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S1219 residue of 53BP1 is phosphorylated by ATM kinase upon DNA damage and required for proper execution of DNA damage response

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ABSTRACT

53BP1 is phosphorylated by the protein kinase ATM upon DNA damage. Even though several ATM phosphorylation sites in 53BP1 have been reported, those sites have little functional implications in the DNA damage response. Here, we show that ATM phosphorylates the S1219 residue of 53BP1 *in vitro* and that the residue is phosphorylated in cells exposed to ionizing radiation (IR). Transfection with siRNA targeting ATM abolished IR-induced phosphorylation at this residue, supporting the theory that this process is mediated by the kinase. To determine the functional relevance of this phosphorylation event, a U2OS cell line expressing S1219A mutant 53BP1 was established. IR-induced foci formation of MDC1 and γH2AX, DNA damage signaling molecules, was reduced in this cell line, implying that S1219 phosphorylation is required for recruitment of these molecules to DNA damage sites. Furthermore, overexpression of the mutant protein impeded IR-induced G2 arrest. In conclusion, we have shown that S1219 phosphorylation by ATM is required for proper execution of DNA damage response.

DNA double-strand breaks (DSBs) are the most severe form of DNA damage. Eukaryotic cells activate a series of events, termed 'DNA damage response', including cell cycle arrest, apoptosis induction and DNA repair, to maintain their genomic integrity. When DSBs occur, cells initially activate a signal transduction cascade composed of sensors that sense DNA damage, signal transducers that generate and amplify the DNA damage signal, and effectors that participate in cell cycle arrest, apoptosis or DNA repair. ATM is the first of the signal transducers to be activated, and phosphorylates another signal transducer, Chk2, as well as various effector proteins, including p53 and BRCA1 [1–4].

53BP1, originally reported as a binding protein of p53 [5], functions in DNA damage signaling processes as an activator protein required to facilitate the precise signaling events from ATM protein kinase to its downstream effector proteins, Chk2, BRCA1 and SMC1 [6,7]. The finding that 53BP1 suppression results in decreased ATM activation led to the hypothesis that 53BP1 is an activator of ATM [8,9]. Recent studies show that 53BP1 functions as a DNA damage sensor that binds to the methylated lysine residue of histone H3 exposed by chromatin remodeling in the vicinity of damaged DNA [10]. Following IR, 53BP1 is recruited to regions of DNA damage via methyl histone-binding activity, where it forms nuclear foci [11].

As opposed to its role as an ATM activator or DNA damage sensor, 53BP1 has been reported to be a downstream phosphorylation substrate of ATM [12]. To overcoming this conceptual difficulty, the hierarchic view of "sensors upstream of the transducer" has been modified to "a cyclic process" whereby the DNA damage signal is amplified by repeated interactions among the sensors and transducers [13,14]. 53BP1 appears to play an important role in maintaining genomic integrity, as evident from the finding that loss of a single *53BP1* allele in mice induces genome instability and tumor formation, particularly lymphoma [15]. Experiments with *53BP1*-null mouse embryo fibroblasts (MEFs) support its involvement in the IR-induced G2 checkpoint [16].

S25 and S29 residues have been identified as the sites in 53BP1 that are phosphorylated by ATM upon ionizing radiation [17]. However, mutation of these sites did not alter the behavior of 53BP1 in DNA damage signaling [17]. Recently, several other sites were identified by mass spectrometric analysis of phosphorylated 53BP1 [18]. However, the functional relevance of these sites to DNA damage response remains to be established.

In this report, we tried to identify an ATM phosphorylation site in the region of 53BP1 required for foci formation and histone H3 binding. As a result, we found that the S1219 residue is phosphorylated by ATM, both *in vitro* and *in vivo*. Furthermore, we present evidence supporting the possibility that phosphorylation of this site is required for the precise execution of DNA damage response, including foci formation by DNA damage signaling participants and cell cycle checkpoint activation.

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Materials and methods

Cells and plasmids. U2OS, HeLa and 293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco) at 37 °C in a humidified incubator supplied with 5% CO_2.

Expression vectors for wild-type and kinase-dead Flag-tagged ATM, pcDNA-Flag-ATMwt and pcDNA-Flag-ATMkd were kindly provided by Dr. M. Kastan [3]. The expression vector for a GST-tagged fragment of 53BP1, pGEX-53BP1(1040-1360), was generated by cloning the amplified 53BP1 cDNA fragment into pGEX5X-1 (GE Healthcare). The 53BP1 expression plasmid, pCMH6K53BP1, was kindly provided by Dr. S. Fields [19].

