Chapter 25

Noncanonical ATM Activation and Signaling in Response to Transcription-Blocking DNA Damage

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Abstract

Environmental genotoxins and metabolic byproducts generate DNA lesions that can cause genomic instability and disrupt tissue homeostasis. To ensure genomic integrity, cells employ mechanisms that convert signals generated by stochastic DNA damage into organized responses, including activation of repair systems, cell cycle checkpoints, and apoptotic mechanisms. DNA damage response (DDR) signaling pathways coordinate these responses and determine cellular fates in part, by transducing signals that modulate RNA metabolism. One of the master DDR coordinators, the Ataxia Telangiectasia Mutated (ATM) kinase, has a fundamental role in mediating DNA damage-induced changes in mRNA synthesis. ATM acts by modulating a variety of RNA metabolic pathways including nascent RNA splicing, a process catalyzed by the spliceosome. Interestingly, ATM and the spliceosome influence each other's activity in a reciprocal manner by a pathway that initiates when transcribing RNA polymerase II (RNAPII) encounters DNA lesions that prohibit forward translocation. In response to stalling of RNAPII assembly of late-stage spliceosomes is disrupted resulting in increased splicing factor mobility. Displacement of spliceosomes from lesion-arrested RNA polymerases facilitates formation of R-loops between the nascent RNA and DNA adjacent to the transcription bubble. R-loops signal for noncanonical ATM activation which in quiescent cells occurs in absence of detectable dsDNA breaks. In turn, activated ATM signals to regulate spliceosome dynamics and AS genome wide.

This chapter describes the use of fluorescence microscopy methods that can be used to evaluate noncanonical ATM activation by transcription-blocking DNA damage. First, we present an immunofluorescence-detection method that can be used to evaluate ATM activation by autophosphorylation, in fixed cells. Second, we present a protocol for Fluorescence Recovery After Photobleaching (FRAP) of GFP-tagged splicing factors, a highly sensitive and reproducible readout to measure in living cells, the ATM influence on the spliceosome. These approaches have been extensively used in our laboratory for a number of cell lines of various origins and are particularly informative when used in primary cells that can be synchronized in quiescence, to avoid generation of replication stress-induced dsDNA breaks and consequent ATM activation through its canonical pathway.

Key words DNA damage, Ultra violet (UV) radiation, ATM signaling, Spliceosome, U2 snRNP, U5 snRNP, Fluorescence recovery after photobleaching (FRAP), Immunofluorescence, Quiescence, Fibroblasts

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1 Introduction

To counteract DNA damage and ensure genomic fidelity, cells employ intricate DNA damage response (DDR) mechanisms that convert stochastic-damage signals into organized responses, including DNA repair systems, cell cycle checkpoints, and apoptosis. DDR is coordinated by signaling networks that utilize post-translational modifications and protein-protein interactions to elicit the initial stages of the cellular response. Later DDR stages depend largely on modulation of RNA metabolism [1, 2].

A fundamental stage of RNA metabolism for the production of mature, protein-coding mRNA is the removal of introns from newly synthesized transcripts [3, 4]. This reaction is catalyzed by the spliceosome, a dynamic megaparticle composed from five structurally distinct ribonucleoprotein particles (snRNPs), each containing a unique small nuclear RNA (U1, U2, U4, U5, or U6 snRNAs), a common core of seven proteins (Sm B/B′, D3, D2, D1, E, F, and G), and a large number of snRNP-specific accessory factors. During each spliceosome cycle, exon/intron definition by U1 and U2 snRNPs stimulates binding of a preassembled U4/U6. U5 tri-particle on the nascent transcript. Following extensive conformational rearrangements and U1/U4 displacement, the two-step splicing reaction is catalyzed by the mature, catalytically active spliceosome composed of U2, U5, and U6 snRNPs.

Splicing reactions are highly complex requiring an estimated 150–200 proteins including, and in addition to snRNPS, numerous auxiliary proteins that facilitate recognition of regulatory splicing sequences and the continuous restructuring of the spliceosome [5, 6]. This complexity is farther enhanced by the fact that the majority of pre-mRNAs are alternatively spliced to produce multiple mRNA variants from a single gene [7]. To maintain splicing fidelity and in parallel accommodate splicing diversity, the spliceosome is subject to complex regulation by developmental and environmental cues. Cumulative evidence supports that splicing is also influenced by the presence of DNA damage [1, 2, 8]. The exact mechanisms by which DNA lesions regulate splicing are not fully understood but likely, most DNA-damage-induced splicing changes result from changes in the transcription elongation rates of RNAPII. Because in the majority of introns, spliceosomes assemble co-transcriptionally so that the two processes are spatiotemporally coupled [9, 10], changes in the elongation rate of RNAPII when it encounters DNA lesions, are likely to influence splicing through several mechanisms; for example by increasing the time the spliceosome has to recognize weak splice sites [11], by disrupting RNAPII interactions with splicing regulators [12–14] or by dissociation of core spliceosomes from nascent transcripts [8]. This

