkpoint after ionizing irradiation ***Molecular and Cellular Biology*** 21: 3445-3450; 2001, **Times Cited:** 253

3. Kim ST, **Xu B** (co-first author) and Kastan MB. Involvement of the Cohesin Protein,Smc1, in ATM– Dependent and –Independent Responses to DNA Damage ***Genes & Development*** 16: 560-570, 2002, **Times Cited:** 213

4. **Xu B,** Kim St, Lim DS, Kastan MB. Two Molecularly Distinct G2/M checkpoints Are Induced by Ionizing Irradiation ***Molecular and Cellular Biology*** 22 (4): 1049-1059;2002, **Times Cited:** 196

5. Taniguchi T, Garcia-Higuera I, **Xu B**, Anderson P, Gregory R, Lane W, Kim S, Kastan MB, D'Andrea A. Convergence of the Fanconi Anemia and Ataxia Telangiectasia Signaling Pathways. ***Cell*** 109: 459-472, 2002, **Times Cited:** 198

6. **Xu B**, O' Donnell AH, Kim ST, Kastan MB. Phosphorylation of Serine 1387 in Brca1 is Specifically Required for the Atm-Mediated S-phase Checkpoint After Ionizing Irradiation. ***Cancer Research***. Aug 15; 62(16):4588-91, 2002, **Times Cited:** 81

7. Kastan MB, Lim DS, Kim ST **, Xu B,** Canman C. Multiple signaling pathways involving ATM ***Cold Spring Harbor Symposia on Quantitative Biology Volume LXV*****,** Cold Spring Harbor Press**,** p.521-526, 2000, **Times Cited:** 28

8. Lee JH, **Xu B**, Lee CH, Ahn JY, Song MS, Lee H, Canman CE, Lee JS, Kastan MB, Lim DS. Distinct functions of NBS1 in ATM-dependent responses to DNA damage. ***Molecular Cancer Research (MCR)*** 1(9):674-81, 2003, **Times Cited:** 40

9. Garg, R., Geng, C.-D., Miller, J. L., Callens, S., Tang, X., Appel, B., **Xu, B**.* Molecular Cloning and Characterization of the Catalytic Domain of Zebrafish Homologue of the Ataxia-Telangiectasia Mutated Gene. ***Molecular Cancer Research (MCR)*** 2: 348-353, 2004. **<u>Also featured the cover page of the issue</u>**. **Times Cited:** 4

10. Garg, R., Callens, S., Lim, D.-S., Canman, C. E., Kastan, M. B., **Xu, B***. Chromatin Association of Rad17 Is Required for an Ataxia Telangiectasia and Rad- Related Kinase-Mediated S-Phase Checkpoint in Response to Low-Dose Ultraviolet Radiation. ***Molecular Cancer Research (MCR)*** 2: 362-369, 2004. **Times Cited:** 11

11. **Xu B***, Kastan MB. Analyzing cell cycle checkpoints after ionizing radiation. ***Methods Mol Biol***.; 281: 283-92, 2004

12. Arlt, M. F., **Xu, B**., Durkin, S. G., Casper, A. M., Kastan, M. B., Glover, T. W. BRCA1 Is Required for Common-Fragile-Site Stability via Its G2/M Checkpoint Function. ***Mol. Cell. Biol. (MCB)*** 24: 6701-6709, 2004, **Times Cited:** 48

13. Wakeman TP, Kim WJ, Callens S, Chiu A, Brown KD, **Xu B*.** The ATM-SMC1 Pathway is Essential for Activation of the Chromium [VI]-induced S-Phase Checkpoint. ***Mutation Research***, 554(1-2):241-51, 2004. **Times Cited:** 13

14. Timothy Wakeman, Dorota Wyczechowska, and **Bo Xu*.** Involvement of the p38 MAP kinase in chromium-induced growth arrest and apoptosis. ***Mol Cell Biochem.*** 2005 Nov; 279(1-2):69-73 **Times Cited:**5.

15. Hong Xie, Sandra Wise, Amie Holmes, **Bo Xu**, Timothy Wakeman, Stephen Pelsue, Narendra Singh, and John Wise. Carcinogenic lead chromate induces DNA double strand breaks in human lung cells. ***Mutation Research*****.** 2005 Oct 3; 586 (2):160-72. **Times Cited:** 24

16. Stephen L. Gasior, Timothy Wakeman, **Bo Xu**, Prescott L. Deininger. The human LINE-1 Retrotransposon Creates DNA DSBs***. J Mol Biol.*** 2006 Apr 14; 357(5):1383-93. **Times Cited:** 33

17. Timothy Wakeman, Renu Garg, and **Bo Xu*,** ATR regulates Cr-induced S-phase checkpoint through dissociation from Rad17. ***Mutation Research*** 2006 Nov 7;610(1-2):14-20. **Times Cited:** 7

18. Mickael Cariveau, Jessi e Tang, Xiaoli Cui, and **Bo Xu***. Conserved NBS1 C-terminal small peptides can inhibit the AT M-mediated DNA damage response and enhance r adiation sensitivity. ***Molecular Pharmacology***, AUG 2007, 72 (2): 320-326. **Times Cited:** 1

19. Mickael J. Cariveau, Murray Stackhouse, Xi ao-li Cu i, Kamal Tiwari, William Waud, John A. Secrist III, **Bo Xu*.** Clofarabine acts synergistic ally wit h ionizing radiation *in vitro* and *in vivo* by interfering with the DNA damage response, ***International Journal of Radiation Oncology, Biology, Physics****,* 2008, 70: 213-220

20. Xi Tang, Zhou-guang Hui, Xiao-li Cu i, Renu Garg, Michael B. Kastan, and **Bo Xu***. A novel ATM-dependent pathway regulates Protein Phosphatase 1 and hist one H3 phosphorylation in response to DNA damage. ***Molecular Cellular Biology (MCB)***. April 4, 2008, 2559-2566 Times cited: 4

21. Xi Tang, Xiao-nan Sun, Renu Garg and **Bo Xu*.** Regulation of ATM phosphorylatio n in the absence of DNA damage. Under review.

