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Detecting ATM-Dependent Chromatin Modification in DNA Damage Response

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Abstract

Loss of function or mutation of the ataxia–telangiectasia mutated gene product (ATM) results in inherited genetic disorders characterized by neurodegeneration, immunodeficiency, and cancer. Ataxia-telangiectasia mutated (ATM) gene product belongs to the PI3K-like protein kinase (PIKKs) family and is functionally implicated in mitogenic signal transduction, chromosome condensation, meiotic recombination, cell-cycle control, and telomere maintenance. The ATM protein kinase is primarily activated in response to DNA double strand breaks (DSBs), the most deleterious form of DNA damage produced by ionizing radiation (IR) or radiomimetic drugs. It is detected at DNA damage sites, where ATM autophosphorylation causes dissociation of the inactive homodimeric form to the activated monomeric form. Interestingly, heat shock can activate ATM independent of the presence of DNA strand breaks. ATM is an integral part of the sensory machinery that detects DSBs during meiosis, mitosis, or DNA breaks mediated by free radicals. These DNA lesions can trigger higher order chromatin reorganization fuelled by posttranslational modifications of histones and histone binding proteins. Our group, and others, have shown that ATM activation is tightly regulated by chromatin modifications. This review summarizes the multiple approaches used to discern the role of ATM and other associated proteins in chromatin modification in response to DNA damage.

Keywords

Ataxia–telangiectasia; Telomerase; Double-stranded DNA breaks; Chromatin modification

1 Introduction

Maintenance of genomic stability depends on appropriate cellular responses to DNA damage [1, 2]. The response is based on a complex network of signaling pathways that activate numerous processes that ultimately lead to damage repair and cell survival or death. The precise sequence of events that follow IR-induced DNA damage in cells is still not well understood but it is increasingly evident that a complex series of proteins are recruited in a time dependent manner at different stages of the cell cycle to maintain genomic stability after DNA damage. The ATM protein plays a key role in this mechanism by sensing, processing, and signaling of IR-induced DNA damage through its interactions with various proteins [3]. Notably, cells deficient in ATM display a high frequency of spontaneous chromosomal aberrations, high rates of intrachromosomal recombination, and error-prone

recombination [4–7]. The cells also have a higher level of initial and residual chromosomal aberrations in G₁ and G₂ phase cells after IR exposure, as determined by analysis of prematurely condensed chromosomes [8–10] and by karyotypic examination of metaphase chromosome spreads [10, 11].

1.1 ATM Activation and DNA Repair

Mammalian cells contain many highly specialized chromatin structures such as telomeres, replication forks, and compact heterochromatin. These structures have specific patterns of histone modifications, differences in nucleosome packing density and varied interactions with binding proteins (reviewed in [12]). On the other hand, proper recognition and recruitment of DNA repair proteins requires efficient access to the damaged site. Hence, the complex and diverse organizational pattern of chromatin poses a series of challenges to the DSB repair machinery which necessitates the ability to rapidly reorganize chromatin and rapidly complete DNA repair with a minimal number of steps [13]. Chromatin structural alterations initiated by posttranslational modification such as phosphorylation, ubiquitination, acetylation, and deacetylation have all been linked to the repair of damaged DNA. Under normal conditions, cellular ATM is found in a homodimeric inactive state but upon IR exposure ATM undergoes rapid autophosphorylation at serine 1981 and dissociates into active monomers, with kinase activity (*see* Note 1). It is proposed that the ATM conversion from dimer to monomer is regulated by the state of the chromatin structure itself [14]. Activated ATM phosphorylates histone H2AX at the carboxy-terminal serine 139 immediately following ionizing radiation exposure [15], or Spoll-induced DSB formation during meiotic prophase [16], and together with dephosphorylation of Tyr 142, binds MDC1 (mediator of DNA damage checkpoint 1 protein). Phosphorylation of H2AX spreads along the chromatin for hundreds of kilobases away from the DSB site in mammalian cells [17, 18]. Analysis of H2AX-deficient mice has demonstrated a role for H2AX in a variety of responses to DSBs, including DNA repair, checkpoint signaling, and immunoglobulin gene class switching [19] (*see* Notes 2 and 3). Mice deficient in H2AX (*H2ax*^{-/-}) exhibit male-specific sterility, likely due to defects in chromatin remodeling during meiosis. In addition to H2AX, activated ATM has been shown to phosphorylate several other substrates [3] including proteins involved in checkpoint activation (p53, and Chk2), and DNA repair such as BRCA1 CtIP, and 53BP1 [20, 21]. ATM also has a critical role in maintaining chromosome condensation in the vicinity of recombination intermediates [4, 22] and ATM phosphorylates the structural maintenance of chromosomes protein (Smc1) necessary for sister chromatid cohesion [4, 23, 24]. Several studies have indicated that the MRN (Mre11/Rad50/NBS1) complex as well as TRF2 either influence activation of ATM or serve to modulate/amplify ATM activity [1, 4, 25–27]. Thus, ATM is a “hierarchical kinase,” capable of initiating many pathways simultaneously [4, 5, 28] (Fig. 1). Studies on DNA and chromosome damage after IR exposure suggested that ATM defective A-T cells more

¹Chromatin structural perturbations induced by DNA double-strand breaks have been proposed to serve as a trigger for ATM activation as determined by ATM autophosphorylation and enhanced kinase activity.

