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# Differential processing of low and high LET radiation induced DNA damage: Investigation of switch from ATM to ATR signaling

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The members of the phosphatidylinositol kinase-like kinase family of proteins namely ataxia-telangiectasia mutated (ATM) and ATM- and Rad3-related (ATR) are directly responsible for the maintenance of genomic integrity by mounting DDR through signaling and facilitating the recruitment of repair factors at the sites of DNA damage along with coordinating the deployment of cell cycle checkpoints to permit repair by phosphorylating Checkpoint kinase Chk1, Chk2 and p53. High LET radiation from GCR (Galactic Cosmic Rays) consisting mainly of protons and high energy and charged (HZE) particles from SPE (Solar Particle Event) pose a major health risk for astronauts on their space flight missions. The determination of these risks and the design of potential safeguards require sound knowledge of the biological consequences of lesion induction and the capability of the cells to counter them. We here strive to determine the coordination of ATM and ATR kinases at the break sites directly affecting checkpoint signaling and DNA repair and whether differential processing of breaks induced by low and high LET radiation leads to possible augmentation of swap of these damage sensors at the sites of DNA damage. Exposure of cells to IR triggers rapid autophosphorylation of serine-1981 that causes dimer dissociation and initiates monomer formation of ATM. ATM kinase activity depends on the disruption of the dimer, which allows access and phosphorylation of downstream ATM substrates like Chk2. Evidence suggests that ATM is activated by the alterations in higher-order chromatin structure although direct binding of ATM to DSB ends may be a crucial step in its activation. On the other hand, in case of ATR, RPA (replication protein A)-coated ssDNA (single-stranded DNA) generated as a result of stalled DNA replication or during processing of chromosomal lesions is crucial for the localization of ATR to sites of DNA damage in association with ATR-interacting protein (ATRIP). Although the majority of RPA-coated ssDNA is generally present only during DNA replication, ATR activation in G1 and G2-phase might still require formation of RPA-coated ssDNA, probably initiated by the MRN-CtIP complex and then extended by the Exo1- or BLM-dependent mechanisms at the sites of DSBs. Evidence accumulates that activation of ATM and ATR are oppositely regulated by the length of single stranded overhangs generated at the break sites by processes mentioned above and these stretches of single stranded overhangs hold the clue for ATM to ATR switch at broken DNA ends. We irradiated 82-6hTERT human fibroblast cells with low LET γ-rays and high LET Fe and Si particles. Preliminary results with cells exposed to 1Gy γ-rays show that the kinetics of pChk2-pT68 foci formation is comparable to that of γ-H2AX although they appear to recede quicker. The number and intensity of observed foci reaches a maximum at 30 min and 60 min post IR for Chk2-pT68 and γ-H2AX foci respectively and all Chk2-pT68 foci colocalize with γ-H2AX foci. The kinetics of Chk1-pS345 and ATRIP are being determined. Results of Chk2-pT68 foci kinetics was also corroborated by western blot experiments, although phosphorylation was detected as early as 10 min and started receding 30 min post IR with 2Gy of γ-rays. On the other hand, level of ATR-pS428 reached its maximum between 60 and 120 min and was maintained until the last measured time point of 4 hours post IR as determined by western blotting. Experiments performed with high LET Fe and Si particles will be reported.

# Differential Processing of Low and High LET Radiation-induced DNA Damage: Investigation of Switch from ATM to ATR Signaling