later mechanism is a two-stage process involving a stochastic step, triggered by RNAPII stalling at DNA lesions, and a signaling-mediated step, controlled by the ATM DDR kinase [8]. Intriguingly, the interaction between spliceosome displacement and ATM signaling is reciprocal. Dissociation of spliceosomes from "stalled" RNAPII at DNA lesions activates ATM that, in turn, signals in a forward fashion to regulate spliceosome mobility, presumably in areas distal to the damage [2, 8].

ATM is a master regulator of the DDR, most extensively studied for its role in repair and downstream signaling of dsDNA breaks (DSBs) [15]. DSBs induce massive ATM activation (known as canonical ATM activation) which initiates after their detection by the MRE11–RAD50–NBS1 (MRN) complex [15, 16]. Under unperturbed conditions ATM exists as an inactive dimer. In response to DSBs, e.g., induced by ionizing radiation, canonical ATM activation requires several steps, including MRN-mediated ATM anchoring to DNA-ends, TIP60/KAT5-mediated acetylation, ATM autophosphorylation, and monomerization of the previously inactive ATM dimer [15, 17]. A large fraction of activated ATM molecules resides at DSBs, forming microscopically discernable foci, where it facilitates local chromatin changes and formation of multiprotein complexes that repair the break. In parallel, ATM signals to activate cell cycle checkpoints and modulate many aspects of the cellular response in a cell-context manner (e.g., apoptosis, DNA-damage-induced senescence).

Despite its robust activation by DSBs, the role of ATM in cellular homeostasis is not restricted to DSB-signaling, but extends to structural chromatin changes and many types of stress, which activate ATM through MRN-independent pathways [15, 17]. This noncanonical ATM activation occurs irrespective of the presence of DSBs. Transcription blocking DNA damage can activate ATM, through canonical and noncanonical pathways depending on the proliferation status of the cells. In nonreplicating cells ATM is activated via a noncanonical R-loop-dependent, but DSB-independent mechanism [8]. It is important to note that when ATM is activated by noncanonical mechanisms (in the absence of DSBs), it is uniformly distributed in the nucleoplasm as opposed to its localization in large foci when DSBs are present [8, 17].

R-loops are three-nucleic-acid structures (DNA:RNA hybrid across a region of unpaired DNA [18]) that can be formed during transcription as a consequence of negative torsional strain behind elongating RNAPII. R-loop formation is suppressed by several mechanisms including: (1) alleviation of torsional strain through the action of topoisomerases (TopoI) or (2) coupling transcription to RNA processing. This latter mechanism can both reduce complementarity and sterically hinder hybridization of nascent transcripts with DNA [2]. Coupling between transcription and splicing can be

disrupted when RNAPII stalls upon encountering DNA-helix distorting lesion (e.g., UV-induced photolesions). In response, mature spliceosomes that normally form stable complexes with nascent transcripts dissociate from the complex, presumably to facilitate backtracking/removal of RNAPII from the damaged bases and subsequent repair. This results in formation of R-loops which signal for ATM activation through an undefined mechanism that in nonreplicating cells is DSB-independent but R-loop dependent. In agreement, ATM can also be activated in the absence of any form of DNA damage through chemical or genetic inhibition of late-stage spliceosome assembly [8].

An intriguing aspect of ATM signaling following its activation by transcription blocking DNA damage is that it can influence alternative splicing and splicing factor mobility, while canonical ATM activation by, e.g., ionizing radiation has no influence on the spliceosome [8]. Currently, it is unclear whether this specificity in ATM function stems from differences in subnuclear localization of active ATM (in DSB-associated foci vs throughout the nucleoplasm). It is clear, however, that ATM does not initiate spliceosome mobilization, but it enhances a preexisting signal originating from stalling of RNAPII at DNA damage sites [8]. This peculiarity of ATM function when ATM is activated by transcription-blocking DNA damage can be used to evaluate noncanonical ATM activation and its influence on splicing factor mobility.