22. Jessie T ang, Chun-y ing Yang and **Bo Xu***. Mi totic–dependent acti vation of A TM i s regulated by Aurora-B and required for the spindle checkpoint. Manuscript in preparation.

23. Mike Lee, Jes sie Tang, Xiao li Cui, Paula Allen, Franç oise Bontemps, William B. Park er, and **Bo Xu***. A nov el mec hanism inv olving ATM and dCK on the syn ergistic eff ect of nucleos ide analogues with radiotherapy. Manuscript in preparation.

24. Xi Tang, Xiaoli Cui, and **Bo Xu***. Brca1 participates in ATM- depedent regulation of P P1 in response to ionizing irradiation. Manuscript in preparation.

25. Xi Tang and **Bo Xu***. Global Hist one H3 phopshorylation leads to ATM-depedent apoptosis. Manuscript in preparation.

---GRANT SUPPORT

1R01CA133093-01A1 (Xu) NCI 04/01/09-03/31/14
Project title: “Mechanisms of Mitotic Activation of the ATM Kinase”
Role on project: Principal Investigator
Goal: Study the molecular mechanisms of mitotic ATM activation and its role in spindle checkpoint.
Status: Active

1R01ES016354-01A2 (Xu) NIEHS/NCI 07/01/09-06/30/13
Project title: "A Novel Pathway Involving ATM, PP1 and I-2"
Role on project: Principal Investigator
Goal: Dissect the signaling pathways regulating protein phosphatase one after DNA damage.
Status: Active

1R21NS061748-01 (Xu), NIH/NINDS, 09/01/2007-08/30/2010
Project title: "An HTS Assay for Inhibitors of NBS1-ATM Interactions"
Role on project: Principal Investigator
Goal: Develop a high throughput assay for identifying small molecules that can disrupt NBS1-ATM interactions.
Status: Active.

W81XWH-05-1-0018 (Xu) Department of Defense. 11/1/2004-11/30/2009
Project title: "Interfering with DNA Damage Signals: Radiosensitizing Prostate Cancer Using Small Peptides"
Role on project: Principal Investigator
Goal: To develop and characterize tumor specific peptides to target critical DNA damage response pathways and sensitize prostate tumor to radiotherapy.
Status: Completed

R03 ES013301-01 (Xu) NIH/NIEHS 09/30/2004-08/31/2008
Project title: "ATM and ATR in Chromium-Induced S-Phase Arrest"
Role on project: principal investigator
Goal: To study the molecular determinants of hexavalent-chromium induced S-phase cell cycle checkpoint
Status: Completed.

P20 RR020152-01 (Deininger) NIH/NCRR 9/16/2004- 7/31/2009
"Mentoring a program in cancer genetics"
Role on project: Principal Investigator of Project No.3 (total cost)
Project title: "The ATR kinase and UV radiation-induced cell cycle checkpoints"
Goal: Elucidate the functional role of the DNA damage checkpoint kinase ATR in regulation of UV-induced cell cycle checkpoints.
Status: Completed.

DAMD17-03-1-0709 (Xu) Department of Defense 8/20/2003-8/19/2005
Project title: "Targeting ATM-mediated Pathways to Sensitize Breast Cancer Cells to Therapeutic Interventions"
Role on project: Principal Investigator
Goal: Develop small molecules to sensitize breast cancer to radiotherapy and chemotherapy.
Status: Completed.

Ladies Leukemia League (Xu) 5/20/2004- 5/19/2005
Project title: "Small Peptides in Leukemia Treatment"

Role on project: Principal Investigator
Goal: Characterize the chemosensitization effect of newly developed fusion peptides on leukemia treatment.
Status: Completed.

---PATENT

Bo Xu and Mickael Cariveau: Targeting NBS1-ATM Interaction to Sensitize Cancer Cells to Radiotherapy and Chemotherapy, PCT/US1007/022886, pending, filed on October 30, 2007

Bo Xu: Methods for treating neoplasia with combination of chemotherapeutic agents and radiation. PCT/US 08/60124, pending, filed on April 14, 2008

---MEETING PRESENTATIONS

1. Xi Tang and **Bo Xu**. Mechanisms governing radiation-induced PP1 activation. Short Talk at the **FASEB Summer Research Meeting** on Protein Phosphatases, July 13, 2008, Snowmass Village, CO

2. Xi Tang, Zhou-guang Hui, Xiao-li Cui, Renu Garg, Michael B. Kastan and **Bo Xu**. A novel ATM-dependent pathway regulates Protein Phosphatase 1 in response to DNA damage. Presented in **Mini-Symposia**, **2008 Annual meeting of American Association for Cancer Research,** San Diego, CA, April 15, 2008—**the AACR-WICR Brigid G. Leventhal Scholar Award winner.**

