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Report

The GAR Motif of 53BP1 is Arginine Methylated by PRMT1 and is Necessary for 53BP1 DNA Binding Activity

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arginine methylation, methylarginine; 53BP1, PRMT1, DNA damage foci, GAR motif, DNA repair

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ABSTRACT

The p53-binding protein 1 (53BP1) is rapidly recruited to sites of DNA double-strand breaks and forms characteristics nuclear foci, demonstrating its role in the early events of detection, signaling and repair of damaged DNA. 53BP1 contains a glycine arginine rich (GAR) motif of unknown function within its kinetochore-binding domain. Herein, we show that the GAR motif of 53BP1 is arginine methylated by protein arginine methyltransferase 1 (PRMT1), the same methyltransferase that methylates MRE11. 53BP1 contains asymmetric dimethylarginines (aDMA) within cells, as detected with methylarginine-specific antibodies. Amino acid substitution of the arginines within the GAR motif of 53BP1 abrogated binding to single and double-stranded DNA, demonstrating that the GAR motif is required for DNA binding activity of 53BP1. Fibroblast cells treated with methylase inhibitors failed to relocalize 53BP1 to sites of DNA damage and formed few γ-H2AX foci, consistent with our previous data that MRE11 fails to relocalize to DNA damage sites in cells treated with methylase inhibitors. Our findings identify the GAR motif as a region required for 53BP1 DNA binding activity and as the site of methylation by PRMT1.

INTRODUCTION

Events generating DNA damage result in a complex cellular response implicating a plethora of factors responsible for detection, signaling and repair of the DNA breaks. This initial response requires the orderly recruitment of many proteins including protein kinases, enzymes and adaptors molecules that will mediate the reparation and signaling of DNA damage.[1,2] One of the first steps consists in the phosphorylation of a multitude of targets by the phosphatidylinositol 3-kinase protein kinase-like (PIKK) family members ATM, ATR and DNA-PK.[3] Immediately following DNA damage, H2AX becomes phosphorylated by PIKK kinases on serine 139 surrounding the DNA break,[4] which is followed by the recruitment of several DNA damage response factor such as BRCA1, 53BP1, MDC1, Rad51 and the MRE11/RAD50/NBS1 complex (MRN complex).[5-10] Although the exact order of events has proven almost impossible to decipher, H2AX deficient mice have demonstrated that phosphorylation of H2AX is one of the earliest event.[9] Indeed, the recruitment of several DNA repair proteins such as NBS1, 53BP1, MDC1 and BRCA1 to the site of DNA damage requires the presence of H2AX.[10] This was further confirmed by the fact that NBS1, 53BP1 and MDC1 could directly interact with phosphorylated H2AX.[11-16] In contrast, H2AX phosphorylation occurred normally in 53BP1 deficient mice,[17] but downstream events such as Chk2 activation was impaired,[16] placing 53BP1 downstream of H2AX in the response to DNA damage. The MRN complex associates with chromatin during DNA replication and acts as a sensor of DNA double strand breaks after exposure to radiation.[18] Although the recruitment to sites of DNA damage of NBS1 is impaired in H2AX deficient mice,[10] the MRN complex was shown to be able to sense breaks in DNA and relay this information to ATM,[19-21] which in turn activates pathways for cell cycle checkpoint activation. Thus the activity of the MRN complex appears to be the first event in the recognition of DNA damage.[22-24]

53BP1 was first identified in a yeast two-hybrid screen for proteins interacting with the tumor suppressor p53.[25] 53BP1 contains a tandem repeat of BRCA1 C-terminus (BRCT) domains and directly binds p53 via this C-terminal BRCT repeat.[26,27] This conserved domain is present at the carboxyl terminus of numerous proteins involved in cellular responses to DNA damage, suggesting a role for 53BP1 in damage response pathways. BRCT domains have also been characterized as phosphopeptide-binding modules.[28]

In response to DNA damage, 53BP1 becomes hyperphosphorylated by ATM and binds to chromatin, forming discrete nuclear foci that colocalize with MRE11.[29-33] 53BP1 is also required for at least a subset of ataxia telangiectasia-mutated (ATM)-dependent phosphorylation events at sites of DNA breaks[34] and has recently been implicated in a novel ATM-dependent pathway, involving Artemis, required for the processing of a subset of DSBs prior to rejoining by nonhomologous end-joining (NHEJ).[35,36] 53BP1 can bind both ss and ds DNA with high affinity, thus possibly acting as an alignment factor for DSBs.[33] 53BP1 contains two tandem tudor domains[33,37,38] that binds to methylated histone H3 in vitro.[38,39] The tudor domain of another protein, SMN, has been shown to bind methylated arginines,[40] suggesting that the 53BP1 tandem tudor domains are methyl-binding domains. The Pax2 transactivation domain-interacting protein (PTIP), an essential gene required for the maintenance of genome stability, also associates with 53BP1 in response to ionizing radiation.[41]

Protein arginine methylation is a post-translational modification that results in the addition of one or two methyl groups to the guanidino nitrogen atoms of arginine resulting in asymmetrically and symmetrically dimethylarginines (aDMA, sDMA).[42] This reaction is catalyzed by a family of protein arginine methyltransferases (PRMTs) that utilize S-adenosyl methionine (AdoMet) as a methyl donor.[43] PRMT1, the first identified arginine methyltransferase, is known to be localized throughout the cell and to preferentially methylate glycine-arginine rich (GAR) motifs within its substrates.[44,45] Proteins associated with nucleic acids such as RNA binding proteins, histones, and the DNA damage response protein MRE11 are known PRMT1 substrates.[42] As such, arginine methylation by PRMT1 has been shown to regulate RNA metabolism, transcription and the DNA damage response.[42,46]

During our proteomic analysis of methylated proteins, we identified the first DNA damage response protein (MRE11) using methylarginine specific antibodies.[47] We subsequently showed that MRE11 does indeed contain methylarginines within its GAR motif by using mass spectrometry and methylarginine-specific antibodies.[48,49] Herein we identify 53BP1 as a methylated protein using methylarginine-specific antibodies. 53BP1 was methylated by PRMT1 at its GAR motif located within the kinetochore-binding domain. Amino acid substitution of the arginines within the kinetochore binding domain abrogated binding to single and double-stranded DNA. These findings suggest that arginine methylation of the GAR motif may regulate the ability of 53BP1 to mediate its role during the DNA damage response.