² γ -H2AX foci are usually the consequence of chromosomal DNA double strand breaks, however, this does not stand true for heat-induced γ -H2AX foci, because hyperthermia does not induce DNA double strand breaks.

³Ionizing radiation induced chromosomal DNA breaks correlate with the number of γ -H2AX foci; however, γ -H2AX foci are seen in S-phase cells without exposure to DNA damaging agents.

efficiently convert DNA damage into chromosome damage [8, 9] presumably due to an inherent chromatin alteration [29]. The chromatin-modifying factor human males absence on the first (hMOF) hMOF influences IR-induced initiation of ATM kinase activity [30]. Furthermore, detailed studies have revealed that heat shock, which induces chromatin alterations independent of DNA damage, also activates ATM [11] (*see* Note 4).

1.2 Chromatin Modifiers and DNA Damage Response

The DNA repair involves alterations in chromatin structure, which are required for direct access to DNA damage sites. This process is facilitated by chromatin modifying factors, which temporarily allow DNA loss or detachment from histones and other bound proteins. Posttranslational histone acetylation has emerged as key regulatory events and a widespread modification in DNA damage responses. Acetylation is regulated by the balance between histone acetyl-transferases (HATs), which transfer an acetyl moiety, and histone deacetylases (HDACs), which reverse the reaction. Reversible acetylation of four lysines (K), at positions 5, 8, 12, and 16 in the amino-terminal tail of histone H4 occurs *in vivo* in all eukaryotes [31, 32]. Hyperacetylation of histone H4 can lead to unfolding of the nucleosomal fiber [33]. Acetylation of histone H4 at K16 occurs on the transcriptionally hyperactive male X chromosome of *Drosophila* polytene chromosomes [34], an indirect indication of greater access to the DNA by RNA polymerase. Studies have shown that histone H4K16 controls chromatin structure and protein interactions [35]. Tip60 (Tat interacting protein) interacts with ATM, acetylates histones (H2A, H3, H4), and also plays a role in DNA repair [36, 37]. In *Drosophila* Tip60 acetylates nucleosomal phospho-H2Av and exchanges it with an unmodified H2Av [38]. Bird et al. [39] reported that the acetylation of histone H4 by Esa1 (essential SAS2-related acetyltransferase) is required for DNA repair in yeast and suggested that a similar modification may function in mammalian cells. Acetylation of H3K56 has been shown to play a crucial role in DNA replication and genomic stability [40, 41]. ATM has been reported to regulate the function of several histone-modifying factors. We previously identified hMOF as an ATM interacting protein and have provided evidence that hMOF participates in the activation of ATM in response to DNA damage [30]. More recently, we have further shown that ATM-mediated MOF phosphorylation modulates the function of 53BP1, an adapter/mediator critical for processing damaged DNA ends, thus establishing a regulatory role for MOF in DSB repair pathway choice in S/G2 phase cells [42, 43].

In spite of accumulating literature concerning histone modifications that participate in specific repair processes, pathway choice, or checkpoint control, mechanistic studies are still limited. Identification of chromatin modifying factors that play a role in the IR response will help to understand the mechanisms by which IR-induced chromatin modifications trigger ATM activation. The protocols presented in this chapter describe how to analyze DNA damage using different methods (*see* Note 5).

⁴Two areas of research that are being actively pursued are: (1) determination of which types of chromatin alterations are responsible for the ATM activation and (2) the mechanism by which such alterations activate ATM function.

⁵To determine cellular ATM activation, cells must be physiologically viable as determined at least by trypan blue dye exclusion assay.

1.3 Determination of ATM Interacting Proteins

Various approaches are used to determine the interactions between chromatin and chromatin binding proteins. For in vivo analysis, the most commonly used techniques are described below.

2 Materials

2.1 Co-immunoprecipitation and immunoprecipitation

1. Total cell extracts or nuclear extracts from cell lines under study. The cells should be growing exponentially at the time of protein extraction using the extraction buffers described below.
2. Protein A/G beads and antibodies targeting the protein of interest.
3. Immunoprecipitation (IP) buffer: (Mild) NP40 buffer; 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 % NP-40, (More Stringent) RIPA buffer; 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5 mM EGTA, 1 % Triton X-100, 0.1 % sodium deoxycholate, 0.1 % SDS, 140 mM NaCl.
4. Protease inhibitor cocktail (100×): 18 mg/ml PMSF, 50 µg/ml aprotinin, 50 µg/ml leupeptin, 70 µg/ml pepstatin in 100 % methanol; store at -20 °C.
5. Sample loading buffer: 50 mM Tris-HCl, pH 6.8, 100 mM dithiothreitol (DTT), 2 % SDS, 0.02 % bromophenol blue, 10 % glycerol.