In this chapter, we will describe fluorescence microscopy methods that can be used to evaluate noncanonical ATM activation and signaling, in response to transcription-blocking DNA damage. ATM activation is evaluated by immunofluorescence-based detection of its autophosphorylated form using antibodies that specifically recognize phospho-Ser1981 ATM. In addition, we will describe in detail a live cell imaging-based approach to measure the effect of ATM on DNA damage-induced changes in spliceosome mobility. Using Fluorescence recovery after Photo bleaching (FRAP) [19], we determine protein mobility of GFP-tagged splicing factors that typically associate with late stage spliceosomes (U2 or U5 snRNP) [8]. While more traditional methodologies have been described in the literature to evaluate changes in splicing factor association with nascent RNA (and thereby, indirectly, with chromatin), such as DNA:RNA immunoprecipitation (DRIP) or fractionation procedures, we find that FRAP is a highly sensitive, rapid, and reproducible approach to evaluate changes in splicing factor behavior [8]. Furthermore, the use of living cells, where all molecules operate in their physiological environment and proper localization eliminates possibility of artifacts that could possibly be introduced by extensive experimental manipulations.

2 Materials

2.1 Serial Subcultivation of Adherent Cells and Treatments

1. Tissue culture plates and disposable pipettes.
2. Phosphate-buffered saline (PBS), pH 7.4: 136.9 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4 , 0.9 mM $CaCl_2$, 0.49 mM $MgCl_2$.
3. Growth media for primary and TERT immortalized diploid fibroblasts: Ham's F10 supplemented with 15% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin.
4. Synchronization media: Serum-free Ham's F10 supplemented with 100 U/mL penicillin, and 100 mg/mL streptomycin.
5. Trypsin/EDTA.
6. Tissue culture incubator set at 37 °C, 5% CO_2 balanced air.
7. Z-series Coulter Counter (Beckman) for cell counting.
8. ISOTON® II Diluent (Beckman).
9. UV-C source (254 nm, TUV Lamp, Philips).
10. Pladienolide B (Santa Cruz Biotechnology). Resuspend to 5 mM with Dimethyl Sulfoxide (DMSO) and use at 1–5 μM final concentration. Store in small aliquots at −20 °C.
11. Camptothecin. Resuspend to 5 mM with Dimethyl Sulfoxide (DMSO) and use at 5–10 μM final concentration. Prepare fresh or store in small aliquots at −20 °C.
12. ATM inhibitor KU55933. Resuspend to 10 mM with Dimethyl Sulfoxide (DMSO) and use at 10 μM final concentration. Store in small aliquots at −70 °C.

2.2 Fluorescence Recovery After Photobleaching (FRAP)

1. Glass coverslips (24-mm diameter).
2. 1× PBS pH 7.4.
3. Leica TCS SP5 AOBS laser scanning confocal microscope (Leica) equipped with an environmental chamber (PeCon GmbH) and a lens warming unit (Temp control mini).

2.3 Immunofluorescence Microscopy

1. Glass coverslips (24-mm diameter).
2. 1× PBS pH 7.4.
3. Paraformaldehyde solution (PFA), 3.7%: Mix 0.37 g PFA in 10 mL 1× PBS, pH 7.5. Heat this mixture until the PFA has dissolved. Confirm pH and adjust if necessary.
4. 0.2% v/v Triton-X 100 in 1× PBS, pH 7.4.
5. 0.1% v/v Tween 20 in 1× PBS, pH 7.4.
6. Blocking solution: 1× PBS containing Bovine Serum Albumin (BSA) at 2% w/v final concentration. Mix 2 g BSA in 100 ml PBS, filter sterilize using 0.22 μm filters and store at 4 °C.

7. Primary antibody: mouse monoclonal anti-phospho-ATM (1981) from Upstate Biotechnology (antibody 05–740). Store at −20 °C in small aliquots. Dilute in blocking solution (1:1000 v/v) just before using.
8. Secondary antibodies: Goat anti-mouse Alexa Fluor555-conjugated IgG, goat anti-rabibit Alexa Fluor488-conjugated IgG (Thermo Fischer Scientific) (stored at 4 °C). Dilute 1:1000 v/v in blocking solution prior to using.
9. 4,6-Diamidino-2-phenylindole(DAPI)-containing ProLong Gold antifade reagent (Molecular Probes/Thermo Fischer Scientific).
10. Microscopic superfrost slides.
11. Zeiss Axio Imager Z2 upright laser-scanning confocal microscope.