MATERIALS AND METHODS

Antibodies. The antibodies recognizing methylated arginines (SYM10, ASYM25), methylated MRE11 (MeMRE11) and MRE11 methylated on arginine 587 have been described previously.[47-50] The polyclonal antibodies were affinity purified over the antigenic peptide coupled to Affigel beads (Bio-Rad), eluted with 100 mM glycine pH 2.5, and concentrated by using Centricon columns (Millipore, Bedford, MA). The 12CA5 HA monoclonal antibodies (mAb) was purchased from the American Type Culture Collection (Manassas, VA). The γ-H2AX monoclonal antibody was from Upstate Biotechnology and the 53BP1 antibody was from Novus Biologicals (Littleton, CO).

Cell culture. The primary human foreskin fibroblasts CRL2097, HeLa and SK-N-SH cells are from the ATCC.

DNA constructs. The PRMT1, PRMT3 and PRMT4/CARM1, PRMT5 and PRMT6 expression plasmids have been described elsewhere.[50,51] The PRMT7 cDNA was amplified by PCR and subcloned into pGEX-4T2 for GST expression. HIS-tagged 53BP1 recombinant protein constructs have been described previously.[33] His-53BP1 mutants have been generated using a mutagenesis kit (QuickChange; Stratagene, La Jolla, CA) with all arginines replaced by alanine (codon GCC). Each multiple mutant was obtained with primers containing the desired mutation in addition to previously generated mutation(s) starting from Arg 1403. Plasmids expressing HA-tagged 53BP1 have been described previously.[33] HA-53BP1 mutants used for foci experiments were generated in the same manner as the His-53BP1 mutants.

Protein expression. HeLa cells were transfected with LipofectAMINE PLUS (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. For immunoprecipitations, cell lysates were incubated on ice with the primary antibody for 1 h. Then 20 µl of a 50% protein A-Sepharose slurry was added and incubated at 4°C for 30 min with constant end-over-end mixing. The beads were washed twice with lysis buffer and once with PBS. Protein samples were analyzed on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The designated primary antibody was followed by goat anti-mouse or goat anti-rabbit antibodies conjugated to horseradish peroxidase (ICN Pharmaceuticals, Costa Mesa, CA) and chemiluminescence was used for protein detection (DuPont, Wilmington, DE).

Purification of wild-type and mutant KBD-M. His-tagged KBD-M wild type and mutants were overexpressed in *E.coli* strain B834 (DE3) pLysS. Cells were grown at 37°C in Luria Broth containing 100 µg/ml ampicillin to an OD_{600} of 0.6. Expression was induced by addition of 1 mM IPTG and cells were incubated at 22°C overnight. Harvested cells were resuspended and sonicated in presence of 100 ml of IMAC buffer A (50 mM Tris pH 7.5, 20 mM Imidazole, 300 mM NaCl, 10% Glycerol, PMSF, benzamidine and a of protease inhibitor cocktail tablet, Complete (Roche Diagnostic). Soluble His-KBD-M was separated from cell debris by centrifugation and the supernatant loaded onto a 25 ml Ni-NTA column (Qiagen). After elution by imidazole (300mM), the protein was dialysed against buffer IEX A (50 mM Tris pH 7.5, 10% Glycerol, PMSF, benzamidine, 5 mM DTT) and loaded onto a 10 ml anionic resin (DEAE, Amersham biotech). Elution was carried out by running a salt gradient (NaCl) and the protein was dialyzed against 50 mM Tris pH 7.5, 150 mM NaCl, 10% Glycerol, PMSF, benzamidine, 5 mM DTT before being stored at 4°C.

Methylation assays. GST-tagged MRE11B (a generous gift from Mark Bedford) corresponding to MRE11 amino acids 554 to 680 or HIS-tagged 53BP1 recombinant proteins corresponding to various region of 53BP1 were incubated with GST-tagged PRMT1, PRMT3, PRMT4, PRMT5, PRMT6 or PRMT7 with 0.55 µCi of [methyl-^{3}H] S-adenosyl-L-methionine (^{3}H-SAM; PerkinElmer Life Sciences, Boston, MA) in the presence of 25 mM Tris-HCl at pH 7.5 for 1 h at 37°C in a final volume of 30 µl. Reactions were stopped by adding 20 µl of 2x SDS-PAGE sample buffer, followed by heating at 100°C for 10 min. Samples were loaded on 10% SDS-polyacrylamide gels and stained with Coomassie Blue. The destained gels were soaked in ENHANCE (PerkinElmer Life Sciences) according to manufacturer instructions and visualized by fluorography. The in vivo methylation assay was performed by metabolically labeling the cells with L-[methyl-^{3}H] methionine directly in methionine-free medium for 3 h in the presence of cycloheximide and chloramphenicol, as described previously.[51] L-[^{35}S] methionine was also used as a control under the same conditions (data not shown). The cell lysates were immunoprecipitated and the ^{3}H-labelled proteins were visualized by fluorography.

Immunofluorescence and in situ fractionation. To visualize 53BP1, transfected cells were fix in 1% paraformaldehyde for 10 minutes, permeabilized in 0.5% Triton X-100 for 10 minutes, blocked in 10% goat serum and labeled for immunofluorescence with primary antibodies against various proteins and the appropriate Alexa488- (green, Molecular Probes) and Alexa546- (red, Molecular Probes) conjugated secondary antibodies. Cells were mounted in Immuno-Mount (Thermo Shandon, Pittsburgh, PA) containing DAPI (Sigma-Aldrich) at 1 mg/ml. Images were collected with an Olympus immunofluorescence microscope.