2.2 Resolution of Proteins with SDS-PAGE and Western Blotting Analysis

2.2.1 SDS-PAGE Separation

1. SDS-PAGE 4–20 % with varying acrylamide concentration depending on the molecular weight of proteins to be resolved. The protocol for casting 8 % and 12 % gels is described below.
 - a. Stacking gel (10-ml total volume): 6.3 ml H₂O, 2.5 ml 0.5 M Tris-HCl, pH 6.8, 1.0 ml 40 % acrylamide solution (37.5:1= acrylamide-bis), 100 µl 10 % SDS, 100 µl 10 % APS (ammonium persulfate), 20 µl TEMED.
 - b. 8 % SDS-PAGE separating gel (10-ml total volume): 5.3 ml H₂O, 2.5 ml 1.5 M Tris-HCl, pH 8.8, 2.0 ml 40 % acrylamide solution, 100 µl 10 % SDS, 100 µl 10 % APS, 10 µl TEMED.
 - c. 12 % SDS-PAGE separating gel (based on 10-ml total volume): 4.3 ml H₂O, 2.5 ml 1.5 M Tris-HCl, pH 8.8, 3.0 ml 40 % acrylamide solution, 100 µl 10 % SDS, 100 µl 10 % APS, 10 µl TEMED.
2. SDS-PAGE running buffer (10×): 30.3 g Tris-HCl, 144 g glycine, 10 g SDS, pH to 8.3 to 1 l with ddH₂O.

2.2.2 Western Blotting

1. Blocking solution (5 % dry milk powder in TBST [pH 7.4]).

2. ECL (enhanced chemiluminescent) detection kit.
3. SDS-PAGE transfer buffer (1×): 700 ml H₂O, 100 ml of 10× SDS-PAGE running buffer without SDS (30.3 g Tris–HCl, 144 g glycine, pH 8.3 to 1 l dH₂O), and 200 ml methanol.
4. 10× Phosphate-buffered saline (10× PBS) (pH 7.4): To prepare 1 l of 10× PBS, dissolve the reagents (80 g NaCl, 2.0 g KCl, 14.4 g Na₂HPO₄, 2.4g KH₂PO₄) in 800 ml of H₂O. Adjust the pH to 7.4 (or 7.2, if required) with HCl, and then add H₂O to 1 l.
5. Primary and secondary (Horseradish peroxidase (HRP)-conjugated) antibodies.
6. TBS-T (pH 7.4) 20 mM Tris–HCl, 150 mM NaCl, and 0.2 % Tween 20.
7. Microcentrifugation tube (1.5-ml).
8. Electrotransfer apparatus.
9. Enhanced chemiluminescent detection equipment.
10. Water bath (for boiling samples).
11. PVDF membrane or nitrocellulose membrane.
12. SDS-polyacrylamide gel electrophoresis equipment.

2.3 Immunofluorescence Staining

1. Specific primary antibodies to proteins of interest, and fluorescence labeled secondary antibodies.
2. Blocking solution (5 % normal goat serum in PBS).
3. Paraformaldehyde (PFA) (4 %) in 1× PBS: 4 % paraformaldehyde solution: Add 4 g of EM grade paraformaldehyde to 100 ml 1× PBS and dissolve with heat carefully.
4. 1× PBS and Hardset mounting medium with DAPI.
5. 2-wells or 4-wells glass chamber slides.
6. Fluorescence microscope or confocal microscope.

2.4 ATM Kinase Assay

1. Lysis buffer: 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 % Tween. 20, 0.2 % NP-40, 1 mM NaF, 1 mM NaVO₄, 50 mM glycerophosphate, 10 % glycerol, 1 mM PMSF, 2 µg/ml pepstatin A, 5 µg/ml leupcetin, 10 µg/ml aprotinin, and 1 mM dithiothreitol (DTT).
2. Anti-ATM antibodies and Protein A/G Sepharose beads.
3. 0.5 M LiCl.
4. Kinase buffer: 50 mM Hepes pH 7.5, 150 mM NaCl, 1 % Tween 20, 0.2 % NP-40, 1 mM NaF, 1 mM NaVO₄, 1 mM DTT, 10 mM MnCl₂.

2.5 Chromatin Immunoprecipitation (ChIP)

1. Swelling buffer: 25 mM Hepes, pH 7.8, 1.5 mM MgCl₂, 10 mM KCl, 0.1 % NP-40, 1 mM DTT, 0.5 mM PMSF, and Protease inhibitor cocktail.
2. Sonication buffer: 50 mM Hepes pH 7.9, 140 mM NaCl, 1 mM EDTA, and 1 % Triton X-100, 0.1 % Na-deoxycholate, 0.1 % SDS, 0.5 mM PMSF, and Protease inhibitor cocktail.
3. Wash buffer A: 50 mM Hepes pH 7.9, 500 mM NaCl, 1 mM EDTA, 1 % Triton X-100, 0.1 % Na-deoxycholate, 0.1 % SDS, 0.5 mM PMSF, and Protease inhibitor cocktail, Wash buffer B: 50 mM Hepes pH 7.9, 250 mM LiCl, 1 % NP-40, 1 % Na-deoxycholate, 1 mM EDTA, and Protease inhibitor cocktail (Roche).
4. Elution buffer: 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 1 % SDS.
5. Sonicator.
6. Antibodies to target proteins.
7. PCR reagents.