3. **Bo Xu**. A novel pathway involving ATM, PP1 and I-2, presented in **Gordon Research Conference** Radiation Oncology, Feb 1, 2008, Ventura, CA

4. Xi Jessie Tang, Xiao-nan Sun, Renu Garg, and **Bo Xu**, Regulation of ATM Phosphorylation in the Absence of DNA Damage. Presented in the 98th Annual meeting of American Association for Cancer Research, Los Angeles, CA, April 2007

5. Mickael J. Cariveau, Xiao-Li Cui and **Bo Xu**, Interfering with NBS1-ATM Interaction to Inhibit the DNA Damage Response and Sensitize Tumor Cells to Radiation. Presented in the 98th Annual meeting of **American Association for Cancer Research**, Los Angeles, CA, April 2007

6. Mickael Cariveau, Murray Stackhouse, Xiao-li Cui, William Waud, William B. Parker, John A. Secrist III, **Bo Xu**. Clofarabine acts synergistically with ionizing radiation in vitro and in vivo by inhibiting the repair of DNA damage. Presented in the 98th Annual meeting of **American Association for Cancer Research**, Los Angeles, CA, April 2007

7. Yali Cui, Shannon Callens, and **Bo Xu.** DNA damage signaling and potential radiotherapeutic targets in prostate cancer treatment. **AACR Meeting Abstracts, Apr 2006; 2006: 1246.**

8. Timothy Wakeman and **Bo Xu.** Carcinogenicity of the chromium intermediates. Presented in the 96th Annual meeting of **American Association for Cancer Research**, Anaheim, CA, April 2005

9. Jessie Tang and **Bo Xu.** Involvement of Protein Phosphatase One in dephosphorylation of Histone H3 and DNA damage response. Presented in the 96th Annual meeting of **American Association for Cancer Research**, Anaheim, CA, April 2005

10. Vinodh Kurella, Toria Obey, Timothy Wakeman, **Bo Xu**. Exposure to Nickel induces DNA double strand breaks and replication blockage, Presented in the 96th Annual meeting of **American Association for Cancer Research**, Anaheim, CA, April 2005

11. Stephen Gasior, Timothy Wakeman, **Bo Xu,** Prescott Deininger, The Human LINE-1 retrotransposon creates DNA double strand breaks. Presented in the 96th Annual meeting of **American Association for Cancer Research**, Anaheim, CA, April 2005

12. Shannon Callens and **Bo Xu**. Targeting ATM- SMC1 pathway: a novel approach to developing radiosensitizers for prostate cancer. Presented in the **Prostate Cancer Symposium**, February 17-19, Orlando, Florida 2005

13. **Bo Xu**. Development of fusion peptides that can interfere with ATM-mediated DNA damage pathways and increase tumor radiosensitivity. **The 3rd International Symposium on Targeted Anticancer Therapies**. March 3 to 5, 2005, Amsterdam, the Netherlands.

14. Xiaoli Cui, Shannon Callens, and **Bo Xu**. A small interfering peptide can interfere with ATM-mediated DNA damage pathways and increase tumor radiosensitivity. Presented in the **Era of Hope 2005**, Philadelphia, PA , June 11, 2005

15. Jessie Tang, Annie Szeto, and **Bo Xu.** The role of protein phosphatase 1 on regulation of histone H3 phosphorylation. Presented in the 44th Annual Meeting of the **American Society for Cell Biology**, Washington, DC, 2004. **Received pre-doctoral travel award.**

16. **Bo Xu** Molecular Determinants of Cr -induced S-phase checkpoint. The 3rd Conference on **Molecular Mechanisms of Metal toxicity and Carcinogenesis**, organized by **NIOSH/CDC,** Morgantown , WV, 2004

17. Renu Garg, Jessie Tang, Bruce Appel and **Bo Xu**. A zebrafish model of Ataxia Telangiectasia. The **Zebrafish 2004 Conference,** Madison WV, 2004

18. Wakeman JP, Kim W J, Callens S, Chiu A, Brown KD, and **Bo Xu.** Exposure to hexavalent chromium activates an ATM and ATR-dependent S-phase checkpoint Presented in **Mini-Symposia** , 95th Annual meeting of **American Association for Cancer Research**, Orlando, FL, March 2004

19. Renu Garg, Shannon Callens, Dae-Sik Lim, Christine E. Canman, Michael B. Kastan and **Bo Xu.** The Rad 17-Atr-Smc1 pathway is essential for ultraviolet radiation induced S-phase

arrest. Presented in the 95th annual meeting of **American Association for Cancer Research,** Orlando, FL, March 2004

20. Timothy P. Wakeman, Arthur Chiu, and **Bo Xu.** An ATM-dependent pathway is essential for activation of cell cycle checkpoints in response to chromium exposure. 43rd Annual Meeting of the **American Society for Cell Biology**, San Francisco, CA, 2003.

21. **Xu B,** Kim ST, Lim DS, Kastan MB Two Molecularly Distinct G2/M checkpoints Are Induced by Ioniz ing Irradiation Presented in a **Mini-Symposia**, **93rd Annual meeting of American Association for Cancer Research,** San Francisco CA, April 2002—**received Young Investigator Award.**

22. **Xu B,** O'Donnell A, Kim ST, Kastan MB The Breast Cancer Susceptibility Gene Product 1, Brca1, functions as a cell cycle checkpoint protein. Presented in **a poster session in 93rd Annual meeting of American Association for Cancer Research,** San Francisco CA, April 2002.