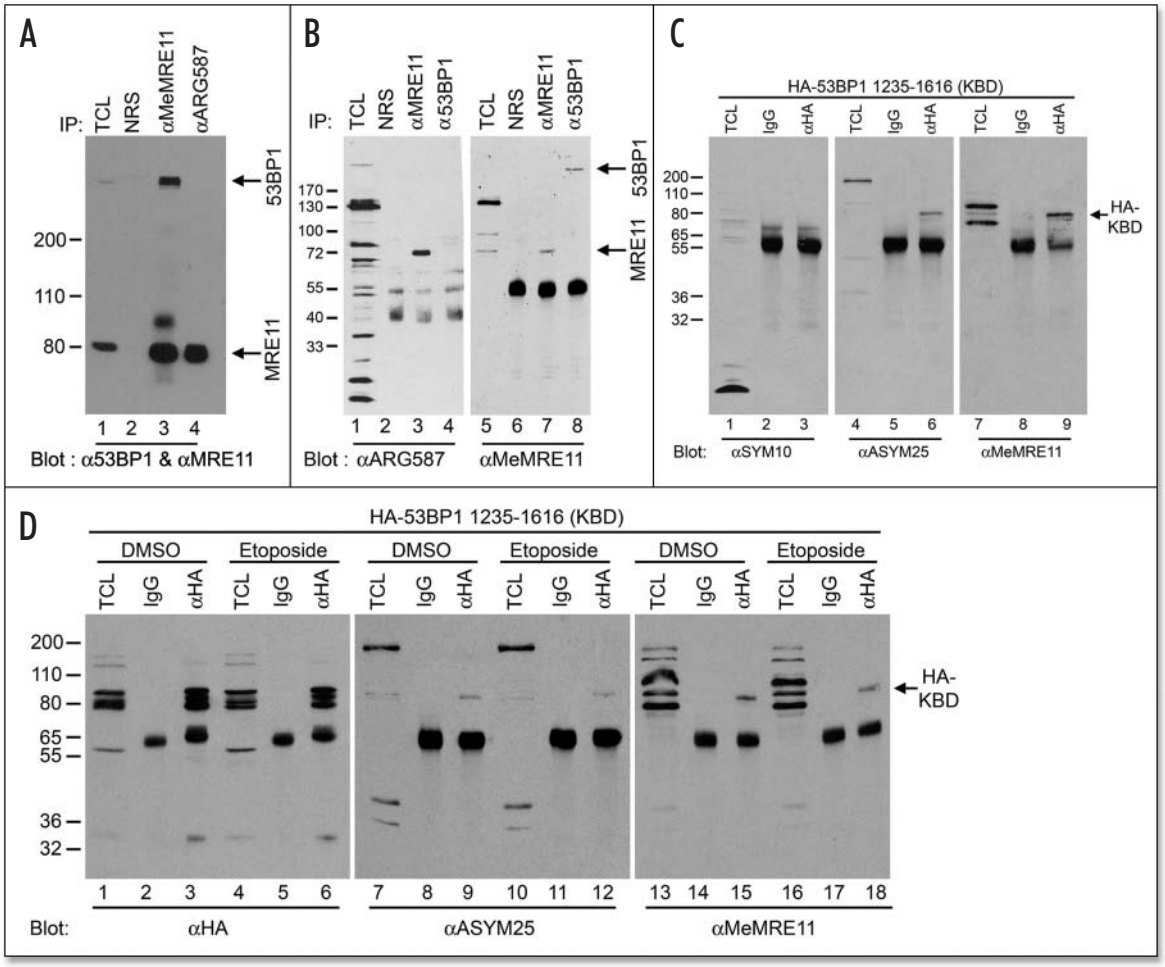

Figure 1. 53BP1 contains asymmetrically dimethylated arginines. (A) Antibodies directed against MRE11 GAR motif (MeMRE11) or methylated arginine 587 of MRE11 (ARG587) were used to perform immunoprecipitations from HeLa cell extracts, and after washing, bound proteins were analyzed by Western blotting to detect 53BP1 and MRE11. The total cell lysate (TCL) shows 10% of the extract used in each binding assay. The migration of 53BP1 and MRE11 is indicated on the right and the migration of the molecular mass markers is shown on the left in kDa. (B) A control antibody (Normal Rabbit Serum, NRS), an antibody directed against MRE11 or an antibody against 53BP1 were used to perform immunoprecipitations from HeLa cell extracts, and after washing, bound proteins were analyzed by Western blotting to detect MRE11 methylated GAR motif (MeMRE11) or methylated arginine 587 of MRE11 (ARG587). The total cell lysate (TCL) shows 10% of the extract used in each binding assay. The migration of 53BP1 and MRE11 is indicated on the right and the migration of the molecular mass markers is shown on the left in kDa. (C) HA-53BP1 KBD domain (amino acids 1235-1616) was transfected in HeLa cells and immunoprecipitated from the cell extracts using an anti-HA antibody, and after washing, bound proteins were analyzed by immunoblotting using an anti-sDMA (SYM10), an anti-aDMA (ASYM25) or an anti-MRE11 GAR (MeMRE11) antibody. Many methylated proteins are visualized in the TCL and the migration of 53BP1 is shown with an arrow. (D) HA-53BP1 KBD domain (amino acids 1235-1616) was transfected in HeLa cells. Following transfection, cells were either mock (DMSO) treated or treated with 20 μM etoposide for 1 hr, washed and allowed to recover for another hr. HA-53BP1 was then immunoprecipitated from the cell extracts using an anti-HA antibody, and after washing, bound proteins were analyzed by immunoblotting using an anti-HA, an anti-aDMA (ASYM25) or an anti-MRE11 GAR motif (MeMRE11) antibody. The migration of 53BP1 is shown with an arrow.