2.6 Histone Acetyl Transferase Assay

1. Histone acetyl transferase kit with reagents and controls.
2. Coenzyme A.
3. DMSO.
4. Recombinant proteins, and acetyl transferases of interest.

2.7 Replication Restart Assay

1. 5-iododeoxyuridine (IdU).
2. Hydroxyurea (HU).
3. 5-chlorodeoxyuridine (CldU).
4. Hypotonic lysis solution: 10 mM Tris-HCl, pH 7.4, 2.5 mM MgCl₂, 1 mM PMSF, and 0.5 % NP-40.
5. 5 % bovine serum albumin (BSA).
6. Primary antibodies against IdU and CldU.
7. Secondary antibodies: anti-rabbit Alexa Fluor 488-conjugated, and anti-mouse Alexa Fluor 568-conjugated.
8. 0.1 % Triton X-100.
9. VECTASHIELD mounting medium without 4',6-diamidino-2-phenylindole (DAPI).
10. Phosphate buffered saline (PBS).
11. ImageJ software for analysis.

2.8 Chromosomal DNA Strand Break Analysis

2.8.1 Pulsed Field Gel Electrophoresis (PFGE)

1. Tris-EDTA buffer [10 mM Tris-HCl, 1 mL EDTA (pH 8)].
2. 0.8 % agarose gels, ethidium bromide, NaCl, and Triton X-100.
3. CHEF DRIII system (Bio-Rad, Hercules, CA).
4. Charge-coupled device camera system under UV transillumination.

2.8.2 Neutral Filter Elution

1. Cells of interest and appropriate growth medium for cells.
2. [Methyl ^3H]thymidine (20 Ci/mmol) or [$2\text{-}^4\text{C}$]thymidine (50 mCi/mmol) for radioactive determination.
3. Unlabeled thymidine and test compound.
4. PBS, ice-cold.
5. Cell lysis solution, pH 9.6: 2 % SDS, 25 mM EDTA, and 0.1 M glycine (optional). Adjust pH to 9.6 with 1 N NaOH.
6. Cell lysis solution (above) containing 0.5 mg/ml proteinase K.
7. Alkaline elution solution, pH 12.3: 0.1 % SDS, 20 mM EDTA, 0.8 % tetrapropylammonium hydroxide. Adjust pH to 12.3 with 20 % (w/v) tetrapropylammonium hydroxide in water.
8. Scintillation fluid (e.g., Ecolume, ICN Biomedicals) containing 0.7 % (v/v) acetic acid 0.2 M tetrasodium EDTA, pH 10 (pH adjusted with 1 N NaOH).
9. 1 N HCl.
10. 0.4 N NaOH.
11. 1 M potassium dihydrogen phosphate.
12. 17 mM KH_2PO_4 .
13. 150 μM Hoechst 33258 stock solution.
14. Tabletop centrifuge and 2.0- μm polycarbonate filter (25-mm diameter).
15. 1-l vacuum flask, 60 °C oven or water bath.
16. Alkaline elution funnel apparatus (e.g., Millipore).
17. Aluminum foil or black paper cylinder.
18. Multichannel peristaltic pump and suitable Tygon tubing.
19. Fraction collector.
20. Glass scintillation vials and liquid scintillation counter or fluorometer.

3 Methods

3.1 Immunoprecipitation and Co-immunoprecipitation

Several approaches are used to determine ATM autophosphorylation and ATM-dependent phosphorylation. These include co-immunoprecipitation, western blotting, and cytological analysis. First phosphorylation sites of protein substrate are identified by MALDI-TOF mass spectroscopy. Once the residue(s) are identified, phosphospecific antibodies are generated. Co-immunoprecipitation is carried out followed by western blotting to resolve and quantitate the phosphorylation status of the interacting proteins, or immunostaining of fixed cells to detect the phosphorylated form of proteins. The beads selected to bind the antibodies depend on the species and subclass of the antibody used (e.g., Protein A beads for rabbit, mouse IgG2a, IgG2b, and IgG3; and Protein G beads for mouse IgG1 and most subclasses of rat IgGs).