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## Abstract

The members of the phosphatidylinositol kinase-like kinase family of proteins namely ataxia-telangiectasia mutated (ATM) and ATM- and Rad3-related (ATR) are directly responsible for the maintenance of genomic integrity by mounting DDR through signaling and facilitating the recruitment of repair factors at the sites of DNA damage along with coordinating the deployment of cell cycle checkpoints to permit repair by phosphorylating Checkpoint kinase Chk1, Chk2 and p53. High LET radiation from GCR (Galactic Cosmic Rays) consisting mainly of protons and high charge and energy (HZE) nuclei, and protons from SPE (Solar Particle Event) pose a major health risk to astronauts. The determination of these risks and the design of potential safeguards require sound knowledge of the biological consequences of damage induction and the capability of the cells to counter them. We here strive to determine the coordination of ATM and ATR kinases at the break sites directly affecting checkpoint signaling and DNA repair and whether differential processing of breaks induced by low and high LET radiation leads to possible augmentation of swap of these damage sensors at the sites of DNA damage. Exposure of cells to IR triggers rapid autophosphorylation of serine-1981 that causes dimer dissociation and initiates monomer formation of ATM. The ATM kinase phosphorylates a large number of downstream molecules including Chk2. In the case of ATR, RPA (replication protein A)-coated ssDNA (single-stranded DNA) generated as a result of stalled DNA replication or during processing of chromosomal lesions is crucial for the localization of ATR to sites of DNA damage in association with ATR-interacting protein (ATRIP). Evidence accumulates that activation of ATM and ATR are oppositely regulated by the length of single stranded overhangs generated at the break sites by processes mentioned above and these stretches of single stranded overhangs hold the clue for ATM to ATR switch at broken DNA ends.

Here, we investigate the ATM to ATR switch in 82-6 hTERT human fibroblast cells after low LET gamma rays and high LET Fe nuclei radiation. Our preliminary data provide evidence of a potential switch from ATM to ATR kinase signaling in cells treated with gamma rays at approximately 2 hours post irradiation when phosphorylation of ATM kinase or its substrates start to wane and ATR kinase or its substrates begin to intensify as observed from western blot experiments and repair foci kinetics. Results from similar experiments performed with high LET 600MeV/u $^{56}$Fe radiation point to a probable delay in switching from ATM to ATR signaling, with phosphorylation of ATM kinase or its substrates staying strong until 8 hours post irradiation. The kinetics of ATR or its substrates show no alteration between high and low LET radiation. These results point strongly to the differential processing of DNA damage induced by low and high LET radiation with a possible essential role of ATM kinase in processing of high LET induced damage.

## Materials and Methods

*Cell culture, chemicals and irradiation.* hTERT-immortalized human fibroblast (82-6 hTERT, a kind gift from Dr. Janice Pluth, LBNL, Berkeley, CA.) were cultured in DMEM medium supplemented with 1x Antibiotic-Antimycotic and 10% FBS. Doses were delivered by BX-3 Cs-137 gamma irradiator (Hopewell Designs Inc.) at Johnson Space Center, NASA, Houston and 600MeV/u $^{56}$Fe (at NSRL, BNL, Long Island, NY.) 10μM ATM kinase inhibitor ku55933 (Tocris), was added to medium 1 hour prior to irradiation and cells were subsequently harvested at 0.5, 1, 2, 4 and 8 hour post irradiation.

*Western blotting and immunofluorescence.* Cells were lysed in NE-PER Nuclear and Cytoplasmic Extraction Reagent supplemented with Halt protease inhibitor cocktail (#78835, Thermo Scientific). Western blotting was used to detect the phosphorylated and native forms of ATM, ATR and Chk2 kinases proteins in the nuclear and cytoplasmic extracts. Mouse anti-pATM(Ser1981) (#600-401-400, Rockland Immunochemicals), Rabbit anti-ATM (#2873S, Cell Signaling), Rabbit anti-pATR(Ser428) (#2853, Cell Signaling), Rabbit anti-ATR (#2790S), Rabbit anti-pChk2(Thr68) (#2661, Cell Signaling), Rabbit anti-pChk1(Ser345) (#2348, Cell Signaling), Mouse anti-γH2AX(Ser139) (#JBW301, Upstate), Mouse anti-TATA box binding protein (#05-1531, Millipore) and Mouse anti-GAPDH (#AB2302, Millipore) were used.

