

Research Paper

Methylation of MRE11 Regulates its Nuclear Compartmentalization

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ABSTRACT

The cellular response to DNA damage includes the orderly recruitment of many protein complexes to DNA lesions. The MRE11-RAD50-NBS1 (MRN) complex is well known to localize early to sites of DNA damage, but the post-translational modifications required to mobilize it to DNA damage sites are poorly understood. Recently, we have shown that MRE11 is arginine methylated in a C-terminal glycine-arginine rich (GAR) domain by protein arginine methyltransferase 1 (PRMT1). Arginine methylation is required for the exonuclease activity of MRE11 and the intra-S phase DNA damage response. Herein, we report that cells treated with methylase inhibitors failed to relocalize MRE11 from PML nuclear bodies to sites of DNA damage and formed few γ -H2AX foci. We also demonstrate that PRMT1 is a component of PML nuclear bodies where it colocalizes with MRE11. Using cellular fractionation, we demonstrate that methylated MRE11 is predominantly associated with nuclear structures and that MRE11 methylated arginines were required for this association. These results suggest that MRE11 methylation regulates its association with nuclear structures such as PML nuclear bodies and sites of DNA damage.

INTRODUCTION

DNA double-strand breaks (DSB) occur naturally when a replication fork collapses, or exogenously when cells are exposed to ionizing radiation or certain chemicals. The initial recruitment of proteins involved in DNA damage signalling and repair to sites of DNA double-strand breaks (DSB) represents the earliest response mechanism that is triggered by DNA damage.¹ The ensuing response to DNA damage includes the accumulation of signalling-repair complexes into nuclear foci in the vicinity of the DSBs.² Following the initial recognition of DNA lesions, histone H2AX becomes rapidly phosphorylated in the chromatin surrounding a DSB.³ Although H2AX deficiency is not essential for cellular survival, H2AX is required for the accumulation of numerous essential proteins into irradiation induced foci (IRIF).^{4,5} The MRE11/ RAD50/ NBS1 (MRN) complex is one of the early DNA repair complex that is recruited at DNA DSBs.^{6,7} Mutations in the *NBS1* and *MRE11* genes lead to genomic instability disorders: the Nijmegen breakage syndrome (NBS)^{8,9} and ataxia-telangiectasia (A-T)-like disease (A-TLD),¹⁰ respectively. MRE11 is a conserved protein with an N-terminal nuclease domain¹¹ as well as a C-terminal DNA binding region^{12,13} and a glycine-arginine rich (GAR) domain regulating both MRE11 exonuclease activity and its ability to signal DNA damage.¹⁴ An intact MRE11 C-terminus, deleted in A-TLD1/2 cells, is also required to assemble signalling proteins at sites of DNA damage.^{15,16} The MRN complex localizes in discrete nuclear foci at DNA DSBs and the MRN complex is involved in DNA repair, DNA damage and checkpoint signalling.¹⁷⁻²⁰

Protein arginine methylation is a post-translational modification that results in the mono- and dimethylation of the guanidino nitrogen atoms of arginine.²¹⁻²³ Arginines can be dimethylated either in a symmetrical or asymmetrical manner (sDMA, aDMA). In humans, protein arginine methyltransferases (PRMT) represent a family of eight known methyltransferases that utilize S-adenosyl methionine as a methyl donor and are divided into type I and type II enzymes. There are five known type I enzymes that catalyze the formation of aDMA including PRMT1,²⁴ PRMT2,²⁵ PRMT3,²⁶ PRMT4 (CARM1)²⁷ and PRMT6.²⁸ Type II enzymes catalyze the formation of sDMA and include PRMT5^{29,30} and PRMT7.^{31,32} Protein arginine methylation has been shown to regulate RNA metabolism, protein-protein interaction, protein localization and transcription.²¹⁻²³

We have previously demonstrated that MRE11 is arginine methylated by PRMT1, and that methylation regulates both MRE11 exonuclease activity and its ability to signal DNA damage.¹⁴ We now report that MRE11 interacts and colocalizes with PRMT1 in PML nuclear bodies. The sites of arginine methylation within MRE11 were replaced with alanines and it was observed that MRE11 R/A had an increased mobility by fluorescence recovery after photobleaching analysis and was loosely associated with nuclear structures unlike wild-type MRE11. Antibodies that recognize arginine methylated MRE11 demonstrated that methylated MRE11 was associated with nuclear structures. Cells treated with methylase inhibitors prevented the recruitment of MRE11 to sites of DNA damage and inhibited phosphorylation of histone H2AX in response to DNA damage. Our findings identify a new role for protein arginine methylation in recruitment of DNA repair proteins to sites of DNA damage.