1. Wash 50 μ l of Protein A/G bead suspension by adding 500 μ l cold IP buffer in a 1.5 ml eppendorf tube and spinning for 5 s, $15,300 \times g$ in a microcentrifuge. Remove supernatant and repeat the washing step twice. Remove supernatant.
2. To the washed beads, add 5–10 μ g of specific antibody or control antibody.
3. Incubate for 1 h at 4 °C rotating gently.
4. Wash beads three times with IP buffer as in **step 1** to remove unbound antibodies. Add cold protein extract in IP buffer with protease inhibitors (500–1,000 μ g). Adjust the volume to 500 μ l with IP buffer. Incubate at gentle rotation at 4 °C for 1–4 h.
5. Carefully remove the supernatant completely after spinning down the beads and wash/pellet the beads three to five times using 500 μ l of IP buffer.
6. After the last wash, aspirate supernatant and add 50 μ l of sample loading buffer to bead pellet. Vortex and heat to 100 °C for 5 min. Spin at $10,000 \times g$ for 5 min collect the supernatant and load onto an SDS-polyacrylamide gel.

3.2 Resolution of Proteins with SDS-PAGE and Western Blotting Analysis

3.2.1 SOS PAGE Analysis

1. Remove medium from the cells, approximately 1×10^6 in 60 mm dish. Rinse the cells once with 1,000 μ l of PBS and add 200 μ l of trypsin-EDTA. Incubate for 1 min at 37 °C; suspend the cells and add 1,000 μ l of DMEM medium to quench the trypsin.
2. Transfer the suspended cells to a chilled 1.5-ml centrifugation tube. Collect the cells by centrifugation at $300 \times g$ for 2 min at 4 °C. Resuspend the cell pellet in 500 μ l ice-cold PBS and centrifuge again.
3. Resuspend the cells in 500 μ l ice cold buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 % NP40, 1 mM DTT, 1 mM EDTA, 50 mM glycerophosphate, 10 % glycerol, 1 mM phenylmethylsulfonyl fluoride, 2 μ g/ml pepstatin A, 5 μ g/ml leupeptin, 10 μ g/ml aprotinin, and 1 mM dithiothreitol (DTT) at 4 °C for 10 min.

then sonicate three times for 10 s each on ice. Centrifuge for 10 min at 15,000 $\times g$ to pellet insoluble cell debris.

4. Recover the supernatant and determine the protein concentration by Bradford protocol. For gel analysis, use 20 μg of supernatant protein in 1 $\times$ Laemmli buffer and incubate the sample for 3 min in a boiling water bath and vortex.
5. Separate the proteins by loading onto an SDS-polyacrylamide gel using an acrylamide concentration appropriate to resolve the molecular weight of the targeted protein.
6. Transfer proteins from the gel to a PVDF or nitrocellulose membrane using an electrotransfer apparatus.

3.2.2 Western Blotting Analysis

1. Incubate the membrane in blocking solution (PBS solution containing 0.05 % Tween 20, and 5 % nonfat dried milk, to block nonspecific binding) for 1 h at 37 $^{\circ}C$.
2. Replenish the blocking solution with fresh blocking solution and add the primary antibody at the appropriate dilution. Incubate for 1–2 h at room temperature or overnight at 4 $^{\circ}C$. Wash the blot four times with TBST for 10 min.
3. For ECL detection, incubate the blot with either HRP-conjugated secondary antibody at a suitable dilution in blocking solution for 1–2 h at room temperature. After washing with TBST four times 10 min each, perform ECL detection.

3.3 Immunostaining

1. Grow cells on coverslips and wash with PBS.
2. Incubate the samples in 4 % PFA 10–20 min at room temperature. Rinse the specimens three times in 1 $\times$ PBS for 5 min each (total time of 15 min).
3. Permeabilize the cells for 20 min in 0.5 % NP-40. Circle the area of interest on each slide with a hydrophobic PAP pen. Gently pipette ~200–300 μl of blocking solution onto each slide. Incubate the slides for 1–2 h at room temperature.
4. Remove the blocking solution by draining the excess. Add ~100–200 μl of primary antibody diluted in blocking solution to each slide. Incubate the slides overnight at 4 $^{\circ}C$.
5. Remove the primary antibody by rinsing the slides three times in PBS for 10 min each at room temperature. Drain the excess PBS, and add 100–200 μl of secondary antibody diluted in blocking solution. Incubate the slides for 1–2 h at room temperature.
6. Remove the secondary antibody by rinsing the slides three times in PBS for 10 min each at room temperature. Drain as much PBS from the slides as possible. Add one drop of mounting medium directly onto each section.

7. Carefully place a coverslip onto each slide. Allow mounting medium to harden for at least 3h at room temperature before imaging. Image the slides on a confocal or fluorescent microscope.

3.4 ATM Kinase Assay

ATM is a serine/threonine kinase and specifically phosphorylates SQ/TQ sites. To determine the substrates of ATM phosphorylation, in vitro kinase assays are performed.