23. D'Andrea AD, Garcia-Higuera I, Lane W S, **Xu B**, Kastan M B, Taniguchi T .Differential activation of the Fanconi Anemia protein, FANCD2, by monoubiquitination and phosphorylation. ***MOLECULAR BIOLOGY OF THE CELL*** 12: 5A-6A 27 Suppl. S, NOV 2001

24. Taniguchi T, Garcia-Higuera I, Lane W S, Kim ST, **Xu B**, Kastan MB, D' Andrea AD Molecular interaction of the Fanconi Anemia (FA) and Ataxia Telangiectasia (AT) pathways. ***BLOOD*** 98 (11): 782A-782A 3252 Part 1, NOV 16 2001

25. **Xu B,** Kastan MB. Dominant negative effects of altered Atm constructs. Presented in Mini Symposia, **91st Annual meeting of American Association for Cancer Research**, San Francisco CA, April 2000- **received Young Investigator Award**

---INVITED SPEECHES:

1. "Ataxia Telangiectasia Mutated protein and cell cycle checkpoints" Monthly Genetics Meeting, CHILDREN'S HOSPITAL, 200 Henry Clay Avenue, New Orleans, LA January 24, 2003.
2. "ATM kinase and DNA damage response" Tulane Cancer Center Seminar Series, March 27, 2003
3. "ATM Kinase and DNA Damage Response", SEMINARS IN INVESTIGATIVE MEDICINE, North Shore-LIJ Research Institute, Manhasset, NY 11030, May 6, 2003.
4. "Mechanisms of Cellular Response To DNA Damage" Biochemistry and Molecular Biology Seminar Series, LSUHSC, August 29, 2003
5. "The ATM kinase and DNA damage-induced Cell Cycle Checkpoints", LSU/Tulane Joint Gene Therapy Seminar, September 9, 2004
6. **"**Molecular Determinants of Cr-induced S-phase checkpoint. The 3rd Conference on Molecular Mechanisms of Metal toxicity and Carcinogenesis, NIOSH/CDC Morgantown, WV, September 12, 2004

7. “DNA damage response and human genetic dis eases”, Tulane Human Genetics Program, September 28, 2004
8. “ The role of ATM in DNA damage respons e and aging”, The National Institute of Aging Workshop, September 30, 2004
9. “The mechanisms of the human G2/M Cell Cycl e Checkpoints", LSU/Tulane Joint Cancer Center Seminar, December 16, 2004
10. “Sensing DNA double strand brea ks”, Tianjin Medical University Cancer Institute, June 3, 2005
11. “Mechanisms of DNA damage-induced cell cycle checkpoints”, Cancer Hospital/Institute, Peking Union Medical College and Chinese Academy of Medical Sciences, June 12, 2005
12. “DNA double strand breaks”, Peking Univer sity Health Sciences Cent er, Cancer Hospital, July 4, 2005
13. “Sensing DNA double strand breaks”, Virginia Commonwealt h University and the Massey Cancer Center, July 13, 2005
14. “Roles of ATM and ATR in chromiu m-induced cell cycle checkpoints”, Workshop on Chromium and Human Health, Portland, ME, August 19, 2005
15. “Regulation of DNA damage-induced cell cycle checkpoints”, Case Western Reserv e University, August 25, 2005
16. “Sensing DNA double strand breaks”, Southern Research Institute, November 22, 2005
17. **“**DNA damage response mechanisms and new approa ches to developing radiosens itizers” Methodist Hospital Residence Seminar Series, Methodist Hospital Houston, March 2006
18. “Mechanisms of DNA damage induced cell cycle checkpoints”, Nelson Institute of Environmental Sciences, NYU School of Medicine, March 21, 2006
19. “Advances in Modern Molecular Radiobiology” Chinese Association of Radiation Oncology, June 22, 2006, Beijing, China
20. “DNA damage response mechanisms, from fundamental biology to cancer drug discovery”, Georgia State University, Atlanta, GA, December 7, 2007,
21. “Recent Advances in Cancer Drug Discov ery”, Samford University School of Pharmacy, March 12, 2008
22. “Modern Molecular Radiobiol ogy: Molecular targeted Radios ensitization”, Department of Radiation Oncology, The Methodist Hospital, Houston, TX. March 19, 2008
23. “DNA damage response mechanisms, from fundamental biology to cancer drug discovery”, The Methodist Hospital Research Institute, Houston, TX. March 20, 2008
24. “Roles of ATM in DNA damage response”, The 2nd International Conference on Frontiers in Biomedical and Environmental Health Sciences: DNA Repair and Cancer Biology. April 16-20, 2008, Hangzhou, China
25. “ATM and Breast Cancer”, Tianjin Medical Univ ersity Cancer Hospital, April 22, Tian jin , China
26. “DNA damage response and human diseases”, Institute of Biophysi cs, Chinese Academy of Sciences, Beijing, China, April 23, 2008
27. “Damage response mechanism s and genetic in stability”, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China, April 24, 2008
28. “Molecular Radiation Biology”, Tianjin Medic al Univ ersity General Hospital, Tianjin, China, April 25, 2008
29. “Targeting DNA damage responses for c ancer drug discov ery”, Nantong Biomics, Nantong, China, May 14, 2008