RESULTS

53BP1 is arginine methylated in vivo. The arginines within glycine-arginine rich (GAR) motifs are known to be post-translationally modified by PRMTs.[42] Recently, the DNA damage response protein MRE11 was shown to be methylated by PRMT1 within its GAR motif.[48] Interestingly, another DNA damage response protein 53BP1 harbors a GAR motif of unknown function within its kinetochore binding domain KBD.[52] We investigated whether arginine methylation of the 53BP1 GAR motif may regulate its function. To examine whether 53BP1 was arginine methylated in vivo, HeLa cell extracts were immunoprecipitated with normal rabbit serum (NRS) as control or with methylarginine-specific antibodies against MRE11 arginine 587 (αARG587) and one that was generated against the methylated MRE11 GAR motif (αMeMRE11). We reasoned that the anti-MRE11 methyl-specific antibodies, especially αMeMRE11, may recognize other arginine methylated proteins besides MRE11. The bound proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with anti-53BP1 and anti-MRE11 antibodies (Fig. 1A). Indeed MRE11 was identified in both αARG587 and αMeMRE11 (Fig. 1A) immunoprecipitations, as reported previously.[48] Interestingly 53BP1 was identified only in the αMeMRE11 immunoprecipitation. The absence of 53BP1 with αARG587 demonstrates that 53BP1 does not coimmunoprecipitate with methylated MRE11 (Fig. 1A, lane 4). Moreover, immunoprecipitated 53BP1 was directly recognized by immunoblotting with αMeMRE11 and not αARG587 antibodies (Fig. 1B). These data confirm that 53BP1 is directly recognized αMeMRE11 and contains asymmetrically dimethylarginines in vivo.

The amino acids 1235 to 1616 of 53BP1 harbor the KBD, which encompasses a GAR motif. To determine whether this region is arginine methylated within cells, HeLa cells were transfected with an expression

vector that expresses a hemaglutin (HA) epitope tagged KBD fusion protein. The transfected cells were lysed and immunoprecipitated with immunoglobulin G (IgG) control or anti-HA antibodies. The bound proteins were separated by SDS-PAGE and immunoblotted with methylarginine-specific antibodies. The HA-KBD fusion protein was recognized by the αMeMRE11 antibodies, and not with an sDMA-specific SYM10 antibodies (Fig. 1C). Moreover, another general methylarginine-specific antibody ASYM25 recognized HA-KBD, demonstrating that this region of 53BP1 contains aDMA. The level of arginine methylation of 53BP1 remained unchanged following exposure to DNA damage, demonstrating that arginine methylation of 53BP1 is not induced following activation of the DNA damage response by treatment with etoposide (Fig. 1D).

Arginine methylation of 53BP1 by PRMT1. Human cells contain at least eight known Protein Arginine Methyl-Transferases (PRMTs).[42] To identify the PRMT responsible for the observed in vivo modification of 53BP1, we first tested a range of recombinant PRMTs for in vitro methylation of recombinant KBD region of 53BP1. We observed that KBD was methylated in vitro in the presence [^{3}H]-AdoMet by PRMT1, but not by PRMT3, PRMT4, PRMT5, PRMT6 or PRMT7 (Fig. 2A). All the methyltransferases were active when incubated with known substrates (data not shown). To narrow down the region of 53BP1 targeted by PRMT1, we performed in vitro methylation assays with different regions of recombinant 53BP1 deletion constructs (Fig. 2B and C). The deletion mapping studies revealed that a central region of KBD, termed KBD-M, but not adjacent regions (KDB-NN or KBD-C) contained the PRMT1 methylation target site of 53BP1 (Fig. 2B–D).

GAR motif of 53BP1 is arginine methylation by PRMT1. The KBD-M region of 53BP1 contains the GAR motif consisting of five arginines (Fig. 3A). Previously, it was reported that this motif is a target site for arginine methylation on a number of proteins, including MRE11.[43] To verify that the arginine residues within the GAR motif were the sites of methylation on 53BP1, we mutated each of these residues in the GAR motif of KBD-M, either individually or in combination, and performed in vitro methylation assays with PRMT1 (Fig. 3B). Single point mutations had no apparent effect of the level of methylation (Fig. 3B, lower panel, lanes 2–6). In contrast, mutation of arginines 1400, 1401 and 1403 (R3) resulted in a significant reduction in the incorporation of [^{3}H]-methyl groups as visualized by in vitro methylation assays with recombinant PRMT1 (Fig. 3B, lower panel, lane 10). The methylation was considerably reduced when 4 or 5 of the arginines are substituted with alanines, R4 and R5 respectively (Fig. 3B, lower panel lanes 11 and 12).

GAR motif of 53BP1 required for DNA binding. As shown above, the methylated GAR motif of 53BP1 resides in the KBD-M fragment of 53BP1. Previously, we reported that this region binds avidly to both double-stranded (ds) and single-stranded (ss) DNA.[33] To test the effect of mutating the arginines of 53BP1 (KBD-M) to alanines on DNA binding, we performed gel electro-mobility shift assays with the various mutants and either ss and ds DNA (Fig. 4A and B). Interestingly, the level of DNA binding directly correlated to the presence of the methylated arginines in the GAR motif (Fig. 3C, lower panel). The single point mutation of the arginines residues in GAR (S1-S5) had little effect on the affinity of KBD-M for DNA and all are PRMT1 methylated (Fig. 4A, lanes 3–7). In contrast, the GAR mutant with three arginines mutated to alanines (R3), that showed reduced methylation by PRMT1 (Fig. 3C, lower panel, lane 10), also demonstrated reduced DNA binding (Fig. 4, lanes 12). Moreover, the two mutants (R4 and R5) that showed almost no methylation by PRMT1 (Fig. 3, lanes 11 and 12) also had no affinity for either ss or ds DNA (Fig. 4, lanes 13 and 14). These data demonstrate that the arginines of 53BP1 that are directly methylated by PRMT1 are also essential for DNA binding.