For immunofluorescence, cells were grown on LabTek 8-well chamber slides, and fixed with 4% paraformaldehyde for 15 min. After permeabilization with 0.3% Triton X-100 in PBS for 15 min at RT, the slides were washed three times in PBS for 5 min and subsequently blocked with PBS containing 10% normal goat serum for 1 hour. The cells were then incubated with indicated primary antibodies in 2% goat serum followed by secondary antibody conjugated with Alexa Fluor 488 and nuclei were counterstained with DAPI. Immunofluorescence was evaluated with a fluorescence microscope Axioplan2 (Zeiss, Germany).

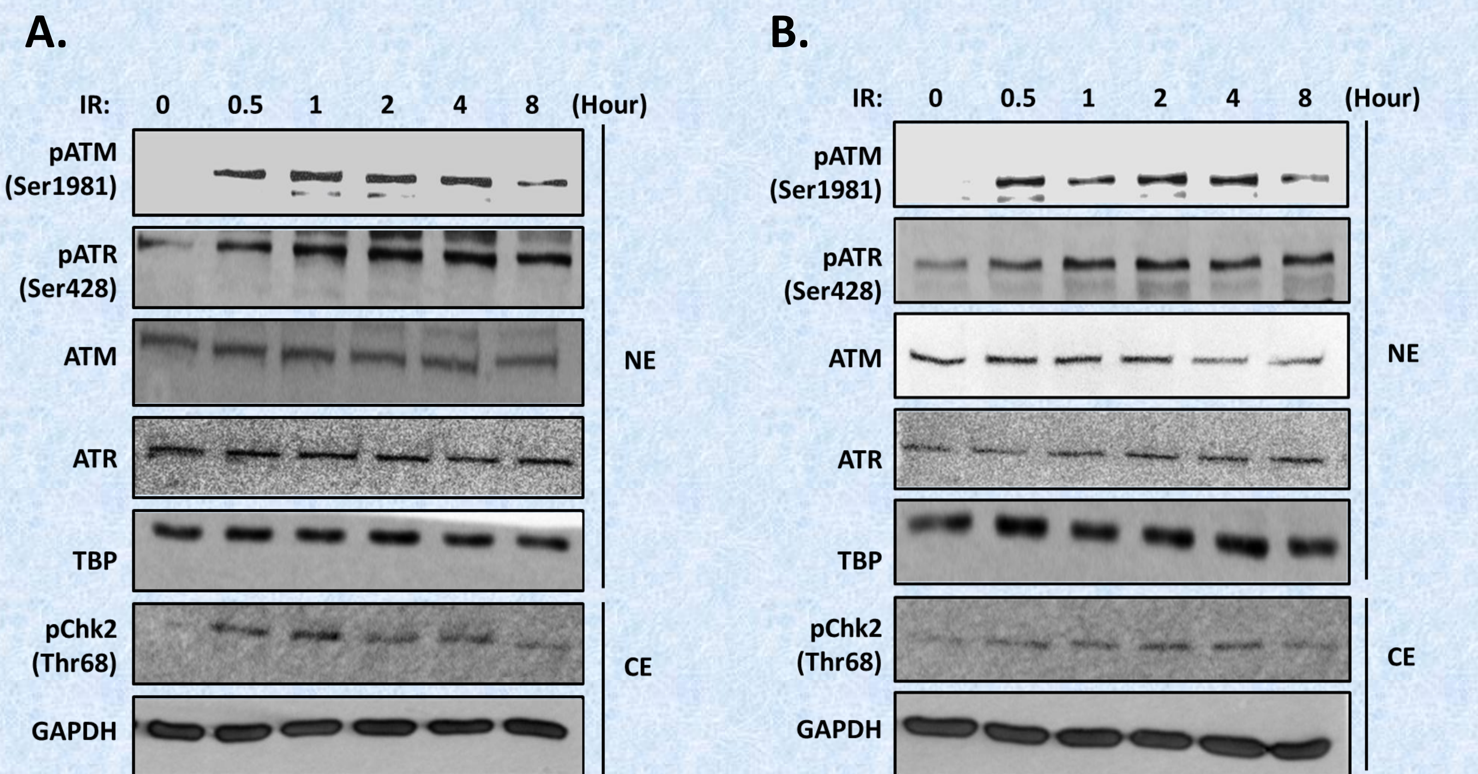



**Figure 1.** Determination of activation kinetics of ATM and ATR kinases along with their downstream substrates in 82-6 hTERT human fibroblast cells after damage induction by western blotting. **A.** 82-6 hTERT human fibroblasts were treated with 2Gy gamma rays and collected at 0, 0.5, 1, 2, 4 and 8 hour post irradiation. Nuclear fractions of cell extracts were blotted for phosphorylated and native forms of ATM and ATR kinases. Cytoplasmic fractions were blotted for phosphorylated Chk2, a downstream substrate of ATM kinase. **B.** Methodology same as in **A.** except that the cells were treated with specific ATM kinase inhibitor ku55933 one hour prior to gamma irradiation.