30. “Molecular Cancer Biology and Molecu lar Targeted Cancer Therapeutics”, Nantong University Medical School, Nantong, China, May 15, 2008
31. “Targeting DNA damage pathw ays by small inhibitory peptides”, 1st Annual World Congress of ibio2008, Hangzhou, China, May 19, 2008 (also served as session co-chair)
32. “DNA damage response mechanisms: imp lications for cancer drug discovery” UAB Comprehensive Cancer Center Experimental Therapeutics Seminar Series, November 22, 2008
33. “Mitotic functions of ATM”, H. Lee Moffitt Cancer Cent er and Res earch Institute, Tampa, FL, Dec 8, 2009
34. “ATM-mediated Signaling Pathways”, The Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA, Jan 6, 2009
35. “DNA damage responsive m echanisms: fr om fundamental biology to cancer drug discovery”, Temple University, Philadelphia, PA, Jan 20, 2009
36. “ATM-mediated pathways: implication in cancer biology and cancer drug discovery” University of Virginia, Charlottesville, VA, May 13, 2009

---CURRENT LAB MEMBERS:

Chunying Yang, PhD, Postdoctoral Fellow
Xiaoli Cui, PhD, Postdoctoral Fellow
Anand Prakash, MD, PhD student
Evan Comeaux, PhD Student
Ning Liu, MD, PhD, Visiting Scientist
Mike Lee, PhD, Visiting Scientist (Assistant Professor at Samford University)

Table 1. Mentoring experience:

Name	position in the lab	time	degree received	current position	mentored awards received
Michael Cariveau	postdotoctal fellow	2005-2007	N/A	assistant professor	Stanley Scott Cancer Center Postdoc Scholar
					2007 UAB Postdoc Research Day Award
Timothy Wakeman	PhD Student	2002-2006	PhD	Postdoctoral Fellow	Stanley S. Scott Cancer Center Graduate Student Scholar (2003)
					CAGNO student research award (2004)
					19th and 20th LSUHSC Annual Research Day Poster presentation awards
					NIOSH ERC Pilot Projects Research Training Program Award
					CAGNO student research award (2005)
Xi "Jessie" Tang	PhD Student	2003-2007	PhD	Postdoctoral Fellow	Student Travel Award to the 44TH ANNUAL MEETING of American Society of Cell Biology (2004)
					CAGNO student research award
					AACR-WICR Brigid G. Leventhal Scholar Award (2008)
Anand Prakash	PhD Student	2007-present			
Srirangan Sampath	Rotation Student	2004-2005			CAGNO student research award
Vinodh Kurella	Rotation Student	2004			

Priyanka Rashmi	Rotation Student	2005			
Zhouguang Hui	postdoctoral fellow	2005-2006	n/a	associate professor	
Renu Garg	postdoctoral fellow	2002-2004	n/a	assistant professor	
Dorota Wyczechowska	postdoctoral fellow	2004-2006	n/a	n/a	
Xiaoli Cui	postdoctoral fellow	2006-present			
Chun-ying Yang	postdoctoral fellow	2007-present			

---ADVISING/SUPERVISING RESPONSIBILITIES:

Postdoctoral fellows:
Renu Garg PhD (2002-2003)
Dorota Wyczechowska PhD (2004-2006)
Mickael Cariveau PhD (2005 to 2007)
Zhou-Guang Hui MD, PhD (2005-2006)
Xiaoli Cui MD, PhD (2004-present)
Chun-ying Yang (2007-present)
Jessie Tang (2007-2008)

Research Associates:
Shannon Callens (2002-2004)
Jason Manning (2004-2006)

Graduate students:
1. Timothy Wakeman, 2002-2006, as PhD dissertation advisor
Awards received while in the lab:
1. Stanley S. Scott Cancer Center Graduate Student Scholar (2003);
2. CAGNO student research award (2004);
3. 19th and 20th LSUHSC Annual Research Day Poster presentation awards;
4. NIOSH ERC Pilot Projects Research Training Program Award;
5. CAGNO student research award (2005);

Note: Tim completed his dissertation resear ch and received his PhD degree in May 2006. He is currently a postdoctoral fello w at Duke University Medica l Center. He is nominated for the 2006 LSUHSC Chancellor award.

2. Xi Jessie Tang, 2002-2007, as PhD dissertation advisor
Awards received while in the lab:
1. Student Travel Award to the 44T H ANNUAL MEETING of American Society of Cell Biology (2004)
2. CAGNO student research award (2005)
3. AACR-WICR Brigid G. Leventhal Scholar Award (2008)

Note: Jessie completed her dissertation rese arch and received her PhD degree in May 2007. She is currently a postdoctoral fe llow in University of Washington in Seattle.

3. Anand Prakash, 2007-present, as PhD dissertation advisor
4. Srirangan Sampath, 2004 to 2006, as rotating student advisor
Award received while in the lab: CAGNO student research award (2005)
6. Vinodh Kurella, 2004, as rotating student advisor
7. Priyanka Rashmi, 2005, as rotating student advisor

Dillon Beardsley, committee member
Daniel Stewart, committee member
Ashley Upton, committee member

Undergraduate students:
Toria Obey (Award received during her su mmer internship in the lab: NCI-summe r internship presentation award)
Annie Szeto (NCI summer intern)
Niah Shanks
Christi Hahn
Kimberly Bridgewater
Anne O'Donnell (NCI summer intern)

High School Students:
Yiwen Peng (NCI summer intern, currently a student at Caltech)
Jessica Xiao (currently a student at MIT)