In vitro mutagenesis. Alanine substitution mutants of possible ATM phosphorylation sites were generated using the QuikChange site-directed mutagenesis kit (Stratagene). The primer sequences used were: CTGTTTCAGCAGCtgCCCAGACTATAAAG (T1171A_F), CTT TATAGTCTGGGcaGCTGCTGAAACAG (T1171A_R), CAGAGTCGC TgCATgc CCAGGGAGAAGAAG (S1219A_F), and CTTCTTCTCCCTGG gcATGcAGCGACTC TG (S1219A_R).

Purification of recombinant 53BP1 fragments. Expression vectors containing GST-tagged 53BP1 fragments were transformed into the *E.coli* BL21 strain. GST-tagged 53BP1 fragments were induced with 1 mM IPTG for 2 h, and were purified through Glutathione–Sepharose chromatography (GE Healthcare).

In vitro kinase reaction. 293T cells were transfected with pcDNA-Flag-ATMwt or pcDNA-Flag-ATMkd using Effectene transfection reagent (Qiagen). After 48 h, cells were lysed in lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1% Tween 20, 0.2% NP-40, 1 mM sodium orthovanadate, and protease inhibitor mix from Roche). Wild-type or kinase-dead Flag-tagged ATM proteins were isolated by immunoprecipitation with an anti-Flag antibody (Sigma) and Protein G-conjugated agarose beads (Invitrogen). After washing beads three times in lysis buffer, twice in high salt buffer (lysis buffer supplemented with 0.5 M NaCl) and twice with kinase buffer (20 mM HEPES, pH 7.5, 50 mM NaCl and 1 mM sodium orthovanadate), GST-tagged 53BP1 fragments were added and the kinase reaction performed in kinase buffer supplemented with 10 mM $MnCl_2$, 1 mM DTT, 10 μM ATP and 2 μCi of [γ-P^{32}] ATP (Perkin Elmer) at 30 °C for 30 min. The reaction was terminated by adding SDS sample buffer, and loaded onto 4–12% Tris–Glycine gels (Invitrogen). After electrophoresis, proteins were transferred to nitrocellulose membranes (S & S), and autoradiography performed. The amounts of Flag-tagged ATM and GST-tagged 53BP1 fragments were evaluated by immunoblotting with anti-Flag and anti-GST antibodies, respectively.

Generation of a phosphospecific antibody against S1219. An immunizing peptide containing the sequence 'Ac-TESLHpSQGEE-C' was used to immunize rabbits. Phosphospecific antibodies were purified by affinity chromatography with phosphopeptide-conjugated resin.

siRNA transfection. Synthetic siRNA sequences targeting ATM (AAGCGCCTGATTCGAGATCCT) and ATR (AAGACGGTGTGCTCATG CGGC) were purchased from QIAGEN. Negative control siRNA (Bioneer Inc.) was used as the transfection control. HeLa cells were transfected with siRNA duplexes using Lipofectamine 2000 (Invitrogen). Briefly, 80 pmol siRNA and 4 μl of Lipofectamine 2000 were added separately to 200 μl of DMEM. After 5 min, the two solutions were mixed and incubated for 20 min at room temperature. Mixtures were added to HeLa monolayers fed with fresh medium.

Generation of U2OS cells expressing 53BP1. To generate U2OS cells expressing wild-type and S1219A mutant 53BP1, cells were co-transfected with pCMH6K53BP1-WT or -S1219A, along with pMamNeo (Clontech) at a ratio of 5:1. At 2 days post-transfection, stably transfected cells were selected with 1 mg/ml G418 sulfate until distinct colonies emerged. Resistant colonies were propagated and examined for the expression of HA-tagged 53BP1 by immunoblotting.