MATERIALS AND METHODS

Antibodies. The sequence of the peptides used to raise antibodies against methylated MRE11 (MeMRE11) and non-methylated MRE11 (UnMRE11) was KGRGR GRGRR GGRGQ NSASR GGSQR GRA where all arginine residues are aDMA or arginines, respectively. The antibodies were affinity purified over the antigenic peptide coupled to Affigel beads (Bio-Rad). PRMT1, ASYM25 and SYM10 antibodies were described previously.³³ γ -H2AX was from Upstate Biotechnology, PML (PG-M3) and PRMT1 (N-19) were from Santa Cruz Biotechnology, MRE11 and NBS1 were from Novus Biologicals (Littleton, CO). ELISAs were performed as described previously.³³

Cell culture. The primary human foreskin fibroblasts CRL2097, HeLa and SK-N-SH cells are from the ATCC and were cultured in DMEM supplemented with 10% calf bovine serum, 2% L-glutamine, 1.2% sodium pyruvate and 1.2% Pen/Strep. DNA transfection and immunoprecipitations have been described elsewhere.³³

DNA constructs. SUMO1 was amplified by PCR from HeLa cells cDNA and cloned into the *EcoRI* site of pEGFP (Clontech). The full length MRE11 and MRE11 R/A were described previously¹⁴ and they were subcloned into the *Kpn I* site of pEYFP-C1 (Clontech).

Immunofluorescence, in situ fractionation and FRAP. To visualize MRE11, PRMT1 and sites of DNA damage, cells were subjected to an extraction protocol prior to fixation, according to a previously reported procedure.^{34,35} Briefly, cells were washed once with PBS, incubated for 5 min on ice in cytoskeleton buffer (10 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES) pH 6.8, 100 mM NaCl, 300 mM Sucrose, 3 mM MgCl₂, 1 mM EGTA and 0.5% Triton X-100) followed by a 5 min incubation on ice in cytoskeleton stripping buffer (10 mM Tris-HCL pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 1% Tween 40 and 0.5% sodium deoxycholate). Cells were then washed three times with PBS, fix in 1% paraformaldehyde for 10 min, permeabilized in 0.5% Triton X-100 for 10 min, 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 μ g/ml. Images were collected with an Olympus immunofluorescence microscope. For FRAP analysis, YFP-MRE11 or R/A transfected cells were maintained at 37°C and analyzed for FRAP as described.³⁶ The average recovery values for 10 cells were plotted.

Cellular fractionation and nuclear structures isolation. Nuclei were prepared essentially as described by.³⁷ In brief, cells were washed in phosphate-buffered saline and subjected to hypotonic lysis in RSB buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 0.5 mM phenylmethylsulfonyl fluoride). Cells were incubated on ice for 10 min, homogenized with a Dounce homogenizer, and centrifuged at 750 x g for 10 min. The supernatant

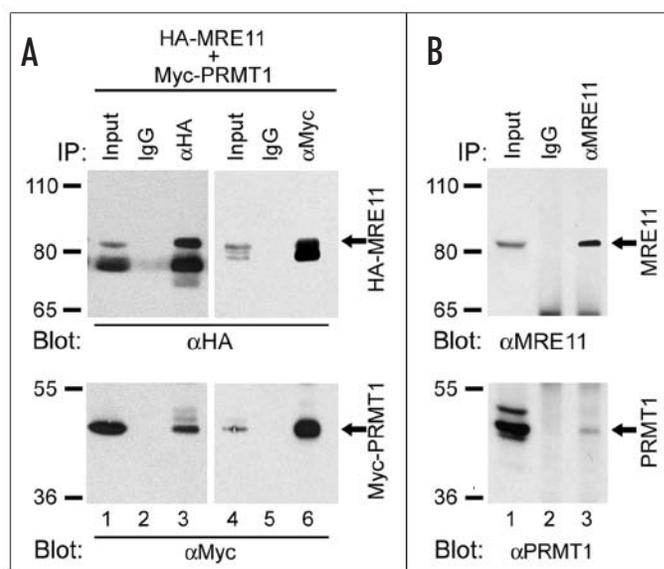


Figure 1. In vivo interaction between MRE11 and PRMT1. (A) Coimmunoprecipitations assays in HeLa cells with HA-MRE11 and Myc-PRMT1 were performed. The immunoprecipitated proteins were analyzed by SDS-PAGE and immunoblotted with anti-HA and anti-Myc antibodies as indicated (lane 1–6). (B) HeLa cell extracts were immunoprecipitated with anti-MRE11 or normal rabbit serum (NRS) antibodies (lane 1–3). The immunoprecipitated proteins were analyzed by SDS-PAGE and Western blotting with anti-MRE11 and PRMT1 antibodies (lane 1–3).