1. Cells are broken open in lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 % Tween 20, 0.2 % NP-40, 1 mM NaF, 1 mM NaVO₄, 50 mM glycerophosphate, 10 % glycerol, 1 mM phenylmethylsulfonyl fluoride, 2 µg/ml pepstatin A, 5 µg/ml leupeptin, 10 µg/ml aprotinin, and 1 mM DTT).
2. Lysates are pre-cleared for 1 h at 4 °C with 1 mg rabbit purified IgG and 40 µl protein A/G beads.
3. Endogenous ATM proteins are immunoprecipitated with anti-ATM antibodies and protein A/G Sepharose. Immunoprecipitants are washed three times with lysis buffer, once with lysis buffer and 0.5 M LiCl, and three times with kinase buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 1 % Tween 20, 0.2 % NP-40, 1 mM NaF, 1 mM NaVO₄, 1 mM DTT, 10 mM MnCl₂).
4. Immunoprecipitant beads are mixed with kinase buffer containing 10 µCi [γ -³²P]ATP and 0.1 mg GST-p53₁₋₁₀₁, incubated for 20 min at 30 °C. The kinase reactions are stopped with SDS-PAGE loading buffer and reaction mixtures are separated by SDS-PAGE.
5. Western transferred ATM and substrates are visualized and quantitated on a PhosphorImager. ATM proteins are detected using anti-ATM antibodies.

3.5 Chromatin immunoprecipitation (ChIP) Assay

The Chromatin Immunoprecipitation (ChIP) assay is used to analyze the association of proteins with specific DNA regions. This technique involves cross-linking of the proteins to the bound DNA then preparing a soluble form of the chromatin followed by immunoprecipitation with an antibody for the protein of interest. The region of DNA associated with the protein is then identified by PCR amplification of the co-immunoprecipitated genomic DNA [43, 44]. The general methodology is described below.

1. Culture cells by growing in 150 mm dishes, containing $2-5 \times 10^7$ cells per plate. Replace media with fresh serum-free medium (27 ml). Cross-link by adding 3 ml formaldehyde (10 %) and mix immediately. Incubate at room temperature for 10 min.
2. Add 3 ml glycine (1.25 M) and mix immediately to stop the reaction and incubate for 5 min at room temperature.
3. Place plates on ice and wash three times with 20 ml ice-cold PBS/0.5 mM PMSF. Scrape cells into 20 ml ice cold PBS/PMSF and centrifuge for 5 min at 1,000 rpm.

4. Resuspend pellet in 10 volumes of swelling buffer and dounce-homogenize to isolate nuclei by centrifugation at 5,000 rpm for 5 min.
5. Resuspend the pellets in 5–10 ml sonication buffer and sonicate ten times for 10–20 s at 80 % setting on ice. Centrifuge the samples twice at 14,000 rpm for 15 min and centrifuge the supernatant again for 5 min.
6. Pre-clear the lysate by incubating by constant rotation with Protein-A or G Sepharose. Redistribute the samples to 1 ml aliquots for IP reaction.
7. Add about 5 µg of antibody and incubate at 4 °C for 2 h; add 40 µl of Protein-A or G Sepharose per IP (pre-equilibrated with appropriate IP buffer) and incubate overnight by constant rotation at 4 °C.
8. Centrifuge the beads at 6,000 rpm for 3 min and wash with 1 ml sonication buffer, and the wash step should be repeated three times with 10 min each time.
9. Wash again two times with buffer A, and two times with buffer B, and then with TE buffer.
10. Add 200 µl of elution buffer to the beads before incubating at 65 °C for 10 min and centrifuging at 14,000 rpm for 1 min.
11. Carry out the de-cross-linking step by adding required amount of NaCl and incubating at 65 °C overnight or for at least 5 h.
12. Add RNase A and incubate at 37 °C for 1 h.
13. Add EDTA and proteinase K and incubate at 42 °C for 2 h.
14. Extract the samples two times with phenol–chloroform–isoamyl alcohol and once with chloroform–isoamyl alcohol. Further, make final concentration of 0.05 µg/µl glycogen, 0.3 M Na-acetate, and 70 % ethanol and incubate at –20 °C overnight.
15. Centrifuge at 14,000 rpm 30 min and wash with 70 % ethanol. Then resuspend the IP and INPUT samples in 100 µl of 10 mM Tris (pH 7.5) and carry out PCR analysis, or submit them for sequencing.

3.6 Histone Acetyl-Transferase Assay

Histone acetylation alters nucleosomal interactions of the chromatin fiber to facilitate decondensation and enhance access to nucleosomal DNA. We have shown that the absence of an acetyl transferase, MOF, influences ATM activation and results in delayed appearance of gamma-H2AX foci. We have further shown that ATM-mediated MOF-T392 phosphorylation is involved in repair pathway choice [43]. A simple, robust in vitro assay to measure histone acetyl transferase activity is described below [45]. The assay can be adapted for other substrates by generating other fusion proteins and acetyl transferases of interest by modifying conditions.

1. Recombinant proteins are immunoprecipitated from 100 µg of nuclear extract using either anti-FLAG or anti-HA antibodies plus Protein A-agarose (Sigma).

2. Immunoprecipitates are washed twice in phosphate buffered saline containing 0.1 % Tween 20 (PBS-T), and once in acetyltransferase assay buffer (50 mM Tris-HCl pH 8.0, 10 % glycerol, 10 mM butyric acid, 0.1 mM EDTA, 1 mM DTT, 1 mM PMSF).
3. Each reaction contains immunoprecipitated proteins, mixed with 10 μ M acetyl CoA, and different quantities of purified substrates as per the kit protocol.
4. Acetylation reactions are incubated for 45 min at 30 °C on a rotating platform, followed by SDS-PAGE sample buffer elution, followed by electrophoresis and western blotting.