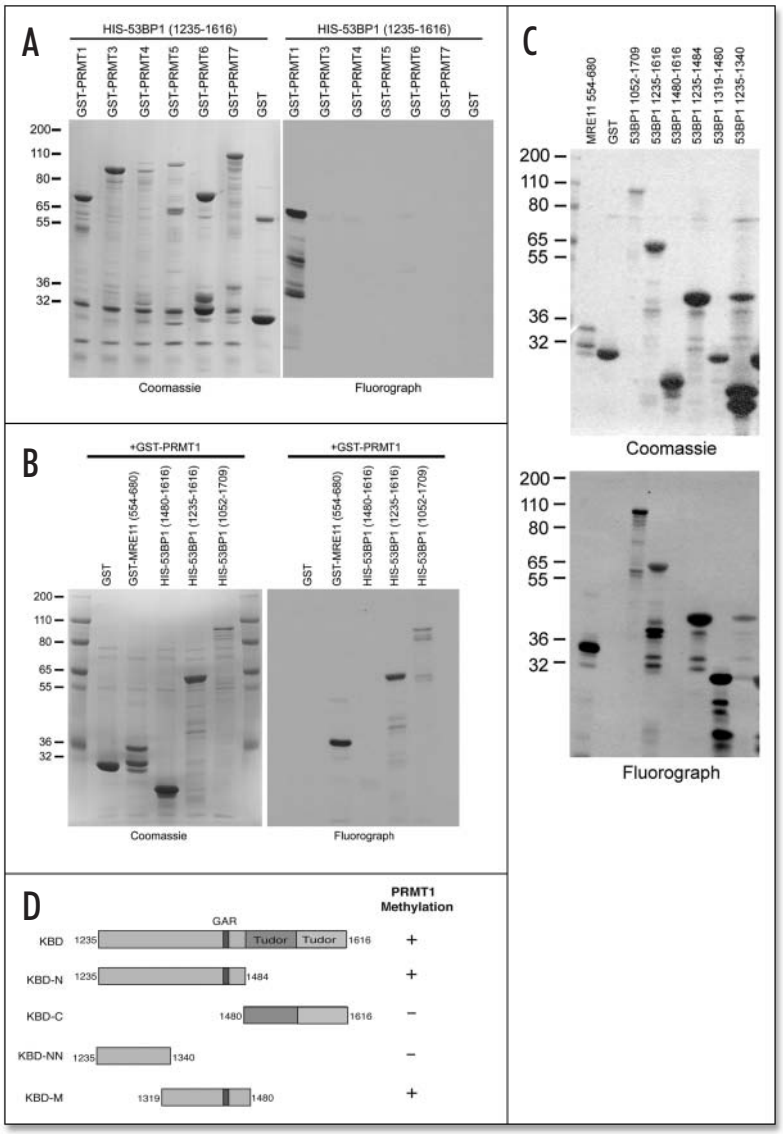

Figure 2. Arginine methylation of 53BP1 by PRMT1. (A) Recombinant GST-PRMTs were incubated with HIS-53BP1 in the presence of [^{3}H]-SAM as the methyl donor. (B) Recombinant GST-PRMT1 was incubated with GST-MRE11 or with HIS-53BP1 amino acids [^{3}H]-SAM. (C) Recombinant GST-PRMT1 was incubated with GST-MRE11 with GST or with HIS-53BP1 with [^{3}H]-SAM. Proteins were separated by SDS-PAGE and visualized by Coomassie staining (left) and the [^{3}H]-labeled proteins visualized by fluorography (right).

Inhibition of methylation prevents 53BP1 localization to sites of DNA damage. To address whether arginine methylation affected the relocalization of 53BP1 following DNA damage, human fibroblasts were either mock treated (Fig. 5A–H) or treated with the methylase inhibitors MTA and adenosine-dialdehyde (Adox) for 24 h (Fig. 3I–P), followed by DNA damage introduced by 1h treatment with 20 μM of the topoisomerase II inhibitor, etoposide. Mock-treated cells exhibited 53BP1 dispersed in the nucleus with few apparent foci and very little γ-H2AX foci (Fig. 5A–D). Etoposide induced the formation of DNA damage induced foci that contained 53BP1 and γ-H2AX (Fig. 3E–H), as observed by using anti-53BP1 (Fig. 5F) and anti-γ-H2AX (Fig. 5G) antibodies. Cells pretreated with methylase inhibitors also contained nuclear 53BP1 and few γ-H2AX foci (Fig. 5I–L). DNA damage by etoposide prevented both the relocalization of 53BP1 to sites of DNA damage (Fig. 5N), as well as the formation of γ-H2AX foci (Fig. 5O). These results indicate that inhibition of methylation using methyltransferase inhibitors prevent the localization of 53BP1 to sites of