corresponding to the cytoplasm-containing fraction was designated fraction number 1. The nuclei recovered in the pellet were washed twice in RSB buffer. The subsequent steps allowing subnuclear fractionation and nuclear structures isolation were performed essentially as described by.³⁸ The washed nuclei were freed of the chromatin by digestion with 20 units of RNase-free DNase-1 (Promega, Madison, WI) per 1×10^6 cells at 30°C for 50 min in digestion buffer (10 mM Pipes, pH 6.8, 50 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 1 mM EGTA, 0.5% (v/v) Triton X-100, 1.2 mM phenylmethylsulfonyl fluoride, 2 μ g/ml aprotinin, 2 μ g/ml leupeptin, and 1 μ g/ml pepstatin A). The digested nuclei were then extracted by the addition of ammonium sulfate from a 1 M stock to a final concentration of 0.25 M. The 750 x g supernatant containing the digested chromatin was designated fraction number 2. The pellet corresponds to nuclear structures containing ribonucleoprotein complexes.³⁹ This nuclear fraction was further fractionated after resuspension of the pellet in digestion buffer and extraction by the addition of NaCl to a final concentration of 2 M from a 5M stock in digestion buffer. The supernatant obtained after a 750 x g centrifugation was designated fraction number 3. The pellet was resuspended in digestion buffer and incubated for 1 h at room temperature with or without RNase A (Sigma-Aldrich) at 100 μ g/ml and RNase T1 (MBI Fermentas, Canada) at 40 units/ml. The fractions were then centrifuged at 750 x g. The supernatant and pellet treated with the RNases were designated fraction numbers 4 and 5, respectively. All steps used for cell fractionation and nuclear structures isolation were performed at 4°C unless otherwise specified.

RESULTS

In vivo interaction between MRE11 and PRMT1. The PRMT1 arginine methyltransferase often associates with its substrates and to determine whether MRE11 and PRMT1 interact, HeLa cells were cotransfected with expression vectors encoding hemagglutinin (HA)-tagged MRE11 and myc-tagged PRMT1. Myc-PRMT1 was readily observed in anti-HA immunoprecipitates, demonstrating coimmunoprecipitation (Fig. 1A, lane 3 bottom panel). The top part of the same gel was immunoblotted with HA