---TEACHING:
Biochem 240: Molecular Biology: Cell signal ing and Gene expr ession, DNA damage and repair
CMB -B: Molecular Genetic Mechanisms: DNA Damage and Repair
Molecular Biology of Cancer: Cell cycle control and apoptosis
Medical Biochemistry: Canc er Biology /Oncogenes, Oncogenes/Tumor Suppressor Genes, and Angiogenesis/Carcinogenesis
Drug Discovery (HMG 705): Cancer Dr ug Disc overy, HHMI Graduate Program, The University of Alabama at Birmingham
Cancer Biology （707）： Radiation Therapy
Toxicology (TOX712): Radiation toxicology

---FACULTY COMMITTEE SERVED:
1. Faculty Member (exam question writer), R adiation Oncology In Service Examination, American College of Radiology (Board Exam), 2008-present
2. Member, The Council of Postdoctoral Educat ion (COPE), University of A labama at Birmingham, April 2007-present
3. Graduate Student Admission Committee, D epartment of Genetics, LSUHSC (2002-2005)
4. Faculty seminar coordinator , LSU/Tulane Cancer Center Joint Seminar Series (2004-2005)

--- REVIEW PANNELS:

Grant reviews

NIH Special Emphasis Panel/Scientific Review Group 2009/10 ZRG1 OBT-A (58) R
DoD CDMRP Breast Cancer Research Program TRN-CBY-B
Division of Biochemistry and Biophysics, Department of Life Sciences, National Nature Science Foundation of China (NSFC)
Alabama Drug Alliance (ADDA)
Ladies leukemia League
Susan G Komen for the Cure

Journal Reviews

Cancer Research
Cancer Science
Cancer Chemotherapy and Pharmacology
Cell Proliferation
EMBO J
International Journal of Biochemistry and Cell Biology
International Journal Of Radiation Biology
Molecular Cancer Research
Molecular Cellular Biochemistry
Molecular Pharmacology
Radiation Research

NEEDLE & ROSENBERG

November 13, 2007

INTELLECTUAL PROPERTY ATTORNEYS
NEEDLE & ROSENBERG PC
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P. Brian Giles, Ph.D.
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Mr. David Mason
Director, Commercialization and Intellectual Property
Southern Research Institute
2000 Ninth Avenue South
Birmingham, AL 35205

RE: New International Application
Based on United States Provisional Patent Application No. 60/863,457
Title: TARGETING NBSI-ATM INTERACTION TO SENSITIZE CANCER CELLS TO RADIOTHERAPY AND CHEMOTHERAPY
Inventors: XU *et al.*
N&R Reference: 19044.0065P1
SRI Reference: 287(P1)

Dear David:

We are pleased to report that the international patent application identified above was filed in the United States Receiving Office for the Patent Cooperation Treaty (PCT) on October 30, 2007, claiming priority to U.S. Patent Application No. 60/863,457, filed on October 30, 2006. Copies of each of the documents as filed, including the application and PCT Request Form, are enclosed for your files.

Please confirm that the residence address and nationality listed on the PCT Request form is current for the named applicants/inventors. Please forward any change of inventorship, address or nationality to us as soon as possible so that amendments to the Request may be timely entered.

Please note that, in general, time periods to respond to actions are shorter for international applications as compared to U.S. applications. Furthermore, the rules governing international applications dictate specific non-extendable due dates for entering both the International Preliminary Examination phase and the National/Regional phases.

The deadline for entering the present international application into National Phase in most designated countries is 30 months from the priority date, or **April 30, 2008.** We will remind you of this deadline as it approaches, however, we recommend that you also docket this date.

Switzerland, Luxembourg, United Republic of Tanzania, and Uganda, still retain a 20 month national phase entry deadline. Direct entry into the national phase for these countries can only be deferred to the more common 30 month date if you file a Chapter II Demand with the Patent Cooperation Treaty (PCT) no later than19 months from the priority date, or by May 30, 2008.

It is important to note that filing a Chapter II Demand at the 19 month date is ONLY necessary if you are not interested in filing national phase patents in Europe or ARIPO at their 31 month national phase entry deadline. If you choose not to file a Demand at the 19 month date, and you will not be entering national phase in Europe or ARIPO, the deadline for entering national phase directly in Switzerland, Luxembourg, United Republic of Tanzania, and Uganda is June 30, 2008.

Please note that we will not file a Demand at the 19 month due date, nor will we directly enter national phase by the 20 month due date in the above referenced countries, unless specifically instructed by you on or before March 30, 2008. No further reminders will be sent regarding the 19 month and 20 month deadlines.

Finally, please be aware that unless you request early publication or withdraw the present international application, the application will be electronically published on or about April 30, 2008. We will report the publication of the application upon notification from the PCT.

If you have any questions or require additional information, please do not hesitate to contact us.

Very truly yours,

NEEDLE & ROSENBERG, P.C.

P. Brian Giles, Ph.D.
Patent Agent

GDS/PBG:acs

Enclosure(s)

cc: Dr. Bo Xu (w/enclosures)

TRANSMITTAL LETTER TO THE UNITED STATES RECEIVING OFFICE

Express Mail mailing number: EV915331710US	Date of deposit: 30 October 2007
File reference no.: 19044.0065P1	International application no. (if known): TBD
Customer Number[1]: 23859	Earliest priority date claimed (Day/Month/Year): 30/10/2006
Title of the invention: TARGETING NBS1-ATM INTERACTION TO SENSITIZE CANCER CELLS TO RADIOTHERAPY AND CHEMOTHERAPY	

☑ **This is a new International Application**

SCREENING DISCLOSURE INFORMATION:

In order to assist in screening the accompanying international application for purposes of determining whether a license for foreign transmittal should and could be granted and for other purposes, the following information is supplied. (check as boxes as apply):

☐ The invention disclose was **not** made in the United States of America.

