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ATMIN is required for the ATM-mediated signaling and recruitment of 53BP1 to DNA damage sites upon replication stress

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ABSTRACT

Unresolved replication intermediates can block the progression of replication forks and become converted into DNA lesions, hence exacerbating genomic instability. The p53-binding protein 1 (53BP1) forms nuclear bodies at sites of unrepaired DNA lesions to shield these regions against erosion, in a manner dependent on the DNA damage kinase ATM. The molecular mechanism by which ATM is activated upon replicative stress to localize the 53BP1 protection complex is unknown. Here we show that the *ATM-IN*teracting protein ATMIN (also known as ASCIZ) is partially required for 53BP1 localization upon replicative stress. Additionally, we demonstrate that ATM activation is impaired in cells lacking ATMIN and we define that ATMIN is required for initiating ATM signaling following replicative stress. Furthermore, loss of ATMIN leads to chromosomal segregation defects. Together these data reveal that chromatin integrity depends on ATMIN upon exposure to replication-induced stress.

1. Introduction

Unresolved replication intermediates can occur during S/G2-phases of the cell cycle and can be converted into DNA lesions in M-phase. It has been shown that 53BP1 forms nuclear bodies at such sites of unrepaired DNA lesions in the subsequent G1-phase to shield these regions against erosion [1]. In the following S-phase 53BP1 nuclear bodies are resolved and DNA lesions are repaired. Interestingly, chromosomal fragile sites (CFS) are enriched within regions of the genome that are sensitive to replication-induced stress and as a consequence such sites, including FRA3B and FRA16D, are commonly mutated in cancers [2,3].

53BP1 nuclear bodies consist of several DNA repair proteins including the ubiquitin ligases RNF8 and RNF168, ATM (autophosphorylated at S1981), NBS (phosphorylated at S343), MDC1 and γH2AX [1]. Moreover, these foci also co-localize with OPT (Oct-1, PTF and transcription) domains that are known to occur in G1 and represent sites of low transcriptional activity, hence functioning to suppress transcription at DNA damage sites [4].

The DNA damage kinase ATM (mutated in the inherited recessive autosomal disease ataxia telangiectasia) [5] has been implicated in 53BP1 localization both in basal conditions and after aphidicolin-induced replicative stress [1,4]. Aphidicolin is an inhibitor of the replicative polymerase α (and also potentially of polymerase δ) and has been shown to specifically increase the breakage of CFS [4,6]. Nuclear body formation of 53BP1 in response to aphidicolin-induced replicative stress is thought to suppress the sensitivity of CFS to breakage by shielding these regions against erosion and degradation [1]. Indeed loss of 53BP1 leads to increased breakage within CFS upon replication stress [1].

This requirement for ATM in 53BP1 localization in response to replicative stress has only recently been reported, in contrast to the intensively investigated roles of ATM in response to the generation of DNA double-strand breaks [7]. Canonical ATM signaling following DNA double-strand breaks has been shown to require NBS [8]. In contrast, little is known about ATM activation following other stresses [9]. The *ATM-IN*teracting protein (ATMIN; also known as ASCIZ) is required for ATM activation after cellular stresses including chloroquine and hypotonic stress [10]. ATMIN has also been shown to be required for the repair of DNA alkylation damage and as it is required for localization of Rad51 it may link base damage repair with the repair of DNA double-strand breaks by homologous recombination [11,12].

Abbreviations: MEFs, mouse embryonic fibroblasts; Aph, aphidicolin; IR, ionizing radiation; Gy, Gray; MMS, methyl methanesulfonate; HU, hydroxyurea; NCS, neocarzinostatin; DAPI, diamidino-2-phenylindole; HR, homologous recombination; NHEJ, non-homologous end-joining; CFS, chromosomal fragile sites.

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The molecular mechanism for ATM activation following aphidicolin-induced replicative stress, and the subsequent localization of 53BP1 to replication sensitive sites is not known. In the present study we define a partial requirement for ATMIN in ATM-mediated localization of 53BP1 following replicative stress. Furthermore, we show that ATMIN is required for ATM-dependent signaling and to suppress chromosomal segregation defects and chromosomal instability. These data define ATMIN as a critical mediator in ATM signaling following replicative stress.

2. Materials and methods

2.1. Cell culture, DNA damage induction and siRNA transfections

Cells were cultured in DMEM (Invitrogen) supplemented with penicillin/streptomycin (Invitrogen), and 10% FCS (Invitrogen). Cells were incubated at 37 °C with 5% CO_2 and 3% O_2. Ionizing irradiation (IR) experiments were performed using a Cs137 Gamma Irradiator at the indicated doses followed by a recovery period of 30 min. Aphidicolin, MMS, NCS, HU and topotecan were purchased from Sigma. siRNAs were purchased from Dharmacon and transfected using the Lullaby transfection reagent as directed by the manufacturer (Oz Biosciences). Cells were analyzed 48–72 h after transfection.