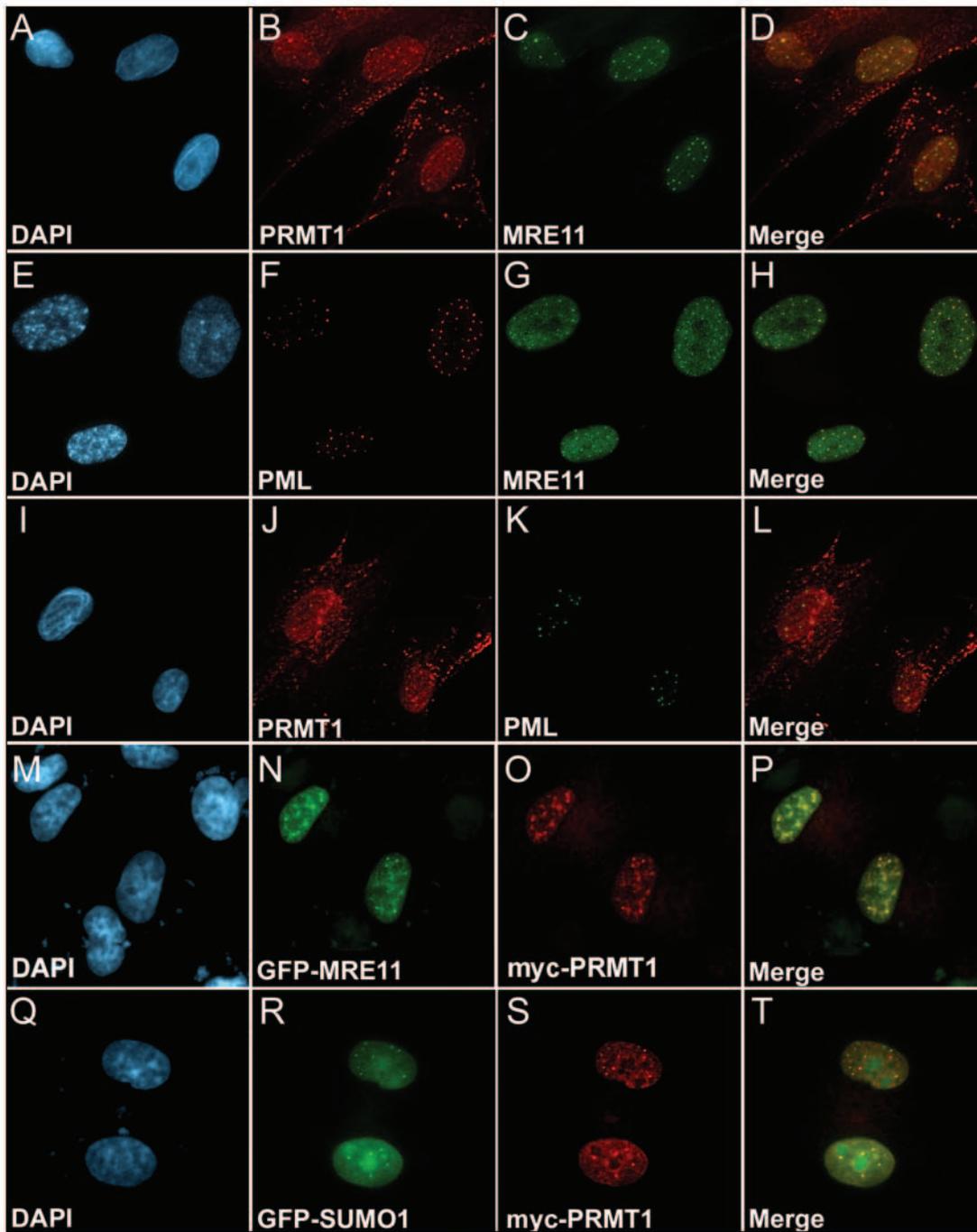


Figure 2. PRMT1 colocalizes with MRE11 in PML nuclear bodies. (A–H) Human normal fibroblasts CRL2097 were labeled for immunofluorescence using anti-MRE11 (C and G), anti-PRMT1 (B and J) and anti-PML (F) antibodies. Nuclei were stained with DAPI (A, E and I) and the merges are shown in panels (D, H and L). (M–P) SK-N-SH neuroblastoma cells transfected with GFP-MRE11 and Myc-PRMT1 or GFP-SUMO1 and Myc-PRMT1 (Q–T) were labeled for immunofluorescence using a Myc antibody (9E10). Nuclei were stained with DAPI (M and Q) and the merges are shown in panels (P and T).

antibodies to visualize the immunoprecipitated HA-MRE11 (Fig. 1A, lane 3 top panel). The converse was also observed as HA-MRE11 was detected in Myc immunoprecipitates (Fig. 1A, lanes 4–6, top panel). The lower part of the gel was immunoblotted with Myc antibodies to visualize Myc-PRMT1 (Fig. 1A, lane 4–6 bottom panel). These data show that overexpressed PRMT1 and MRE11 associate in transfected cells. We next examined whether or not endogenous PRMT1 and MRE11 interact. Untransfected HeLa cell extracts were immunoprecipitated with either control immunoglobulin G (IgG) or anti-MRE11 antibodies. Anti-MRE11

immunoprecipitates, but not control immunoprecipitates contained PRMT1, as detected by immunoblotting with anti-PRMT1 antibodies (Fig. 1B, lanes 1–3, lower panel). The upper part of the same gel was immunoblotted with MRE11 antibodies, confirming the presence of MRE11 (Fig. 1B). These data demonstrate that PRMT1 and MRE11 interact *in vivo* in human cells.

PRMT1 localizes within PML nuclear bodies with MRE11. PRMT1 is a ubiquitously expressed and localized within the cytoplasm and the nucleus.²⁶ MRE11 resides within the nucleus and a fraction of MRE11 is associated with the nuclear structure within PML nuclear bodies.^{35,40} This PML nuclear body localization of MRE11 is visualized by using a detergent extraction step before the immunostaining which reduces the signal contributed by diffuse nucleoplasmic MRE11 and reveals the PML nuclear bodies.^{35,40} To examine whether endogenous PRMT1 was also present in PML nuclear bodies, CRL2097 human normal diploid fibroblasts were detergent extracted and the localization of endogenous PRMT1 was visualized by indirect immunofluorescence. Indeed PRMT1 localized within discrete nuclear foci (Fig. 2B) that colocalized with MRE11 (Fig. 2C and D). In addition, PRMT1 also stained in cytoplasmic and peripheral foci that may represent focal adhesion contacts (Fig. 2B). To confirm the localization of MRE11 in PML nuclear bodies, we performed coimmunostaining using anti-PML (Fig. 2F) and anti-MRE11 (Fig. 2G) antibodies. Indeed, MRE11 was found in PML nuclear bodies in

CRL2097 human primary fibroblasts (Fig. 2H). To confirm that PRMT1 was also present in PML nuclear bodies, coimmunostaining was performed with anti-PRMT1 (Fig. 2J) and anti-PML (Fig. 2K) antibodies. Endogenous PRMT1 colocalized with PML (Fig. 2L), confirming the presence of PRMT1 in PML nuclear bodies.