☐ There is no prior U.S. application relating to this invention.

☑ The following prior U.S. application(s) contain subject matter which is related to the invention disclosed in the attached international application. (*NOTE: priority to these applications may or may not be claimed on the Request (form PCT/RO/101) and this listing does not constitute a claim for priority.)*

application no.	60/863,457	filed on	30 October 2006
application no.		filed on	

☐ The present international application contains additional subject matter not found in the prior U.S. application(s) identified above. The additional subject matter is found on pages _____ and ☐ **DOES NOT ALTER** ☐ **MIGHT BE CONSIDERED TO ALTER** the general nature of the invention in a manner which would require the U.S. application to have been made available for inspection by the appropriate defense agencies under 35 U.S.C. 181 and 37 C.F.R. 5.15.

Itemized list of contents

Sheets of Request form: 5	Check no.: Form 2038
Sheets of description (excluding sequence listing): 107	Return receipt postcard: Yes
Sheets of claims: 3	Power of attorney:
Sheets of abstract: 1	Certified copy of priority document (specify):
Sheets of drawings: 18	Other (specify):
Sheets of sequence listing: 25	Fee Calculation Sheet
Sequence listing diskette/CD:	
Tables related to sequence listing CD:	

The person signing this form is:	☐ Applicant	
	☑ Attorney/Agent (Reg. No.)	Name of person signing P. Brian Giles (57,896)
	☐ Common Representative	Signature Brian Giles

[1] **Customer Number will allow access to the application in Private PAIR but cannot be used to establish or change the correspondence address.**

Applicant's Entitlement to Reduced Search Fee Under 37 CFR 1.445(a)(2) Corresponding prior US national application filed under 35 USC 111(a)	
Filing date:	Application no. (if known):
Inventor's Name(s):	
Title of the invention:	
Application docket no. (if applicable):	

This collection of information is required by 37 CFR 1.10 and 1.412. The information is required to obtain or retain a benefit by the public, which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 15 minutes to complete, including gathering information, preparing, and submitting the completed form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS.
SEND TO: Mail Stop PCT, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

International Application No.

International Filing Date

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference *(if desired) (12 characters maximum)* 19066.0065P1

Box No. I TITLE OF INVENTION

TARGETING NBS1-ATM INTERACTION TO SENSITIZE CANCER CELLS TO RADIOTHERAPY AND CHEMOTHERAPY

Box No. II APPLICANT ☐ This person is also inventor

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

SOUTHERN RESEARCH INSTITUTE
2000 Ninth Avenue South
Birmingham, Alabama 35205
US

Telephone No.

Facsimile No.

Applicant's registration No. with the Office

State *(that is, country)* of nationality: US

State *(that is, country)* of residence: US

This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

☒ Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

SPRATT, Gwendolyn D.
NEEDLE & ROSENBERG, P.C.
Suite 1000, 999 Peachtree Street
Atlanta, Georgia 30309-3915
United States of America

Telephone No.
(678) 420-9300

Facsimile No.
(678) 420-9301

Agent's registration No. with the Office
36,016

☐ **Address for correspondence:** Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

If none of the following sub-boxes is used, this sheet should not be included in the request.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

XU, Bo
5308 Hickory Trace
Hoover, Alabama 35226
US

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office

State *(that is, country)* of nationality: CN

State *(that is, country)* of residence: US

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

CARIVEAU, Michael J.
1094 Waller Rd.
Mount Olive, NC 28365
US

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office

State *(that is, country)* of nationality: US

State *(that is, country)* of residence: US

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:
- ☐ applicant only
- ☐ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:
- ☐ applicant only
- ☐ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

☐ Further applicants and/or (further) inventors are indicated on another continuation sheet.

Supplemental Box *If the Supplemental Box is not used, this sheet should not be included in the request.*

1. *If, in any of the Boxes, except Boxes Nos. VIII(i) to (v) for which a special continuation box is provided, the space is insufficient to furnish all the information: in such case, write "Continuation of Box No...." (indicate the number of the Box) and furnish the information in the same manner as required according to the captions of the Box in which the space was insufficient, in particular:*

   (i) ***if more than one person is to be indicated as applicant and/or inventor*** *and no "continuation sheet" is available: in such case, write "Continuation of Box No. III" and indicate for each additional person the same type of information as required in Box No. III. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below;*

   (ii) *if, in Box No. II or in any of the sub-boxes of Box No. III, the indication* ***"the States indicated in the Supplemental Box"*** *is checked: in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the applicant(s) involved and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is applicant;*

   (iii) *if, in Box No. II or in any of the sub-boxes of Box No. III,* ***the inventor or the inventor/applicant is not inventor for the purposes of all designated States or for the purposes of the United States of America:*** *in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the inventor(s) and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is inventor;*

   (iv) *if, in addition to the agent(s) indicated in Box No. IV, there are* ***further agents:*** *in such case, write "Continuation of Box No. IV" and indicate for each further agent the same type of information as required in Box No. IV;*

   (v) *if, in Box No. VI, there are* ***more than three earlier applications whose priority is claimed:*** *in such case, write "Continuation of Box No. VI" and indicate for each additional earlier application the same type of information as required in Box No. VI.*