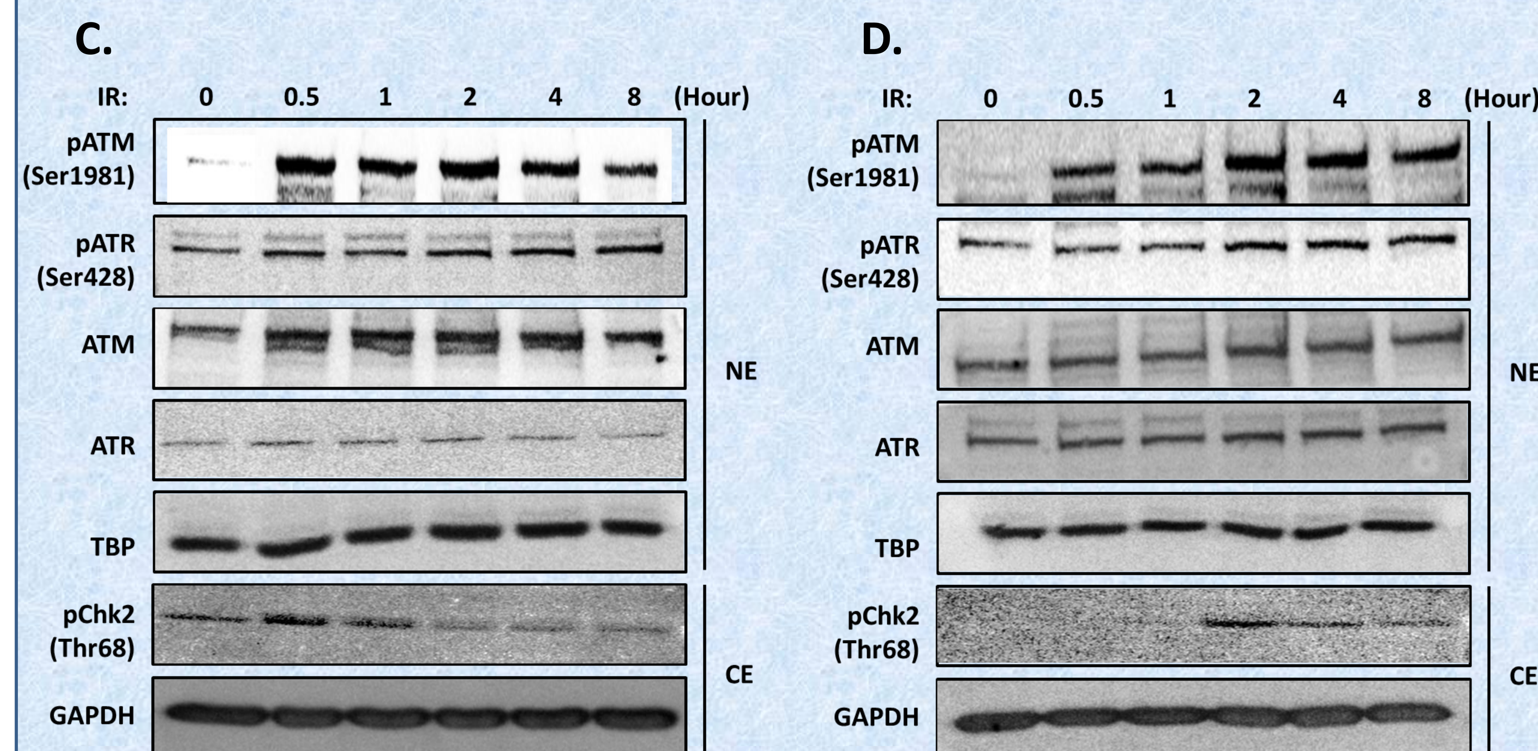