The localization of PRMT1 within PML nuclear bodies was also confirmed using transfected PRMT1. HeLa cells were cotransfected with GFP-MRE11 and myc-tagged PRMT1. The cells were labeled for immunofluorescence using an anti-myc antibody. GFP-MRE11 was visible in nuclear foci and

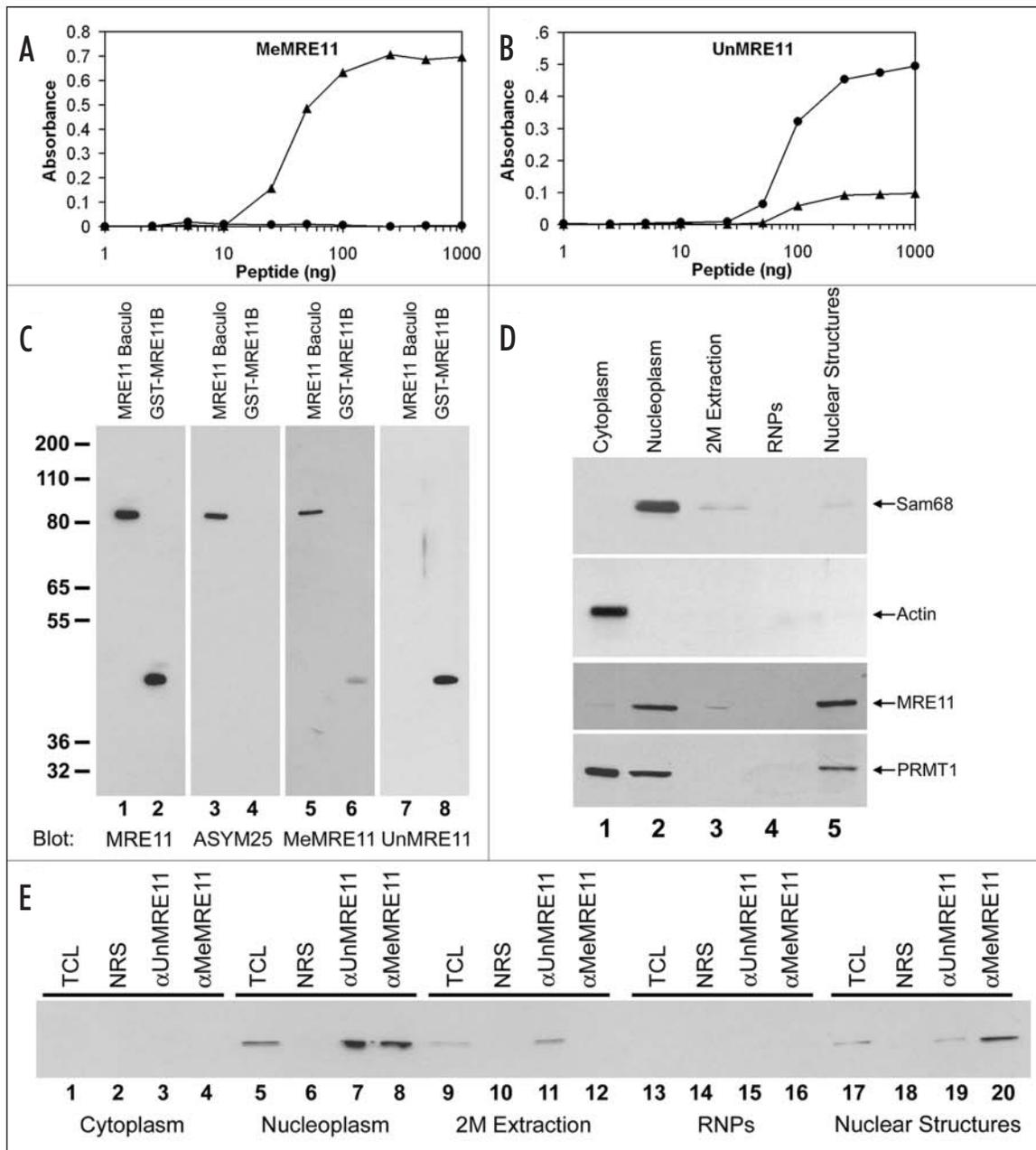


Figure 3. Arginine methylation of MRE11 influences its nuclear localization. (A and B) The ELISA demonstrates the specificity of the methyl-specific (MeMRE11) or the non-methyl-specific (UnMRE11) anti-MRE11 antibodies using a methylated MRE11 peptide (triangles) and an unmethylated peptide (circles). (C) Baculovirus human methylated MRE11 and unmethylated GST-MRE11 residues 554-680 were separated by SDS-PAGE and immunoblotted with the indicated antibodies. (D) Fractionated HeLa cells were resolved by SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-actin, -Sam68, -PRMT1 and -MRE11 antibodies. (E) Fractionated HeLa cell lysates were immunoprecipitated with NRS, UnMRE11 and MeMRE11 antibody. Immunoprecipitates were resolved by SDS-PAGE, transferred to nitrocellulose and immunoblotted with MRE11 antibodies.