Confocal microscopy. U2OS cells were grown on coverslips to 50% confluence, and irradiated with gamma rays. Cells were fixed in 3.7% paraformaldehyde in PBS for 15 min, permeabilized with 0.2% Triton X-100 in PBS for 10 min and blocked with 1% BSA in PBS for 1 h. Fixed cells were incubated for 2 h with primary antibodies, washed in PBS, and incubated with Alexa 488-conjugated donkey anti-rabbit and Alexa 546-conjugated donkey anti-mouse antibodies for 1 h. The primary antibodies used were anti-HA (Covance), anti-MDC1 (Bethyl) and anti-γH2AX (Upstate). Cells were washed in PBS, stained with DAPI, mounted on glass slides, and observed with a Zeiss Axiovert LSM510 microscope.

Cell cycle analysis. Cells were washed with PBS, and fixed in 50% ethanol. Fixed cells were washed once in PBS supplemented with 0.1% Triton X-100, and resuspended in 300 μl of cold PBS. RNase A (5 μl; 10 mg/ml) was added, and the cell suspensions incubated for 15 min at room temperature. Propidium iodide was added at a concentration of 77 μg/ml, and incubated for 30 min on ice. The cellular DNA content was analyzed with FACSCalibur (Becton Dickinson).

Results and discussion

S1219 of 53BP1 is phosphorylated by ATM in vitro

To determine the ATM phosphorylation site of 53BP1 that is functionally relevant to signaling events involved in the DNA damage response, we analyzed serine and threonine residues from positions 1052-1639 (encompassing the region sufficient for IR-induced foci formation) [17]. Among the residues conforming to ATM phosphorylation consensus site [20], only T1171 and S1219 were conserved between *Xenopus laevis*, mouse and human (Fig. 1A).

To determine whether T1171 or S1219 serve as ATM phosphorylation sites, GST-tagged 53BP1 fragments with alanine substitutions at these positions were generated and used as substrates for *in vitro* kinase reactions (Fig. 1B). In the S1219A mutant, phosphorylation of GST-53BP1 was almost abrogated (lanes 1 and 5), clearly implying phosphorylation at this position by ATM.

S1219 is phosphorylated upon DNA damage in vivo

A phosphospecific antibody against phosphorylated S1219 (P-S1219) was raised in rabbits and purified. Using this antibody, S1219 phosphorylation of 53BP1 was readily detected in IR-treated 293T and U2OS cells (Fig. 2A). Furthermore, phosphorylation upon IR exposure was detected by means of immunofluorescence microscopy with the phosphospecific antibody (Fig. 2B). These data confirm that S1219 phosphorylation occurs upon DNA damage.

In an effort to characterize the sites of DNA damage-induced 53BP1 phosphorylation by mass spectrometry, the Rouse laboratory recently identified several phosphorylation sites following IR exposure, including S1219 [18]. However, they did not confirm S1219 phosphorylation with a phosphospecific antibody, nor ascertain the functional relevance of these sites to DNA damage response.

S1219 phosphorylation accompanying DNA damage is primarily mediated by ATM

To check whether S1219 phosphorylation is mediated by ATM, the phosphorylation status of S1219 was monitored after inhibition of ATM or ATR expression by siRNA transfection (Fig. 2C). S1219 phosphorylation was clearly suppressed by siRNA targeting ATM (lanes 2 and 4), but not ATR-specific siRNA (lane 6). However,