3 Methods

3.1 Serial Cell Subcultivation of Adherent Cells and Treatments

We routinely use primary and TERT immortalized human fibroblasts but any cell type can be used (*see* **Note 1**). Noncanonical, R-loop-dependent ATM activation is triggered by transcription-blocking DNA damage induced by UVC irradiation using a 254 nm, TUV Lamp, Philips. Spliceosome inhibitors added directly in the culture media (e.g., Pladienolide B) can also be used to activate ATM through a noncanonical R-loop-dependent pathway. Alternatively, DNA damage can be induced by topoisomerase inhibitors that can both interfere with transcription, and promote R-loop formation (e.g., camptothecin and its derivatives). For FRAP experiments we use cells stably expressing GFP-tagged splicing factors that participate in the U2 (e.g., SF3a1, SF3b2) or U5 snRNP complexes (e.g., PRP8, SNRNP40). For immunofluorescence experiments we use either transfected or untransfected cells.

3.1.1 Serial Subcultivation of Primary and TERT Immortalized Human Dermal Fibroblasts

1. Aspirate growth media.
2. Wash cells twice with 1× PBS.
3. Aspirate last drops of PBS.
4. Add enough Trypsin/EDTA to cover cell monolayer (e.g., 1 ml per 100 mm plate).
5. Incubate for 2–3 min at room temperature or until cells are detached. This can be verified by microscopic examination.
6. Add 9 ml of serum-containing growth media to stop trypsinization and repeatedly pipette to ensure single-cell suspension. Verify by microscopic examination that there are no cell "clumps" present.
7. Dilute 0.5–1 ml cell suspension with Isoton II to a total volume of 10 ml (20× or 10× dilution respectively).

8. Count cells using Coulter Counter.
9. Calculate cell numbers per ml and seed cells to a density of 1×10^4 per cm^2 of growth surface area (*see* **Note 2**) in appropriate volume of growth media.
10. Place cells in a tissue culture incubator at 37 °C, 5% CO_2 balanced air.
11. Subculture when cells reach confluency.

3.1.2 Preparing Cells for Immunofluorescence Experiments and FRAP

1. Place sterilized glass coverslips into 35-mm cell culture dishes (or in 6-well plates). Seed an extra coverslip that can be used to verify the quiescent status of the cells (*see* **Note 1**).
2. Seed cells at $2–3 \times 10^5$ cells per dish.
3. Incubate for 24–48 h in a tissue culture incubator.
4. Wash cells twice with 1× PBS.
5. Add 2 ml of serum-free media (*see* **Note 3**).
6. Incubate cells for an additional 72 h prior to the experiment.

3.1.3 Cell Treatments

We always include in each experiment: (1) cells that do not receive the ATM inhibitor nor UV irradiation, (2) cells that are treated with the ATM inhibitor but are not irradiated, (3) cells that are not treated with the ATM inhibitor but are UV irradiated, and (4) cells that are treated with the ATM inhibitor and are UV irradiated.

1. To inhibit ATM activity add directly in the culture media 1 μl/ml from a 10 mM stock of the ATM inhibitor KU55933 (*see* **Note 4**).
2. In negative control plate, add 1 μl/ml DMSO.
3. Incubate cells for 1 h in a tissue culture incubator.
4. Transfer media into a sterile dish.
5. Wash cells twice with 1× PBS.
6. Aspirate PBS.
7. Irradiate cells with desired UVC dose. We typically irradiate cells so that they receive either 10 or 20 J/m^2 UVC (*see* **Note 5**).
8. Return media to cells and incubate for 1 h in tissue culture incubator.

3.2 Measurement of Splicing Factor Mobility by Fluorescence Recovery after Photobleaching (FRAP)

We measure changes in splicing factor mobility upon genotoxic stress and transcription inhibition, by FRAP, using cells stably expressing GFP-tagged splicing factors. For FRAP experiments we use a Leica SP5 AOBS confocal microscope equipped with three spectral PMT (Photo Multiplier Tube) detectors in combination with 458, 476, 488, 496, 514, 561, and 633 nm laser lines (Leica Microsystems). The confocal microscope is equipped with a motorized and heated stage with a coverslip holder, in combination with

a PeCon small chamber incubator with heating, CO_2 and O_2 regulation (*see* **Note 6**). In addition, a lens warming unit (Temp control mini) is used to heat the lens used, to 37 °C. For our experiments we use a HCX PL APO CS 63x Magnification oil Immersion lens, with a numerical aperture of 1.40 and a working distance of 0.14 mm.

3.2.1 Switching "on" the Confocal Microscope

1. At least 15 min before starting the experiment, preheat the incubator and the heated stage at 37 °C and connect the lens heater around the 63× lens. The CO_2 level should be set at 5%.
2. Switch on the microscope, lasers, and detectors.
3. Start the LAS AF software (Leica Microsystems) which controls the confocal microscope.
4. Turn on the 488 nm laser so that its fluorescent output signal can stabilize.