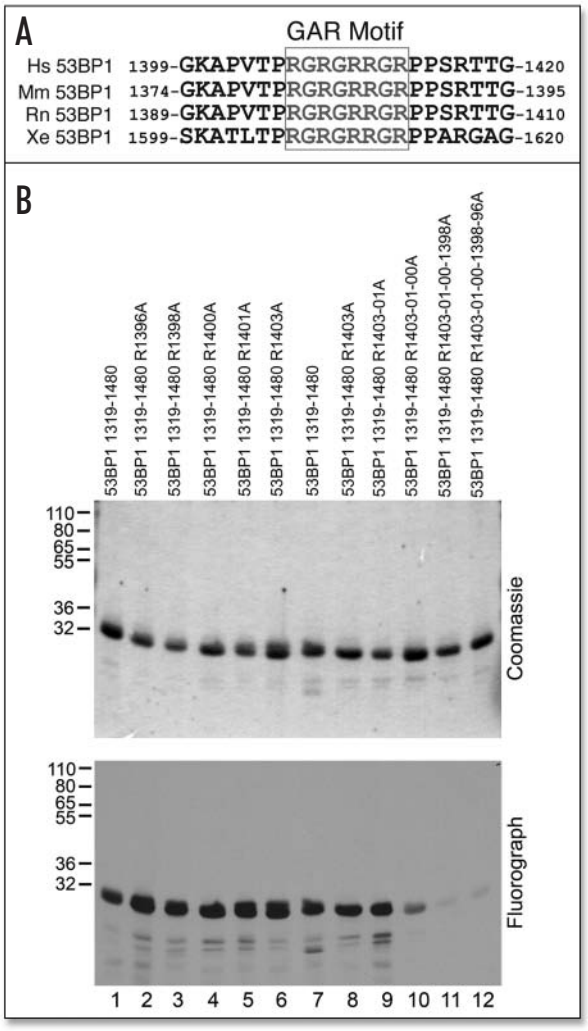

Figure 3. Mutations of arginines within the GAR motif of 53BP1. (A) The amino acids sequence of 53BP1 GAR motif. (B) Recombinant GST-PRMT1 was incubated with HIS-53BP1 amino acids 1319-1480 or with His-53BP1 amino acids 1319-1480 with the indicated arginines substitutions to alanines in the presence of [^{3}H]-SAM as the methyl donor. Proteins were separated by SDS-PAGE and visualized by coomassie staining (top) and the [^{3}H]-labeled proteins visualized by fluorography (bottom).

DNA damage and these findings are consistent with our previously published data that show defects in MRE11 relocalization after DNA damage.[49]

Mutation of GAR motif does not effect foci formation. To determine whether the arginines of 53BP1 are directly involved in its recruitment to sites of DNA damage, we mutated the five arginines within 53BP1 GAR motif to alanines. Human SK-N-SH cells were then transfected with either HA-tagged wild-type 53BP1 KBD domain (KBD-WT), or the KBD domain with the five arginines mutated to alanines (KBD-M5). Twenty-four hours following transfections, DNA damage was introduced by 1 h treatment with 20 μM of the topoisomerase II inhibitor, etoposide. Both KBD-WT and KBD-M5 were dispersed in the nucleus with few apparent foci (Fig. 5B and J) that colocalized perfectly with endogenous 53BP1 (Fig. 5D and L). Etoposide induced the formation of DNA damage induced foci that contained both KBD-WT and 53BP1 (Fig. 5H). Mutations of the arginines within the GAR motif did not prevent the relocalization of KBD-M5 to sites of DNA damage (Fig. 5P), indicating that these arginines are not essential for recruitment of 53BP1 to sites of DNA damage. Similar results were obtained with 53BP1$^{-/-}$ immortalized MEFs cells transfected with either the wild-type KBD, or the mutants carrying all five arginines mutated to alanines (data not shown).

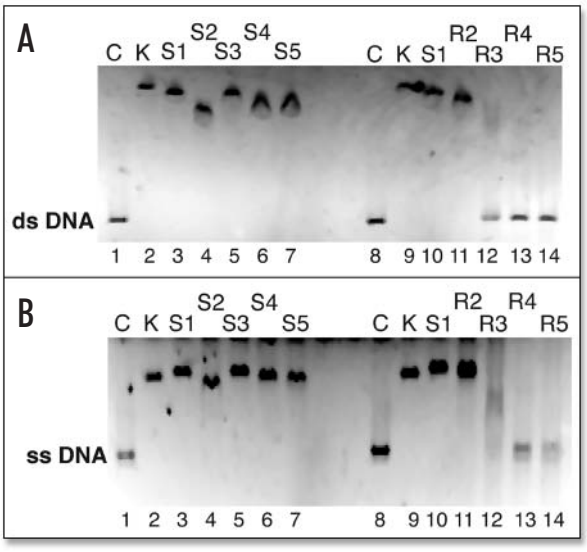

Figure 4. The GAR motif is required for DNA binding. Recombinant HIS-53BP1 amino acids 1319-1480 or with HIS-53BP1 amino acids 1319–1480 with the indicated arginines substitutions to alanines were incubated with either dsDNA (A) or ssDNA (B). Protein:DNA complexes were separated by electrophoresis and stained with ethidium bromide. C, no protein; K, KBD-M; S1, single mutant 1403 R-A; S2, Single mutant 1401 R-A; S3, Single mutant 1400 R-A; S4, Single mutant 1398, R-A ; S5, Single mutant 1396; R-A; R2, double mutant 1403,1401 R-A; R3, triple mutant 1403, 1401, 1400 R-A; R4, quadruple mutant 1403, 1401, 1400, 1398, R-A; R5, quintuple muatant 1403, 1401, 1400, 1398, 1396 R-A.

GAR and tudor domains both required for recruitment of 53BP1 to sites of DNA damage. Recently, histone H3 methylation on lysine 79 has been shown to be required for recruitment of 53BP1 to sites of DNA damage,[38] possibly through direct interaction between the methylated lysine of histone H3 and the Tudor domains of 53BP1. To determine which minimal region was required for recruitment of 53BP1 to sites of DNA damage, we tested different regions of the KBD domain in their ability to form foci following DNA damage. Human SK-N-SH cells were transfected with either HA-tagged 53BP1 KBD domain (KBD, Fig. 7A–H) or regions of the KBD domain containing either the GAR motif (KBD-N, (Fig. 7I–P) or the Tudor domains (KBD-C, (Fig. 7Q–X). 24 hr following transfections, DNA damage was introduced by 1h treatment with 20 μM of the topoisomerase II inhibitor, etoposide. All the different constructs were localized dispersed in the nucleus with few apparent foci (Fig. 7B, J and R). Etoposide induced the the formation of DNA damage induced foci that contain both KBD and 53BP1 (Fig. 7H). However, the GAR motif or the Tudor domains alone did not relocalized to sites of DNA damage (Fig. 7N and V), indicating that these domains are not sufficient by themselves for recruitment of 53BP1 to DNA break sites. Interestingly, the presence of excess KBD-N appears to partially prevent endogenous 53BP1 foci formation (Fig. 7O).