3.7 Replication Restart Assay

DNA replication occurs during the synthesis (S) phase of the cell cycle and starts at predefined replication origins or origin firing [46, 47]. Once the origins of replication have fired, the DNA replication proteins organize into a structure called the replication fork. These forks are highly susceptible to stalling or collapse when they encounter obstacles such as unrepaired DNA damage, chromatin bound proteins, or secondary structures of the DNA. Chemicals such as hydroxyurea (HU) also lead to fork stalling or collapse. While the stalled forks are capable of reversing when the stress is removed, the collapsed forks are essentially irreversible due to generation of double strand breaks or dissociation of replication machinery [48]. In recent years, the single-molecule analysis of DNA replication has become the standard assay for replication fork restart efficiency as a response to inhibition of DSB repair pathways. While several lines of clear evidence point to the role of ATR and its substrates [49–52], and nuclear structural proteins [53] in modulating the replication fork restart, only limited evidence exists that shows ATM involvement in replication restart in cooperation with ATR [54, 55]. Nevertheless, this assay system accurately analyzes the involvement of DNA helicases, nucleases, and homologous recombination factors during replicative stress. The general methodology is described below.

1. The exponentially growing cells are pulsed with 50 mM 5-iododeoxyuridine (IdU) for 20 min, and then washed three times with phosphate-buffered saline (PBS).
2. Then the cells are treated with 2 mM HU for the appropriate intervals, washed three times with PBS.
3. Then the cells are incubated in fresh medium containing 50 mM 5-chlorodeoxyuridine (CldU) for 20 min, and then washed three times in PBS.
4. The cells labeled with IdU and CldU are mixed with unlabeled cells in a ratio of 1:10, and 2 μ l cell suspensions are dropped onto a glass slide and then mixed with a 20 μ l hypotonic lysis solution (10 mM Tris-HCl, pH 7.4, 2.5 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride [PMSF], and 0.5 % Nonidet P-40) for 8 min.
5. The slides are air-dried and fixed with methanol, followed by 70 % ethanol, and washed with 1 $\times$ PBS.
6. The slides are then blocked with 5 % bovine serum albumin (BSA) for 15 min, and incubated with primary antibodies against IdU and CldU and secondary antibodies,

anti-rabbit Alexa Fluor 488-conjugated, and anti-mouse Alexa Fluor 568-conjugated for 1 h.

7. The slides are washed with 1× PBS with 0.1 % Triton X-100 and mounted with VECTASHIELD mounting medium without 4',6-diamidino-2-phenylindole (DAPI).
8. ImageJ software is used to analyze and quantitate the DNA fibers.

3.8 Chromosomal DNA Strand Break Analysis

Two different approaches are used to measure DNA strand breaks after treatment with heat shock or IR. exposure or heat shock + IR exposure. Biochemical methods include (a) pulsed field gel electrophoresis (PFGE) [11] and (b) Neutral filter elution [9, 30] while cytological approaches involve either the analysis of chromosomes at metaphase or interphase using the premature chromosome condensation technique as described previously [8, 56]. The biochemical approaches are described here mainly.

3.8.1 Pulse Field Gel Electrophoresis

1. In PFGE, cells are harvested, embedded in agarose plugs, and lysed in situ.
2. Plugs are washed in Tris-EDTA buffer [10 mM Tris-HCl, 1 mM EDTA (pH 8)] and PFGE is carried out with a CHEF DRIII system in 0.8 % agarose gels.
3. The gels are run at 14 °C with linearly increasing pulse times from 50 to 5,000 s for 192 h at field strength of 1.5 V/cm.
4. Subsequently, gels are ethidium bromide stained and photographed with a charge-coupled device camera system under UV transillumination.
5. Quantitative analysis to determine the fraction of DNA entering the gel provides a measure for the relative number of double-strand breaks. Control cell DNA is normalized to zero and DNA of cells treated with 10 Gy is given a value of 1.

3.8.2 Neutral Filter Elution—Filter elution is a commonly used technique for measuring DNA strand breakage (*see* Note 6). The assay uses a filter to mechanically impede passage of long (unbroken) DNA strands during elution. Double-strand DNA breaks are detected with “neutral” filter elution (performed at pH 9.6). This pH is nondenaturing, so no DNA strand unwinding occurs. The eluting solution is composed of 0.05 M Tris-Cl/0.05 M glycine/0.025 M EDTA/2 % (w/v) sodium dodecyl sulfate. The pH of the eluting solution is adjusted to pH 9.6 with 10 N NaOH. DNA quantitation is conducted by prelabeling cells with radiolabeled thymidine or by using fluorometry.