3.2.2 Preparing Cells for FRAP

1. At the appropriate time-point after the treatment of the cells the 24-mm diameter coverslip with attached cells is taken out of the tissue culture plate using forceps and placed in a stainless-steel preheated (37 °C) coverslip holder.
2. Close the coverslip holder and add 1 ml of the culture medium on the top of the coverslip.
3. Take care that the coverslip holder is not leaking and that the cells do not dry out during this procedure.
4. Apply a small drop of immersion oil to the 63× lens.
5. Close the environmental chamber and wait for 5 min so that temperature (37 °C) and CO_2 (5%) are stabilized.
6. Locate cells and focus, using transmission light. Do not use Metal-Halide/Mercury lamp as this may cause bleaching of the fluorescent signal of the GFP-tagged proteins.

3.2.3 Obtaining a Representative Image of Each Cell That Will Be Analyzed by FRAP

1. Switch Argon Laser from "idle" to 24% output power.
2. Start the "FRAP Wizard" and work in the "Setup mode."
3. Switch on the "FRAP Booster."
4. Put the 488-nm laser-line output on 3% (*see* **Note 7**). All other laser-lines remain at 0% (off).
5. Select "bidirectional imaging."
6. "Activate Pinhole" and set to 2 airy units (191.1 μm).
7. Set line and frame averaging to 1.
8. Set Scan speed at 200 Hz.
9. Set imaging format at 512 × 512 lines.
10. Activate PMT1 and select emission bandwidth (begin–end) to 500–600 nm to image GFP. If needed the PMT-Trans can be activated to enable a transmission light image.

11. PMT sensitivity is set between 950 and 1100 in such a way that Mean Fluorescence intensity is above 40.
12. Using "live mode" the cells are focused using the *Z*-axis controller in fine mode. Cells with strict nuclear expression and with a clear localization of GFP-tagged splicing proteins in both the nucleoplasm and in "speckles" are selected for further analysis (*see* **Note 8**).
13. Press "capture" to obtain image.

3.2.4 Fluorescent Recovery After Photobleaching (FRAP) Procedure

1. A cell of choice is placed in exactly in the center of the imaging field using the x- and y-stage. Press CRTL + T (Target) to determine the exact center of the imaging field.
2. Set zoom at 8.0, in such a way that the selected cell is in the middle of the image and the whole nucleus is visible.
3. Rotate the cell in such a way that the oval-shaped nucleus in always exactly horizontally orientated (see below). By keeping the same orientation for all cells a similar volume of the cell is bleached (*see* Fig. 1).
4. Set scan speed at 1400 Hz.
5. Set imaging format at 512 × 32 lines representing a physical length of 30.75 μm by 1.87 μm.
6. All other settings remain the same as in Subheading 3.2.3.
7. Press "live "and position the cell in such a way that no nucleoli are present in the region of interest. Press "stop" afterward.
8. Select the "bleach setup" tab, and put the 488 nm laser power at 100% during bleaching. Using these setting the complete 512 × 32 strip will be measured and bleached (*see* Fig. 1 and **Note 9**).
9. In the "time setup" tab, we define 200 images prebleach (*see* **Note 10**), five bleach pulses (*see* **Note 11**) followed by 2000 postbleach measurements, all with a 20-ms interval. The num-

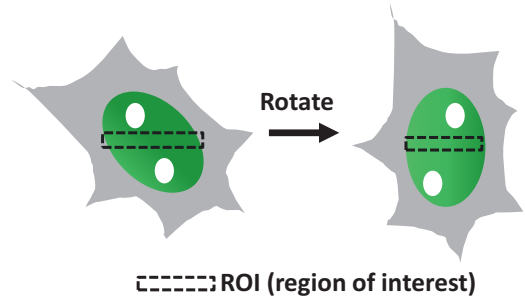

Fig. 1 Cartoon of a cell expressing a GFP-tagged SF in its nucleus (*green*). Prior to FRAP the cell is rotated and centered in the imaging field so that a 512 × 32 strip is photobleached. GFP signal intensity during FRAP is quantified in a user-defined region of interest (ROI/*dashed box*) spanning the entire length of the nucleus

ber of postbleach images can be varied depending on the mobility of the studied protein, but images should be acquired until the fluorescent signal reaches steady-state levels.

10. Press "start experiment" to start the FRAP procedure in this cell line.
11. Select a new cell and perform the whole procedure again. For each experimental condition a minimum of ten cells are analyzed. Typically, we image 15 cells per condition.