DISCUSSION

The results presented here establish for the first time that 53BP1 is arginine methylated and demonstrate another molecular function for methylation in DNA repair. Herein we demonstrate that 53BP1 contains aDMA within its GAR motif and that these modifications are catalyzed by PRMT1. By using antibodies recognizing aDMA, we examined whether arginine methylation was regulated following DNA damage and no difference in 53BP1 methylation or overall methylation was observed following genotoxic treatments with our methylarginine-specific antibodies. Recently, histone H3 methylation on lysine 79 has been shown to be required for recruitment of

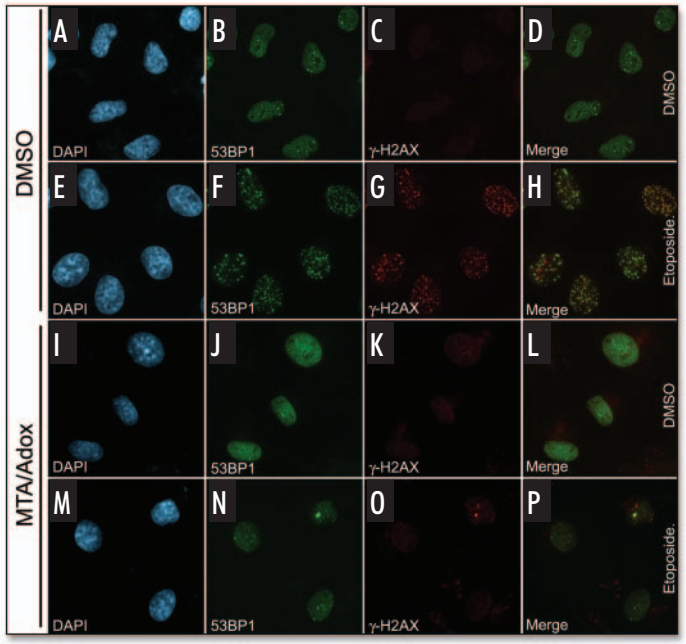

Figure 5. Methylase inhibitors prevent the localization of 53BP1 at sites of DNA damage. The human primary fibroblasts CRL2097 were mock treated with DMSO (A-H) or pretreated for 24 hrs with the methyltransferase inhibitors MTA and Adox (I-P). Cells were then treated with etoposide at 20 μM (E-H and M-P) for 1 hr, allowed to recover for 2 hr, fixed and labeled for immunofluorescence with anti-53BP1 (Novus Biologicals) and anti-γ-H2AX (Upstate) antibodies and the nuclei stained with DAPI.

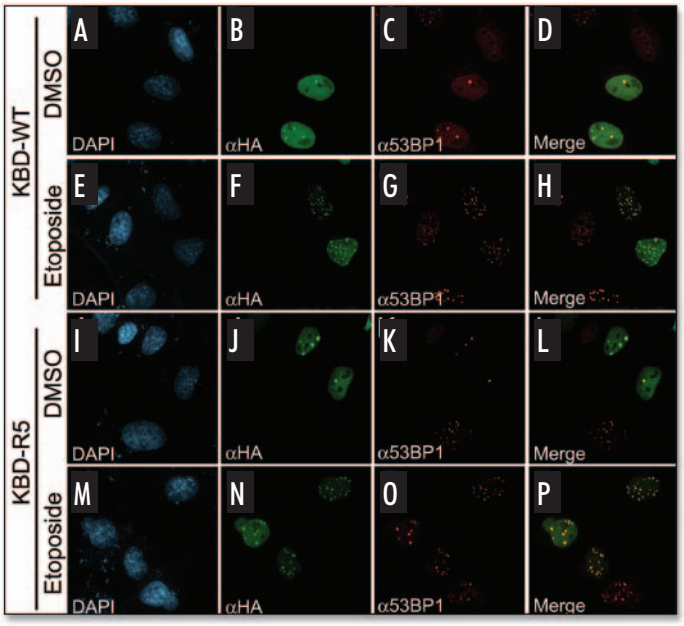

Figure 6. 53BP1 GAR motif is dispensable for recruitment of 53BP1 to sites of DNA damage. SK-N-SH cells were transfected with either HA-tagged wild-type 53BP1 KBD domain (KBD-WT, A-H) or with a mutant where the five arginines within the GAR motif were substituted to alanines (KBD-M5, I–P). Twenty-four hours following transfection, cells were either mock-treated (DMSO, A–D and I–L) or treated with etoposide at 20 μM (E–H and M–P) for 1 hr, allowed to recover for 2 hr, fixed and labeled for immunofluorescence with anti-53BP1 (Novus Biologicals) and anti-HA (12CA5, ATCC) antibodies and the nuclei stained with DAPI.

53BP1 to sites of DNA damage.[38] However, methylation of lysine 79 was also not increased in response to DNA damage, and it was suggested that changes in higher-order chromatin structure could allow exposure of a new binding site for 53BP1. So far, methylation of both arginines and lysines does not represent a DNA-damage-induced signal, but rather appears to be a necessary modification that must be present for an appropriate cellular response to DNA damage.

Most GAR motifs are found in nuclear proteins involved in interactions with RNA, such as hnRNPs, Sam68, fibrillarin and nucleolin. However, arginine methylation is not restricted to RNA binding protein as it has also been identified in myelin basic protein, fibroblast growth factor-2, histones and some viral proteins. At present, there is no clear understanding of the exact role that arginine methylation may play in nucleic acid–protein interactions. Valentini et al as well as Raman et al reported that methylation does not specifically affect RNA binding.[53,54] On the other hand, Rajpurohit et al. reported that binding of recombinant hnRNP A1 protein to single-stranded nucleic acid is slightly reduced following arginine methylation.[55] The GAR motifs by themselves do not appear to mediate nonspecific binding to nucleic acids and in some cases may mediate sequence-specific RNA binding as demonstrated for nucleolin, hnRNP A1, hnRNP U and ICP27.[56-59] 53BP1 has been shown to bind to both ssDNA and dsDNA.[33] The region of binding has been mapped to the amino acids 1319-1480, containing a GAR motif but no other conventional DNA binding motifs.[33] Our results demonstrate that the arginines within 53BP1 GAR motif are required for both ssDNA and dsDNA binding and that arginine methylation may regulate this activity.