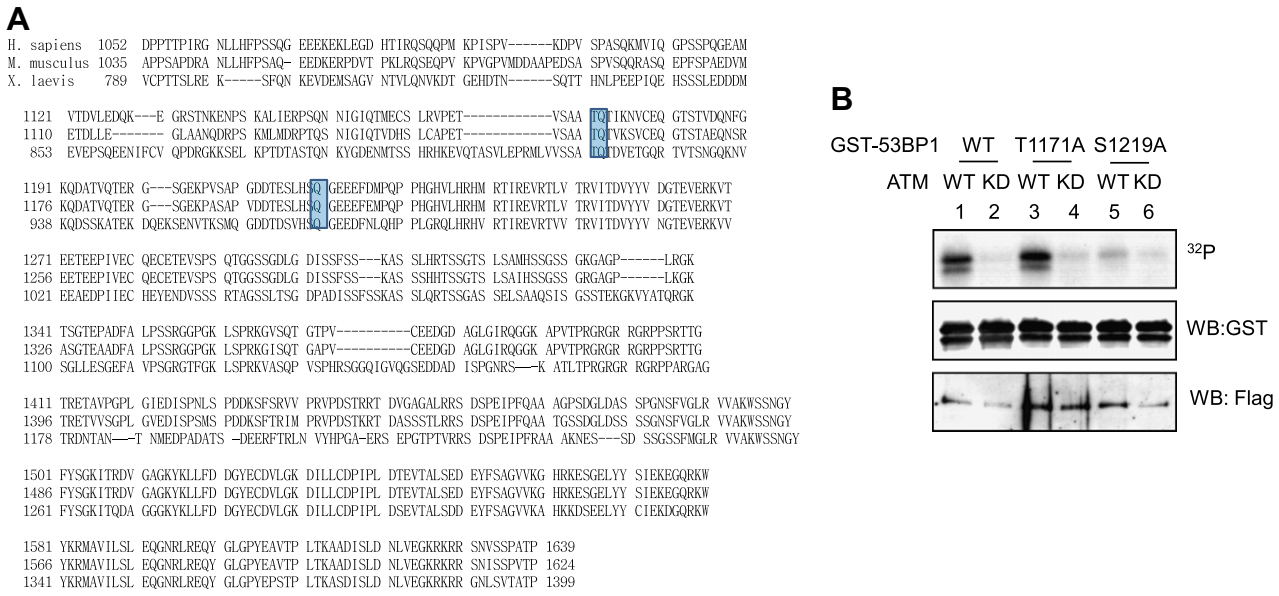

Fig. 1. Determination of the ATM phosphorylation site in the region required for IR-induced foci formation of 53BP1. (A) Alignment of the regions required for IR-induced foci formation of 53BP1 from human, mouse and Xenopus laevis. T1171 and S1219 are presented in shaded boxes. (B) Phosphorylation of GST-tagged 53BP1 (1040–1360) by ATM. Flag-tagged ATM was expressed in 293T cells, and purified using anti-Flag affinity chromatography. Wild-type and mutant GST-53BP1 (1040–1360) (T1171A or S1219A) were used as substrates in the kinase reaction. Reactions with kinase-dead (KD) ATM were performed as negative controls. Western blotting with anti-GST and anti-Flag antibodies were performed to confirm similar amounts of substrates and ATM in the reactions.