3.2.5 Quantification of FRAP and Data Analysis

1. Select "Quantification" tab in the FRAP wizard of the LAS AF software to quantify levels of fluorescence prior to, and after photobleaching, for each cell analyzed.
2. Define two regions of interests (ROI); ROI1 and ROI2. Place ROI1 outside of the cell to measure background signal. ROI2 should cover the complete width of the nucleus (Fig. 2).
3. The fluorescence nuclear signal (ROI2) should be background-corrected, by subtracting the background fluorescent signal (measured in ROI1) at the same time point (corrected signal = signal ROI2 − Signal ROI1).
4. The fluorescence signal value for each measurement should be normalized to the average fluorescence signal values of the last 50 (out of 200) prebleach measurements.
5. The background-corrected and pre-bleach-normalized values of all cells analyzed for each condition should be averaged for each FRAP point, and plotted.
6. The change in mobility (Δmobility) is calculated as the average fluorescence of treated cells—average fluorescence of untreated cells of 50 images at approximately 1 min after postbleaching. This time is chosen arbitrarily and can be varied as long as the recovery of the fluorescent signal has reached a plateau and no farther increase is observed (Fig. 3).

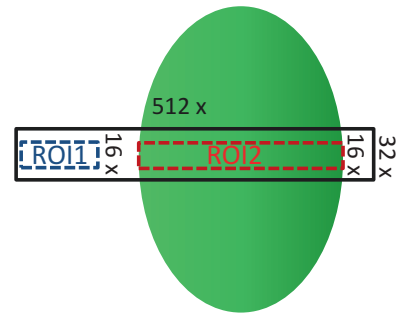

Fig. 2 Cartoon of a cell nucleus expressing a GFP-tagged SF (*green*). The bleached area (*black solid line*), background region of interest (ROI1—*blue dashed line*), and nuclear region of interest (ROI2—*red dashed line*) are indicated

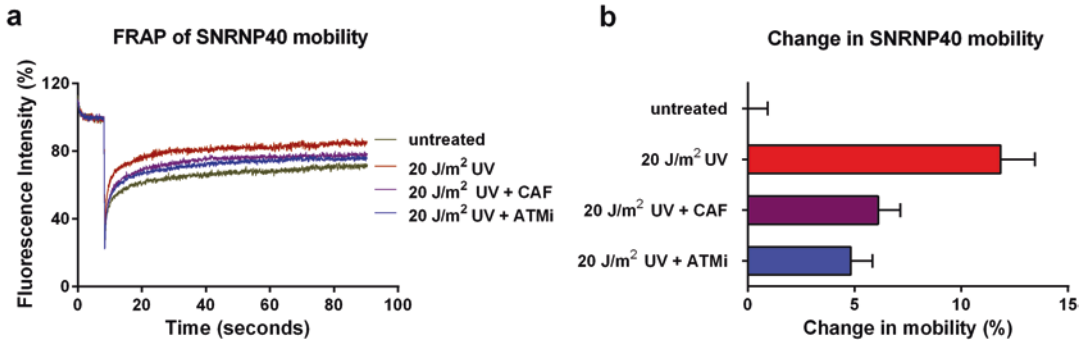

Fig. 3 ATM modulates the UV-induced spliceosome mobilization. FRAP of the U5 snRNP splicing factor SNRNP40, in quiescent human dermal fibroblasts (HDFs). (**a**) FRAP curves of SNRNP40-GFP in untreated, UV irradiated, and UV irradiated cells in the presence of a specific (ATMi KU55933, 10 μM) and nonspecific (CAF, Caffeine, 10 mM) ATM inhibitor. SNRNP40 mobility was measured 1 h post-irradiation. (**b**) Graph of SNRNP40 change in mobility calculated as the fluorescence of irradiated cells minus the fluorescence of non-irradiated cells at 1 min post-bleaching ($n = 12$, mean ± s.e.m., one-way ANOVA with Bonferroni post-test, ***P*, 0.01, ****P*, 0.001)

3.3 Assay of ATM Activation by Immunofluorescence

The following protocol describes the immunofluorescence-based detection of activated ATM in adherent mammalian cells. As a measure of ATM activity, we detect ATM autophosphorylation at Ser-1981 using an antibody that specifically recognizes this ATM modification. This approach is highly sensitive as we can detect low levels of ATM phosphorylation, but also particularly informative as it provides information about the localization of activated ATM. For example, in response to DSBs the majority of p-ATM signal localizes in large foci, together with other "markers" of DSBs. ATM kinase activated by noncanonical pathways shows more "diffused" localization.

3.3.1 Cell Fixation

Cells are seeded on coverslips, serum deprived and treated as detailed above (*see* Subheading 3.1.3).

1. Aspirate media and wash cells twice with 1× PBS.
2. Fix cells by incubating with 1 ml 3.7% PFA/PBS for 15 min at RT.
3. Wash twice with 1× PBS.
4. Permeabilize cells by incubating in 0.1% Triton X-100/PBS, for 20 min at RT.
5. Wash cells three times with 1× PBS.