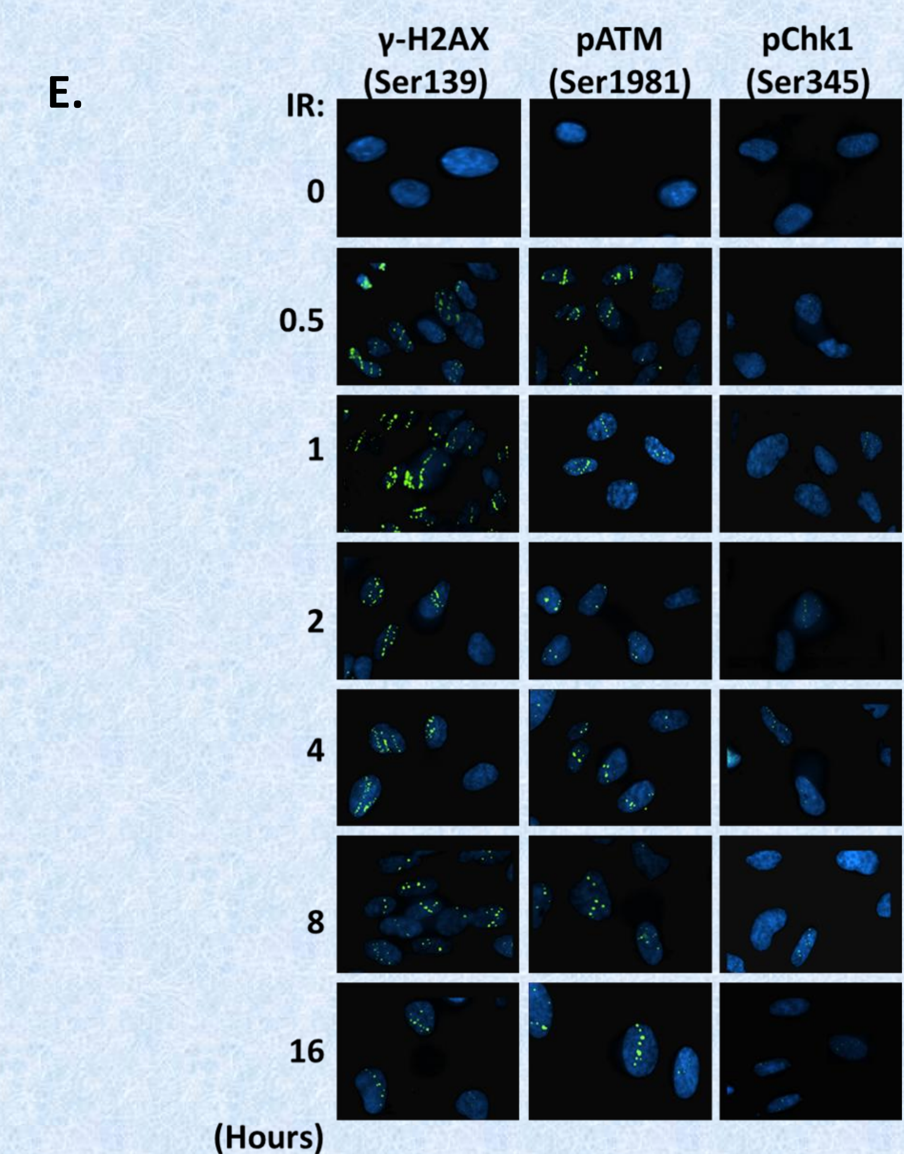