indeed myc-PRMT1 colocalized with MRE11 within the nuclear foci that resembled PML nuclear bodies (Fig. 2M–P). To further confirm the authenticity of the PML nuclear bodies, we cotransfected HeLa cells with myc-PRMT1 and GFP-SUMO1 expression plasmids. GFP-SUMO-1, a PML nuclear body marker,⁴¹ colocalized with myc-PRMT1 within PML nuclear bodies (Fig. 2Q–T). However, PRMT1 did not relocalize from PML nuclear bodies to sites of DNA breaks with MRE11 following DNA damage (data not shown). Our results suggest that PML nuclear bodies may represent a nuclear site for the arginine methylation of MRE11 or its sequestration.

Arginine methylated MRE11 associates with nuclear structures. To determine whether arginine methylated MRE11 was present in PML nuclear bodies, we generated antibodies that recognize fully methylated MRE11

(MeMRE11) and unmethylated MRE11 (UnMRE11). The specificity of each antibody was confirmed by ELISA (Fig. 3A and B). The antibodies were examined for their ability to detect unmethylated bacterial produced GST-MRE11 amino acid 554 to 680 or methylated baculovirus produced hMRE11 by immunoblotting. GST-MRE11 was recognized by UnMRE11, and slightly recognized by MeMRE11 (Fig. 3C). Baculovirus hMRE11 was recognized by a general αDMA-specific antibody ASYM25 and by MeMRE11 (Fig. 3C), confirming their specificity. We next proceeded to cell fractionation followed by immunoprecipitation studies to identify the fractions that contain unmethylated and methylated MRE11.

The cytoplasm was separated from the nuclei of HeLa cells by hypotonic lysis. The washed nuclei were lysed and freed of the chromatin using Triton

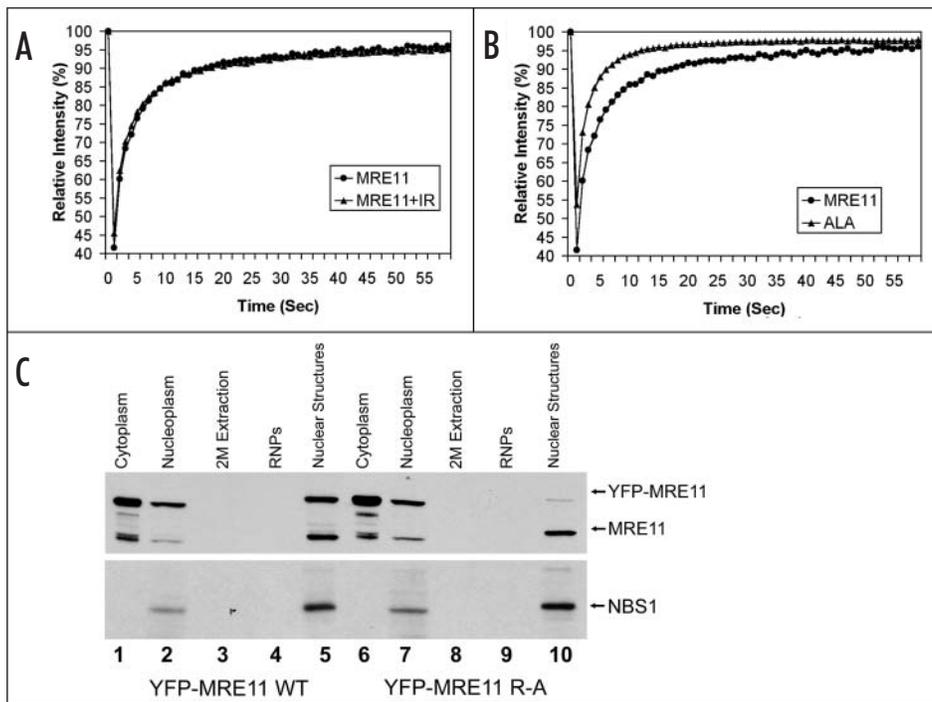


Figure 4. Arginines in the MRE11 C-terminus regulate nuclear structure association (A) FRAP analysis of YFP-MRE11 in HeLa cells in the absence or presence γ -irradiation. (B) FRAP analysis of YFP-MRE11 and -MRE11R/A. (C) Cellular fractionation of HeLa cells transfected with YFP-MRE11 (lane 1–5) or YFP-MRE11 R/A (lane 6–10). Proteins were visualized by immunoblotting with anti-MRE11 and anti-NBS1 antibodies.