Arginine methylation of Sm proteins by PRMT5 has been shown to enhance its interaction with SMN[60,61] promoting the assembly of the Sm proteins into spliceosomal UsnRNPs prior to nuclear import.[62] The regulation of the interaction between SMN and dimethylarginine-modified proteins was shown to be mediated by the tudor domain of SMN.[40,65] 53BP1, and its yeast homologue Crb2, both contain a tandem repeat of tudor domains that can bind to methylated lysine 79 of histone H3[38] and to methylated lysine 20 of histone H4, respectively.[39] The tudor domains are located less than 100 residues away from the GAR motif on 53BP1. The proximity of these conserved domains evokes the possibility that there is an intra-molecular interaction between the tudors and the methylated residues of the GAR motif. To test for such an interaction, we immobilized histidine tagged KBD-C (tandem tudor repeat) on a Biacore nickel affinity chip and subsequently, passed over either unmodified or asymmetrically methylated GAR peptides. We detected only a modest interaction (low millimolar range) between the tudor domains and the unmodified GAR peptide (data not shown), in line with a previous report.[37] This weak interaction was not enhanced by arginine methylation of the arginines within the GAR motif. Inhibition of methylation using broad methyltransferase inhibitors prevented 53BP1 from relocalizing to sites of DNA damage, perhaps by blocking, in part, methylation of histone H3 on lysine 79. Although our results demonstrate that mutations of the arginines required for DNA binding within 53BP1 did not prevent the relocalization of 53BP1 to sites of DNA damage (Fig. 6), the tandem-repeat of Tudor domains (KBC-C) were not sufficient for foci formation (Fig. 7). Instead, both the GAR DNA binding domain (KBD-M) and the methyl-binding Tudor domains together form the minimal foci-forming region of 53BP1, suggesting that indeed histone recognition and DNA binding through 53BP1 methylated arginines are coupled

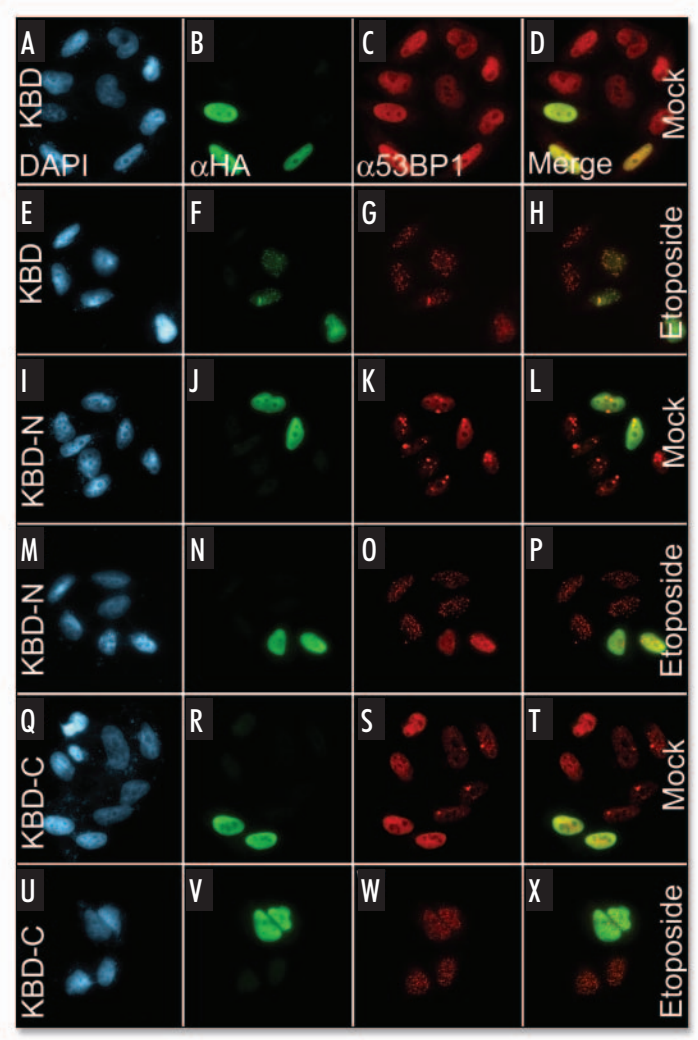

Figure 7. GAR and Tudor domains of 53BP1 are both required for recruitment of 53BP1 to sites of DNA damage. SK-N-SH cells were transfected with either HA-tagged 53BP1 KBD domain (KBD, A-H) or regions of the KBD domain containing either the GAR motif (KBD-N, I-P) or the Tudor domains (KBD-C, Q-X). 24 hr following transfection, cells were either mock-treated (DMSO, A-D, I-L and Q-T) or treated with etoposide at 20μM (E-H, M-P and U-X) for 1 hr, allowed to recover for 2 hr, fixed and labeled for immunofluorescence with anti-53BP1 (Novus Biologicals) and anti-HA (12CA5, ATCC) antibodies and the nuclei stained with DAPI.

to allow for stable foci formation following the initiation of the damage response in cells. Thus, methylation may play a role in regulating the 53BP1 tudor domains interaction with histones as well as regulating the interaction of the 53BP1 GAR motif with DNA.

In conclusion, the DNA damage response proteins MRE11 and 53BP1 are substrates of PRMT1 within their GAR motifs. The arginine methylation of each protein is required for their relocalization to DNA damage sites[49] and this study, suggesting that this post-translational modification is required for their mobility to DNA damage sites. In the case of MRE11, the GAR motif is required for an active nuclease[48] and in the case of 53BP1 the GAR motif regulates its ability to bind DNA. The challenge ahead will be to define the regulation of arginine methylation in the DNA damage response and to identify GAR motif interacting proteins that may regulate the accessibility of these proteins to sites of DNA damage.

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