**Figure 2.** Determination of activation kinetics of ATM and ATR kinases along with their downstream substrates in 82-6 hTERT human fibroblast cells by western blotting. **C.** 82-6 hTERT human fibroblasts were treated with 2Gy 600MeV/u $^{56}$Fe radiation and collected at 0, 0.5, 1, 2, 4 and 8 hour post damage induction. Nuclear fractions of cell extracts were blotted for phosphorylated and native forms of ATM and ATR kinases. Cytoplasmic fractions were blotted for phosphorylated Chk2, a downstream substrate of ATM kinase. **D.** Methodology same as in **C.** except that the cells were treated with specific ATM kinase inhibitor ku55933 one hour prior to irradiation.



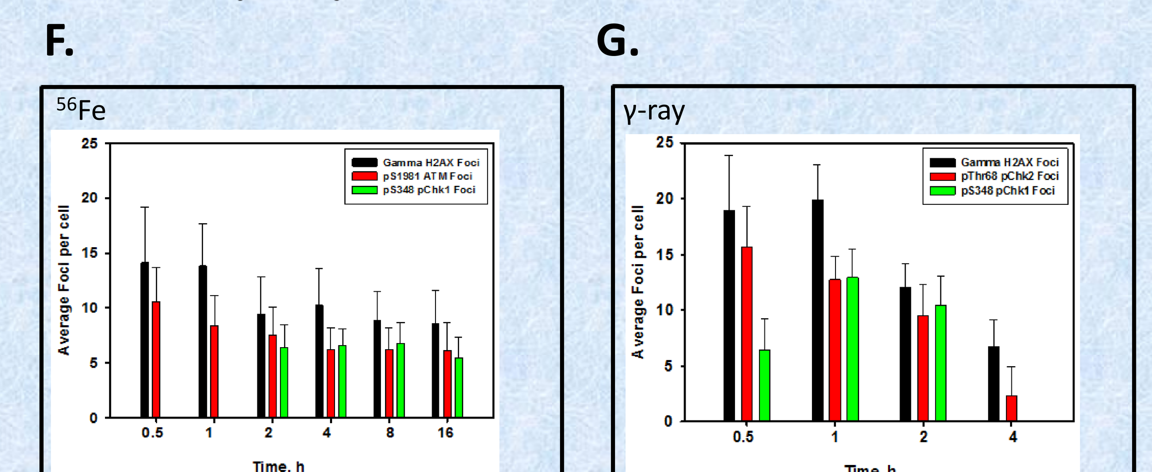




**Figure 3.** Determination of repair foci kinetics of ATM and ATR or their downstream substrates along with DNA damage marker γ-H2AX in 82-6 hTERT human fibroblast cells after high and low LET radiation. **E.** Fluorescence microscopy images depicting foci kinetics of γ-H2AX, pATM and pChk1 in 82-6 hTERT human fibroblast cells irradiated with 1Gy 600MeV/u $^{56}$Fe nuclei and fixed at 0, 0.5, 1, 2, 4, 8 and 16 hours post irradiation. **F.** Bar graph depicting the foci kinetics of γ-H2AX, pATM and pChk1 in 82-6 hTERT human fibroblast cells treated with 1Gy 600MeV/u $^{56}$Fe radiation. **G.** Bar graph depicting the foci kinetics of γ-H2AX, pChk2 and pChk1 in 82-6 hTERT human fibroblast cells treated with 1Gy gamma rays.

## Conclusions

1. A switch from ATM to ATR kinase signaling is evident in human fibroblasts treated with gamma rays.
2. The switching is delayed in cells treated with high LET radiation.
3. ATM kinase plays an essential role in repair of high LET radiation induced damage.
4. Chemical inhibition of ATM kinase did not alter activation kinetics of ATR kinase.

This research is supported by NASA Space Radiation Program. We are indebted to Medical Department and NSRL at BNL, New York.