X-100 and digestion with DNaseI. The digested nuclei were extracted by the addition of ammonium sulfate to a final concentration of 0.25 M. The supernatant containing the digested chromatin was designated the nucleoplasm. The proteins weakly associated with the insoluble nuclear fraction were washed with a 2 M NaCl solution (2 M extraction). The pellet was then incubated with RNase A and T1 to elute RNA bound proteins. The supernatant and pellet were designated ribonucleoproteins (RNPs) and nuclear structure, respectively. Aliquots of each fraction were immunoblotted with anti-Actin, anti-Sam68, anti-MRE11 and anti-PRMT1. Actin was detected only in the cytoplasmic fraction and the majority of the Sam68 RNA binding protein was observed in the nucleoplasm as expected (Fig. 3D). MRE11 was detected in both the nucleoplasm and the fraction that corresponds to nuclear structures (Fig. 3D). Consistent with the localization by immunofluorescence, PRMT1 was found in both the cytoplasm and the nucleoplasm, but a fraction was also associated with the nuclear structure (Fig. 3D). To identify where methylated and unmethylated MRE11 reside, each fraction was divided equally and immunoprecipitated with control, UnMRE11 and MeMRE11 antibodies. The bound proteins were separated by SDS-PAGE and immunoblotted with an MRE11 antibody that detects both forms. Our data revealed that the nucleoplasm contained equal amounts of methylated and unmethylated MRE11 (Fig. 3E, lanes 5–8). In addition, the unmethylated MRE11 was detected in the 2 M extraction fraction (lanes 9–12), whereas the methylated MRE11 was predominantly detected in the nuclear structure fraction (Fig. 3E, lanes 17–20). These data suggest that arginine methylation of MRE11 regulates the association with nuclear structures.

The MRE11 GAR domain is required for association with nuclear structures. MRE11 is arginine methylated within its glycine arginine rich (GAR) domain.¹⁴ To investigate whether the arginines within the GAR domain affected MRE11 cellular localization, wild-type MRE11 and a mutant that contains the arginines within the GAR domain substituted for alanines (MRE11 R/A) were expressed in HeLa cells as YFP-fusion proteins. We first examined the ability of the YFP-MRE11 fusion protein to move in

the presence or absence of DNA damage (10 Gy) by fluorescence recovery after photo-bleaching (FRAP) analysis. The recovery of the fluorescence in a photo-bleached area was determined and the normalized intensity of recovery of ten different cells was plotted against time. The recovery of YFP-MRE11 occurred in seconds (Fig. 4A) and as previously observed with another MRN protein, NBS1,⁴² and was independent of γ -irradiation (Fig. 4A). To address whether the arginines or methylarginines within the MRE11 GAR domain were involved in MRE11 mobility, FRAP analysis was performed with the fusion protein. Indeed, the kinetics of recovery of the YFP-MRE11 R/A was rapid and appeared to consist of a single kinetic population (Fig. 4B) in which all the fluorescence is rapidly redistributed, with little slow recovery phase afterwards. In contrast, the wild-type YFP-MRE11 after photo-bleaching had a significantly slower recovery and was found in both fast and slow recovering populations (Fig. 4B). These findings are indicative of an association of wild-type MRE11 with a nuclear structure and this association is absent or reduced with MRE11 R/A. These data suggest that the MRE11 GAR domain is required for association with nuclear structures consistent with our fractionation studies of Figure 3.

The cellular localization of the YFP-MRE11 fusion protein was examined by immunoblotting after fractionation. Both YFP-MRE11 fusion proteins and endogenous MRE11 were observed in both the nucleus and the cytoplasm (Fig. 4C, YFP-MRE11 and MRE11). Transfected MRE11 is known to localize in the cytoplasm as well as the nucleus due to limiting NBS1.⁸ However, as expected endogenous MRE11 and YFP-MRE11 were strongly associated with nuclear structures (Fig. 4C). In contrast, YFP-MRE11 R/A behaved differently, as it was weakly associated with nuclear structures (Fig. 4C, compare lanes 5 and 10). Endogenous NBS1 was only present in nucleoplasm and within nuclear structures (Fig. 4C), as expected for this protein.⁴² Collectively, these data identify a role for the MRE11 GAR domain in protein localization and mobility.