3.3.2 Immunodetection

1. Block nonspecific antigens by incubating cells in 2% BSA/PBS for 60 min at RT.
2. Incubate with primary antibody (anti-phosphoATM) diluted 1 to 500 in 2% BSA/PBS. Add 100–120 μl on top of each coverslip and incubate overnight at 4 °C in a humidified chamber to avoid evaporation.

3. Wash cells with 0.1 Tween-20/1× PBS three times, 5 min each wash, at RT.
4. Incubate with Alexa-labeled secondary antibody (goat anti-mouse) diluted 1 to 1000 in 2% BSA/PBS. Add 100–120 μl on top of each coverslip and incubate for 1 h at RT in a humidified chamber.
5. Wash cells with 0.1 Tween-20/1× PBS three times, 5 min each wash, at RT.
6. Drain PBS (without allowing the coverslips to dry) and mount on a glass slide using Prolong Gold antifade reagent with DAPI.
7. Seal the edges of the coverslip with clear nail polish.
8. Visualize cells and record images using fluorescence microscope. We use a Zeiss LSM 700 Axio Imager Z2 upright laser-scanning confocal microscope but any fluorescence microscope can be used (Fig. 4).

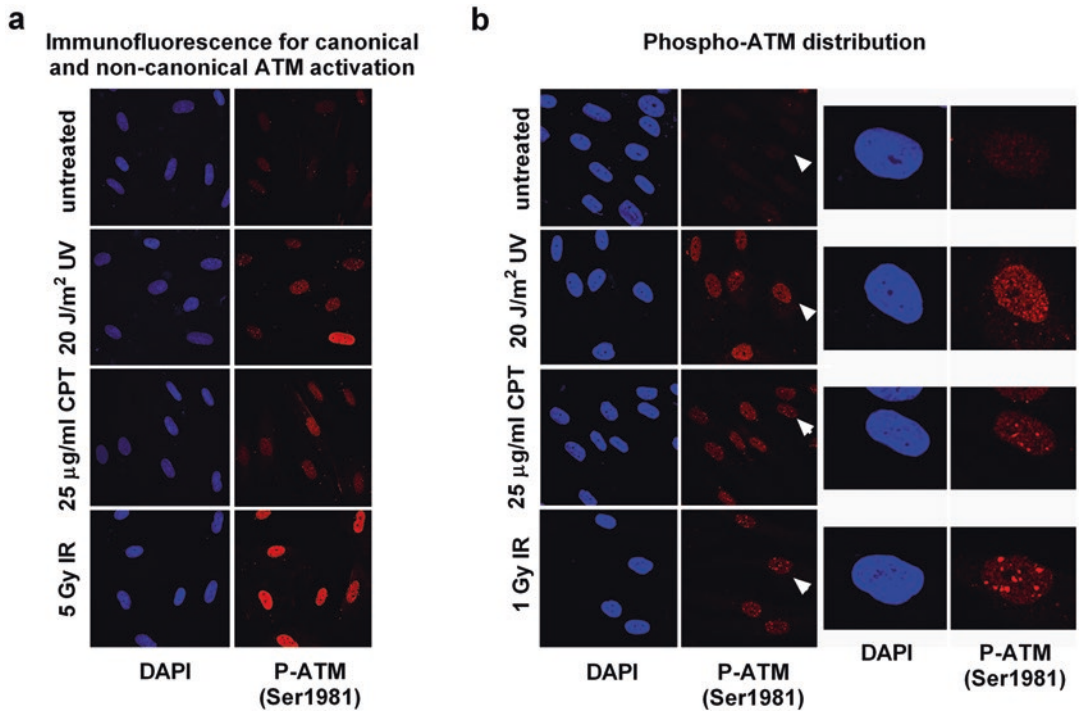

Fig. 4 Canonical and noncanonical ATM activation. (**a**) ATM autophosphorylation (Ser1981) was assayed in quiescent human dermal fibroblasts (HDFs) 1 h after the indicated treatments. In non-replicating cells UV irradiation activates ATM via a noncanonical pathway. (**b**) Differences in autophosphorylated ATM distribution in quiescent HDFs treated with various ATM activators. *Left*: multiple cells; *right*: single-cell magnification illustrating pan-nuclear localization of phosphorylated ATM after UV irradiation and focal accumulation after CPT or ionizing radiation treatments. Magnified cells are indicated by *arrows* (*left panel*). Images were obtained with a 40× and 63×, lens, respectively. Figure was adapted from Tresini et al., doi:10.1038/nature14512