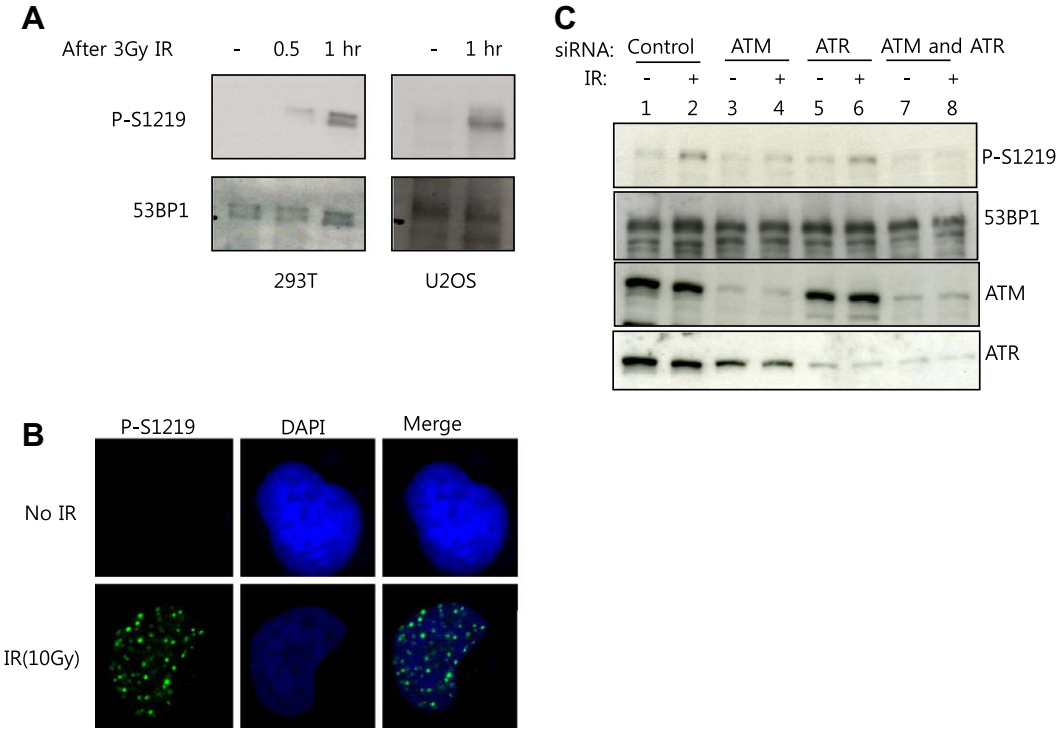

Fig. 2. Confirmation of IR-induced S1219 phosphorylation by ATM *in vivo*. (A) 293T (left panel) and U2OS (right panel) cells were irradiated with 3 Gy of gamma rays, and incubated for the indicated times. Phosphorylated S1219 (P-S1219) levels were detected with a phosphospecific antibody. (B) IR-induced phosphorylation of S1219 was confirmed by immunofluorescence microscopy. U2OS cells were irradiated with 10 Gy of gamma rays, stained with anti-P-S1219, and counterstained with DAPI. (C) ATM kinase is required for phosphorylation of S1219 *in vivo*. HeLa cells were transfected with ATM-specific and/or ATR-specific siRNAs, and irradiated with gamma rays. Phosphorylation of S1219 was detected by immunoblotting with an anti-P-S1219 antibody. Expression levels of 53BP1, ATM and ATR were additionally examined.

complete abrogation of the phosphorylation occurred only after introduction of both siRNAs (lane 8). These findings demonstrate that IR-induced S1219 phosphorylation is primarily mediated by ATM, even though there might be functional redundancy between ATM and ATR on this phosphorylation event.

Functional relevance of S1219 phosphorylation in the DNA damage response

To examine the functional significance of S1219 phosphorylation in DNA damage, U2OS cells were stably transfected with

wild-type or S1219A mutant 53BP1 expression plasmids, and the DNA damage response was examined in these stable cell lines. Even though the stable cell lines still express endogenous 53BP1, we anticipated that overexpressed S1219A mutant 53BP1 might inhibit the function of 53BP1 through dominant-negative effects. The most prominent phenomenon in the early phase of the DNA damage signaling is the formation of IR-induced foci by various DNA damage sensor and effector molecules. We assessed the formation of foci by MDC1, an early participant of DNA damage signaling [21]. In cells expressing S1219A mutant 53BP1, foci formation of MDC1 was partially, but significantly inhibited (Fig. 3A). Similarly, formation of phosphorylated form of H2AX, a histone H2 variant (γ-H2AX) was suppressed by overexpression of S1219A mutant 53BP1 (Fig. 3B). These results imply that 53BP1 S1219 phosphorylation is required for the foci formation of the early participants in DNA damage signaling.

Next, we investigated whether proper G2 arrest occurs after IR in the mutant S1219A 53BP1-expressing stable cell line (Fig. 4). The cell population in the G2 phase (38.1% after 5 Gy of IR) was lower in S1219A-expressing cultures, compared to control cells (64.9%), strongly implying that S1219 phosphorylation mediates the signaling events required for proper cell cycle arrest.

In terms of the sequence of recruitment of signaling molecules to the DNA damage sites for nuclear foci formation, Mre11-Rad50-Nbs1 (MRN complex) is the first factor that binds to the damaged sites and acts as a DNA damage sensor [22,23]. And phosphorylation of H2AX by ATM is required for the recruitment and retention of DNA repair and checkpoint proteins, including 53BP1 and MDC1, early participants in DNA damage signaling, to the sites of DNA damage [21,24,25]. Furthermore, it has been reported that MDC1

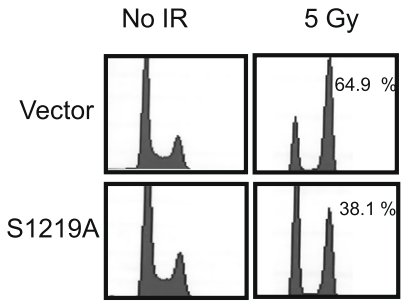

Fig. 4. Overexpression of the S1219A mutant 53BP1 protein impedes IR-induced G2 arrest. IR-stimulated G2 arrest was analyzed in U2OS stable cell lines expressing the vector only and S1219A mutant protein. Cell cycle distribution was examined by flow cytometry after propidium iodide staining at 16 h after IR with 5 Gy. The numbers within the graphs indicate percentages of the G2 population.