2. *If the applicant intends to make an indication of the wish that the international application be treated, in certain designated States, as an application for a patent of addition, certificate of addition, inventor's certificate of addition or utility certificate of addition: in such a case, write the name or two-letter code of each designated State concerned and the indication* ***"patent of addition," "certificate of addition," "inventor's certificate of addition"*** *or* ***"utility certificate of addition,"*** *the number of the parent application or parent patent or other parent grant and the date of grant of the parent patent or other parent grant or the date of filing of the parent application (Rules 4.11(a)(iii) and 49bis.1(a) or (b)).*

3. *If the applicant intends to make an indication of the wish that the international application be treated, in the United States of America, as a continuation or continuation-in-part of an earlier application: in such a case, write "United States of America" or "US" and the indication* ***"continuation"*** *or* ***"continuation-in-part"*** *and the number and the filing date of the parent application (Rules 4.11(a)(iv) and 49bis.1(d)).*

Continuation of Box IV:

NEEDLE, William H. (26,209); ROSENBERG, Sumner C. (28,753); KATZ, Mitchell A. (33,919); KIRSCH, Gregory J. (35,572); CAMPBELL, Cathryn A. (31,815); SPRATT, Gwendolyn D. (36,016); BECKER, Bruce H. (48,884); DECARLO, Kean J. (39, 956); HODGES, Robert A. (41,074); HUIZENGA, David E. (49,026); MEADOWS, Brian C. (50,848); MURPHY, Mark A. (42,915); CLEVELAND, Janell T. (53,848); GILES, P. Brian (57,896); HATHCOCK, Kevin W. (52,998); LANIER, J. Gibson (57,519); HALL, Miles E. (58,128); JACKSON, Jason S. (56, 733); DOVALE, Anthony J. (50,349); MARTY, Scott D. (53,277); SHORTELL, D. Brian (56,020); BROWN,Charley F. (52,658); CURFMAN, Christopher L. (52,787); ECHLER, Richard S. (41, 006); CORNETT, David A. (48,417); ANDREWS, Cecilia M. (57,970); and ANDERSON, J. Scott (48, 563) all of NEEDLE & ROSENBERG, P.C., Suite 1000, 999 Peachtree Street, Atlanta, Georgia 30309-3915 United States of America

Box No. V DESIGNATIONS

The filing of this request **constitutes under Rule 4.9(a), the designation** of all Contracting States bound by the PCT on the international filing date, for the grant of every kind of protection available and, where applicable, for the grant of both regional and national patents.

However,

☐ DE Germany **is not designated** for any kind of national protection

☐ JP Japan **is not designated** for any kind of national protection

☐ KR Republic of Korea **is not designated** for any kind of national protection

☐ RU Russian Federation **is not designated** for any kind of national protection

(The check-boxes above may only be used to exclude (irrevocably) the designations concerned if, at the time of filing or subsequently under Rule 26bis.1, the international application contains in Box No. VI a priority claim to an earlier national application filed in the particular State concerned, in order to avoid the ceasing of the effect, under the national law, of this earlier national application.)

Box No. VI PRIORITY CLAIM

The priority of the following earlier application(s) is hereby claimed:

Filing date of earlier application *(day/month/year)*	Number of earlier application	Where earlier application is:		
		national application: country or Member of WTO	regional application: regional Office	international application: receiving Office
item (1) 30 October 2006	60/863,457	US		
item (2)				
item (3)				

☐ Further priority claims are indicated in the Supplemental Box.

Transmit certified copy: the receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) *(only if the earlier application was filed with the Office which for the purposes of this international application is the receiving Office)* identified above as:

☐ all items ☒ item (1) ☐ item (2) ☐ item (3) ☐ other, see Supplemental Box

Restore the right of priority: the receiving Office is requested to restore the right of priority for the earlier application(s) identified above or in the Supplemental Box as item(s) (_____). *(See also the Notes to Box No. VI; further information **must** be provided to support a request to restore the right of priority.)*

Incorporation by reference: where an element of the international application referred to in Article 11(1)(iii)(d) or (e) or a part of the description, claims or drawings referred to in Rule 20.5(a) is not otherwise contained in this international application but is completely contained in an earlier application whose priority is claimed on the date on which one or more elements referred to in Article 11(1)(iii) were first received by the receiving Office, that element or part is, subject to confirmation under Rule 20.6, incorporated by reference in this international application for the purposes of Rule 20.6.

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(if two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used)*:

ISA / EP

Request to use results of earlier search; reference to that search *(if an earlier search has been carried out by or requested from the International Searching Authority)*:

Date *(day/month/year)* Number Country *(or regional Office)*

Box No. VIII DECLARATIONS

The following **declarations** are contained in Boxes Nos. VIII (i) to (v) *(mark the applicable check-boxes below and indicate in the right column the number of each type of declaration)*:		Number of declarations
☐ Box No. VIII (i)	Declaration as to the identity of the inventor :	
☐ Box No. VIII (ii)	Declaration as to the applicant's entitlement, as at the international filing date, to apply for and be granted a patent :	
☐ Box No. VIII (iii)	Declaration as to the applicant's entitlement, as at the international filing date, to claim the priority of the earlier application :	
☐ Box No. VIII (iv)	Declaration of inventorship (only for the purposes of the designation of the United States of America) :	
☐ Box No. VIII (v)	Declaration as to non-prejudicial disclosures or exceptions to lack of novelty :	