4 Notes

1. The majority of our experiments are performed with quiescent cells as to control for influences of the cell cycle phase. This is particularly important for avoiding replication stress and formation of DSBs. In replicating cells (S phase) collision of replication forks with damage-arrested transcription complexes or R-loops, may result in replication fork collapse and DSBs. DSBs result in robust canonical ATM activation masking subtle changes in ATM activity by noncanonical pathways. Quiescence can be verified by immunofluorescence detection of the Ki67 antigen, a protein present in all active phases of the cell cycle but is absent in G_0 (quiescent) cells. A mouse monoclonal antibody against Ki67 is available from Abgent (ab833).
2. Seeding numbers of adherent cells are adjusted to the surface area of the tissue culture plate (i.e., the size of surface available for cells to attach). This ensures uniform and predictable growth kinetics, independent of the size of plate used. Size of surface area is provided by the manufacturer of the plates.
3. If similar experiments are performed with murine cells, complete absence of serum will result in massive cell death. Addition of 0.1–0.2% FBS in the culture media does not sustain proliferation but is sufficient to ensure cell viability.
4. Alternatively, ATM can be inhibited using Caffeine, a solution of which must be prepared fresh prior to the experiment (100 mM in PBS) and added in the cell culture medium to a 10 mM final concentration. It is important to note, however, that Caffeine is a nonspecific ATM inhibitor; it also inhibits ATR and DNA-PK, as well as other cellular kinases.
5. Quiescent human fibroblasts (both primary and TERT immortalized) can tolerate well relatively high UV doses without significant changes in viability. However, this may vary between cell lines.
6. To successfully image cellular processes in living cells, it is critical that cells are imaged in an environment that does not induce additional stress responses that may influence the experimental outcome. This is achieved by maintaining the temperature at 37 °C, keeping the pH at physiological levels (by the use of 5% CO_2 that buffers bicarbonate-based culture medium) and by minimizing evaporation to prevent changes in medium osmolality.
7. While high laser power generally results in higher signal-to-noise ratio and better images, when laser power is set too high, it will cause "monitor bleaching," i.e., bleaching of the fluorescent GFP signal while monitoring the postbleach signal. This is particularly important during FRAP experiments as during

this procedure small subcellular areas are monitored with high frequency (every 20 ms) using a high zoom factor. Monitor bleaching can be recognized by a slow decrease over-time in signal intensity during the FRAP procedure. The best method to test for monitor bleaching is to image fixed cells (e.g., using 2% PFA, 15 min, RT) under the same experimental conditions used for FRAP, but without a bleaching step. Fixation prevents diffusion of GFP-tagged proteins and the recorded signal should remain stable. "Monitor bleaching" can be avoided by decreasing the laser power, increasing the PMT settings or by imaging less frequently.

8. GFP-tagged splicing factors tend to cluster in nuclear "foci" known as splicing speckles or interchromatin granule clusters or SC35 domains. Splicing speckles can also be visualized by immunofluorescence using a monoclonal antibody against the spliceosome assembly factor SC35.
9. During imaging, presence in ROI2 of non-photobleached splicing speckles due to cell migration or chromatin movement may result in aberrant measurements. To minimize the relocalization of non-photobleached proteins into ROI2, these ROIs are selected so that they are half the width of the bleached region. ROIs are 16 lines wide, while bleached area is 32 lines wide (*see* Fig. 2). If cells or chromatin move during the FRAP analysis, ROIs are shifted upward or downward within the "32 bleached lines" in such a position that no unbleached GFP-tagged proteins are present in the ROIs during the FRAP measurement.
10. The measured GFP fluorescence intensity should be stable prior to photobleaching. GFP fluorescence normally decreases when acquiring the first few images using the above-described settings (1400 Hz, image acquisition every 20 ms). Since during data analysis of the quantified images all values post-bleaching are normalized to the prebleach values, it is crucial to acquire enough prebleach images until the GFP fluorescence stabilizes. We typically acquire 200 images prior to photobleaching and have observed that the GFP signal stabilizes after 50–60 images. The number of prebleach images should be increased if GFP fluorescence has not stabilized.
11. To accurately study changes in protein mobility, a large enough fraction of the fluorescent proteins residing in the area of interest should be photobleached. This fraction is expressed as percentage and is defined as "bleach depth." We typically bleach, using enough pulses, so that the fluorescence signal is decreased to a maximum of 30% of the prebleach levels. If this bleach depth is not achieved, the number of bleach pulses should be increased. If required, the "FRAP booster" can be switched on in the "FRAP wizard"; this increases the laser power and bleaches the GFP-tagged proteins more efficiently.

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