2.2. Quantitative RT-PCR

RNA was extracted using TRIzol reagent (Life Technologies). RNA was treated with 1 μl DNase (TURBO™ DNase, Lifetechnologies) in a 0.1 volume TURBO DNase Buffer for 30 min at 37 °C. The reaction was terminated with 0.1 volume of DNase inactivation reagent (Life Technologies), incubated for 5 min at room temperature and centrifuged at 10,000 × *g* for 1.5 min before transferring RNA into fresh tubes. RNA was reverse transcribed with the SuperScript III Reverse Transcriptase protocol (Invitrogen) to obtain cDNA. An amount of 50 ng of cDNA template was used with the reverse transcription SYBR Green qPCR Mastermix (QIAGEN). The 7900HT Fast Real-Time PCR System (Applied Biosystems) was used for the quantitative RT-PCR reaction. RNF168: forward primer - GCCTGTGGTGCCGAATG, reverse primer – CCCATGATTGCTTGGTCTTGT. GAPDH: forward primer – CGAGCCACATCGCTCAGACA, reverse primer – GGCGCCCAATACGACCAAAT. The data was normalized to GAPDH.

2.3. I-Sce1 assay

Reporter cell lines were used to assess homologous recombination (DR-GFP) and non-homologous end joining (EJ5-GFP) [13]. For transfections, I-SceI expression vector (pCBASce), GFP expression vector (pCAGGS-NZEGFP) and a control empty vector (pCAGGS-BSKX) were used [13]. In detail, 2×10^5 cells were plated into a 12-well dish and transfected with non-targeting or siRNA targeting ATMIN (Dharmacon) using Lullaby reagent (OZ Biosciences). After 48 h, cells were transfected with I-SceI or control plasmids. Transfection complexes were prepared by mixing Lipofectamine 2000 (Life Technologies) in OptiMEM (Invitrogen) with 0.8 μg of expression vectors for I-SceI or control vectors per sample. Samples were analyzed 3 days after transfection by immunoblotting to assess the knockdown efficiency. The frequency of GFP^+ cells was determined on a Fortessa II flow cytometer (BD Bioscience).

2.4. Protein extracts and immunoblotting

Cells were extracted in RIPA lysis buffer (NEB) supplemented with protease inhibitors (Sigma) and phosphatase inhibitors (Sigma, NEB). Immunoblots were performed using standard procedures. Protein samples were separated by SDS–PAGE (3–8% gradient gels; Invitrogen), and subsequently transferred onto nitrocellulose membranes. All primary antibodies were used at 1:1000 dilution, except for P-S957-SMC1 that was used at 1:400, and secondary antibodies at 1:5000. The following antibodies were used: ATM 2C1 (Santa Cruz), P-S1981-ATM (10H11.E12; NEB), ASCIZ (Millipore), P-S824-KAP1 (Bethyl Laboratories, Inc), KAP1 (Bethyl Laboratories, Inc), P-S15-p53 (16G8; NEB), P-S957-SMC1 (5D11G5; Millipore), SMC1 (Abcam), P-S317-CHK1 (NEB), CHK1 (DCS-310; Santa Cruz), FANCD2 (EPR2302; Abcam), p95/NBS (NEB), β-actin (Sigma), 53BP1 (H300; Santa Cruz), Chk1 (DCS-310; Santa Cruz), HRP-conjugated goat anti-mouse/rabbit IgG (Sigma).

2.5. Immunofluorescence, microscopy and statistics

Cells were adhered onto coverslips and stained as described previously [20]. The antibodies used were 53BP1 (H300; Santa Cruz) and Alexa Fluor® 546 goat anti-rabbit or Alexa Fluor® 488 goat anti-rabbit (Invitrogen). Fixed cells were counterstained with diamidino-2-phenylindole (DAPI). Images of cells were acquired on a Deconvolution Microscope (Leica). Cell Profiler cell image analysis software (developed by the Broad Institute) was used for the quantification of 53BP1 focus formation. Intensity of 53BP1 was measured using the Thermo Scientific Cellomics high content screening platform where the intensity of individual foci per nucleus was assessed. Statistical significance was calculated using Fisher's exact test.

2.6. Cell proliferation

$ATMIN^{+/+}$ and $ATMIN^{\Delta/\Delta}$ MEFs were seeded at a density of 2×10^5 per well in a 24-well plate. Cells were collected after 48 h, counted and 2×10^5 of cells were replated. Cells were counted at 3 consecutive passages.

2.7. Cell cycle analysis

After treatments as indicated, cells were fixed with 70% ethanol, rehydrated in PBS, stained with propidium iodide and analyzed on a FACScalibur flow cytometer. Following cell acquisition, analysis was performed using FlowJo software (Tree Star).

2.8. Cell survival analysis

Cells were seeded at a density of 7×10^3 cells per well in a 96-well-plate. On the following day, cells were treated as indicated and consequently grown in drug-free media until control cells reached 90% confluence. Aphidicolin was used for 24 h, MMS was used for 1 h and HU was used for 24 h. Cells were washed twice with PBS upon which 50 μl of CellTiter-Glo® Reagent (Promega) was added. Following 30 min of gentle agitation, luminescence was recorded using a Victor™ X3 Multilabel Plate Reader (PerkinElmer). Data was analyzed using GraphPad Prism® software.

2.9. Colony formation assay

Cells were seeded at a density of 250 cells per 6-well and treated 24 h later with aphidicolin, HU, NCS, topotecan or MMS at the indicated concentrations. Aphidicolin, NCS and topotecan were left on for 5 days. HU treatment was for 24 h and MMS treatment was for 1 h after which cells were fixed 7 days later. Cells were then washed with PBS, fixed with 3.7% Formaldehyde solution and stained with 0.1% Crystal Violet (Sigma–Aldrich, diluted in ethanol). After scanning the plates, the Crystal Violet was extracted from cells with