is required for sustained binding of the MRN complex and 53BP1 to damaged chromatin [23,26]. However, there are several reports suggesting that 53BP1 is one of earliest DNA damage participants, along with the MRN complex. First, 53BP1 has been suggested to function as a sensor molecule through its methylated histone binding activity [10] and to be involved in activation of ATM [8]. Second, the initial migration of 53BP1 to DNA damage sites does not require γ-H2AX [27]. Recently, it has been reported that tethering MDC1 to chromatin led to the recruitment of MRN complex and vice versa, suggesting a positive feedback loop between these repair factors [28]. Therefore, it is quite possible that repeated interactions between early participants of DNA damage signaling pathway, γ-H2AX, MDC1, 53BP1 and MRN complex, instead of

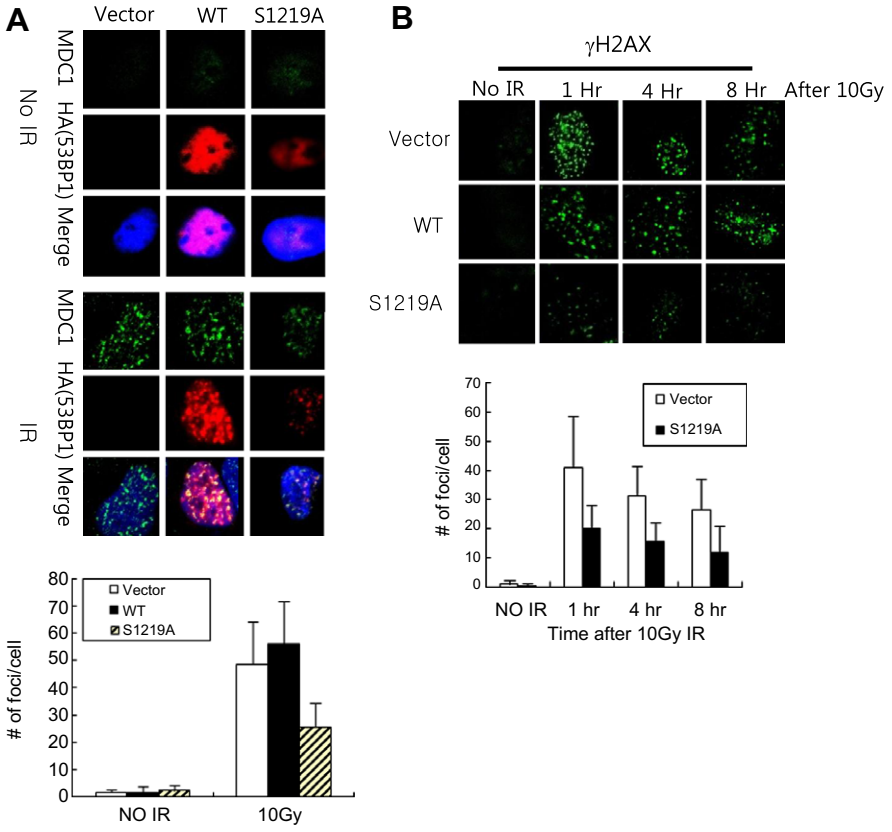

Fig. 3. Mutant S1219A 53BP1 displays defects in forming foci upon IR, and its expression impairs foci formation by MDC1 and γ H2AX. Foci formation of DNA damage response regulators, MDC1 (panel A) and γH2AX (panel B), was monitored in U2OS cells stably expressing wild-type (WT) or mutant protein. Control cells were transfected with empty vector (Vector). After irradiation, cells were stained with anti-HA, along with anti-MDC1 (panel A) or anti-γ H2AX (panel B) antibodies, and examined under a Zeiss Axiovert LSM510 microscope. The number of foci per cell was counted for 20 cells, and plotted in the graphs below.

sequential recruitments of these factors, occur to maximize DNA damage signaling. During these interactions, phosphorylation by ATM kinase may play an important role in signal amplification. In this regard, S1219 phosphorylation of 53BP1 might be one of the key signaling events required for complete recruitment of DNA damage signaling molecules.

In conclusion, we have identified an ATM phosphorylation site within the region required for IR-induced foci formation of 53BP1. Furthermore, we present evidence that the phosphorylation of this site is required for IR-induced G2 checkpoint activation and full activation of DNA damage signaling molecules, such as γ−H2AX and MDC1.

Acknowledgments

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