1. *Label cells with [¹⁴C] or [H]thymidine*—Expose 10⁵ cells/ml in suspension or a monolayer culture of 10⁶ cells to 0.1 µCi/ml [methyl ³H]-thymidine plus 10^{−5} M unlabeled thymidine or 0.01–0.02 µCi/ml of [2-¹⁴C]-thymidine for 24 h.

⁶Both neutral filter elution and pulse field gel electrophoresis allow measurement of gross chromosomal DNA double strand breaks, whereas cytogenetic approaches allow measurement of DNA double strand breaks of individual chromosomes of each cell. Thus the cytogenetic approach to quantitate chromosomal DNA damage is more sensitive.

2. Recover cells in suspension (by centrifuging 5 min at 200 μ g, 4 °C) or in monolayer (by rinsing twice with PBS before trypsinization, and centrifuging 5 min at 200 μ g, 4 °C) and remove the supernatant.
3. Rinse pellets with ice-cold PBS, centrifuge again, remove the supernatant, and transfer an appropriate number of cells into the appropriate medium for treatment with test agent.
4. *Assemble and prepare filter apparatus* — Secure the filter within the funnel apparatus and attach to a suction flask that is in turn connected to a vacuum line. Add 20 ml of ice-cold PBS to the funnel. Apply a full vacuum for a few seconds to expel air from the filter. Adjust vacuum to a slow flow rate.
5. *Apply cells to filter* — Add $0.5\text{--}2 \times 10^6$ cells, diluted in 20 ml ice-cold PBS, to the funnel. Continue to apply a gentle vacuum to pull the cells onto the filter.
6. Just before the solution level reaches the filter, add 5–10 ml of additional ice-cold PBS to wash the cells. Repeat twice. During the last wash, turn off the vacuum when the fluid is ~1 cm above the filter and allow the last of the solution to flow through the filter by gravity. Cover the funnel apparatus, with foil or a black paper cylinder.
7. *Lyse the cells* — Disconnect the funnel/filter apparatus from the suction flask. Fill the upper chamber of the filter holder with cell lysis solution, pH 9.6, by using a pipettor with a plastic tip that will fit through the filter orifice. Withdraw the tip slightly to allow for air bubbles to escape from the upper chamber. Pipet 1 ml at a time, slowly. Add a total of 5 ml of cell lysis solution. Allow the lysis solution to drip by gravity into a scintillation vial.
8. *Digest protein* — Connect the outflow tubes to tubing leading to a peristaltic pump (with the pump turned off at this point). Fill the upper chamber of the filter holder with 2 ml of cell lysis solution containing 0.5 mg/ml of proteinase K, using the procedure described above for adding the cell lysis solution. Pump through the filter at 0.035 ml/min. Turn off the pump.
9. *Elute damaged DNA* — Aspirate excess proteinase K solution from the filter funnel and add 5 ml of alkaline elution solution, pH 12.3, by slowly running the solution down the wall of the funnel. Pour an additional 30–40 ml of the solution into the funnel. Turn on the pump at a speed of 0.035 ml per min and collect fractions into scintillation vials at 90- or 180-min intervals for 15 h. Turn pump off.
10. *Quantitate DNA using scintillation counting of radiolabeled DNA* — Pour off any remaining elution solution.
11. Turn pump on to maximum speed to empty solution remaining in the line and filter holder. Collect this fraction into a scintillation vial.
12. Turn off the pump and remove the filter. Place it in a scintillation vial containing 0.4 ml of 1 N HCl. Cap the vial and heat at 60 °C in oven or water bath for 1 h to depurinate the DNA.

13. Add 2.5 ml of 0.4 N NaOH, shake vigorously. Allow to stand for 1 h at room temperature. Shake again.
14. Flush the filter holder and line tubing with 10 ml of 0.4 N NaOH by pumping at high speed. Collect into another scintillation vial. Wash again with 2.5 ml 0.4 N NaOH and count to check if all radioactivities have been removed.
15. Add 10 ml of scintillation fluid (e.g., Ecolume, ICN Biomedicals) containing 0.7 % (v/v) acetic acid to each scintillation vial.
16. Count the radioactivity in each of the vials containing the filters, rinse solution, and eluted fractions.
17. Calculate results by adding the radioactivity (cpm) from the filter to the radioactivity measured in the wash of the filter holder. Add this amount to the cumulative amount of radioactivity in the fractions.
18. Plot data on a semilogarithmic plot of DNA retention [i.e., the fraction of total DNA retained on the filter (log scale)] versus elution time.

3.9 Cytogenetic Approach

This method is cell-based analysis of individual chromosome damage, and provides the most sensitive assay for determining the induction of DNA strand breaks after stress. Essentially, cells are treated with colcemid after IR exposure. Chromosome aberrations are assessed by counting chromatid breaks and gaps per cell. The details of cytogenetical approaches for assessing chromosomal DNA damage have been well described [6, 8, 9, 30, 56–62].

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Fig 1.

A schematic showing the various signaling pathways modulated by ATM kinase in response to DNA damage by ionizing radiation. Several phosphorylation as well as acetylation events are regulated by ATM, enabling the appropriate response to DNA damage