Methylase inhibitors prevent the recruitment of MRE11 to sites of DNA damage. To address whether arginine methylation affected the relocalization of MRE11 following DNA damage, CRL2097 human primary fibroblasts were mock treated (Fig. 5A) or treated with a moderate (Fig. 5B) or high (Fig. 5C) dose of methylase inhibitors MTA (5-deoxy-5-methylthioadenosine) and Adox (adenosine-2',3'-dialdehyde) for 24 h, followed by DNA damage introduced by 1 h treatment of 50 μ M of the topoisomerase II inhibitor, etoposide. MRE11 methylation, as visualized using an antibody recognizing methylated arginine 587,¹⁴ was greatly reduced using the methylase inhibitors (data not shown). Moreover, MRE11 methylated peptides as analyzed by mass spectrometry were not detected anymore following treatment of cells with the methylase inhibitors, confirming the hypomethylated state of MRE11 (data not shown). The phosphorylation of serine 139 of the histone variant H2AX (γ -H2AX) is induced rapidly following DNA damage and antibodies against γ -H2AX serve as a marker of DNA damage foci.³ The phosphorylation of H2AX is not required for the initial recruitment of DNA damage complexes, but rather is necessary for association with chromatin regions distal to the break and the initiation of the DNA damage response.⁵ Mock-treated cells contained MRE11 in PML nuclear bodies with few γ -H2AX foci, as visualized by indirect immunofluorescence with anti-MRE11 and anti- γ -H2AX antibodies (Fig. 5A, top row). Etoposide treatment resulted in the formation of DNA damage induced foci that contained both MRE11 and γ -H2AX, (Fig. 5A, bottom row) but not

PRMT1 (data not shown). Cells pretreated with a moderate dose of methylase inhibitors also contained MRE11 within PML nuclear bodies and few γ -H2AX foci (Fig. 5B, top row), suggesting that methylase inhibitors do not disrupt the integrity of PML nuclear bodies. Etoposide treatment stimulated the appearance of γ -H2AX foci, but interestingly the recruitment of MRE11 to the sites of DNA damage was severely inhibited (Fig. 5, compare bottom rows of Figure 5A and B). The MRE11 nuclear foci that did not colocalize with γ -H2AX foci appeared to be PML nuclear bodies, suggesting that the relocalization of MRE11 to sites of DNA damage is partially impaired at this methylase inhibitor concentration. The number of cells with DNA damage foci was quantified following treatment with MTA alone at 750 μ M, or following treatment with MTA at 750 μ M and Adox at 250 μ M (Fig. 6). Cells treated with the higher concentration of methylase inhibitors contained only weakly visible γ -H2AX foci and MRE11 remained localized in PML nuclear bodies (Fig. 5C). At this concentration, DNA damage using etoposide was still induced by looking at DNA fragmentation by pulse-field electrophoresis (data not shown). Visualization of a larger field confirmed that γ -H2AX foci were greatly diminished in cells treated with methylase inhibitors (Fig. 7H) compared to mock-treated cells (Fig. 7D). These findings demonstrate that methylation is required to localize MRE11 to sites of DNA damage and for the appearance of γ -H2AX foci.

DISCUSSION

In the present study, we establish a new role for protein methylation in the DNA damage response. Inhibition of methylation prevented the recruitment of the double-strand repair protein MRE11 to sites of DNA damage and prevented the formation of γ -H2AX foci. MRE11 associated *in vivo* with the arginine methyltransferase PRMT1 and the two proteins colocalized within PML nuclear bodies in normal diploid fibroblasts. Using our newly generated MRE11 methylarginine-specific antibodies, we show that arginine methylated MRE11 is preferentially associated with nuclear structures and that amino acid substitutions replacing the arginines within the MRE11 glycine arginine rich (GAR) domain prevented association with nuclear structures. These findings suggest that arginine methylation of the MRE11 GAR domain is required for its association with nuclear structures including sites of DNA damage.

We showed previously that MRE11 contains aDMA by using mass spectrometry and aDMA-specific antibodies. We also demonstrated that MRE11 is a substrate of PRMT1 *in vitro* and *in vivo*.¹⁴ Now we report that MRE11 and PRMT1 associate and colocalize in PML nuclear bodies. PRMT1 was shown previously to reside in

both the cytoplasm and the nucleus.²⁶ By reducing the nucleoplasmic signal using a detergent extraction step prior to immunostaining, we observed endogenous PRMT1 in cytoplasmic foci that may represent focal adhesion contacts and we identified PRMT1 in PML nuclear bodies. Biochemical experiments and *in situ* fractionation show that many DNA repair proteins are part of a dynamic nuclear structure complex that requires PML^{35,43} and dispersal of PML nuclear bodies following DNA damage may facilitate the enhanced release of DNA repair proteins in order to respond adequately to extensive DNA damage.⁴⁴ PML nuclear bodies are known to be

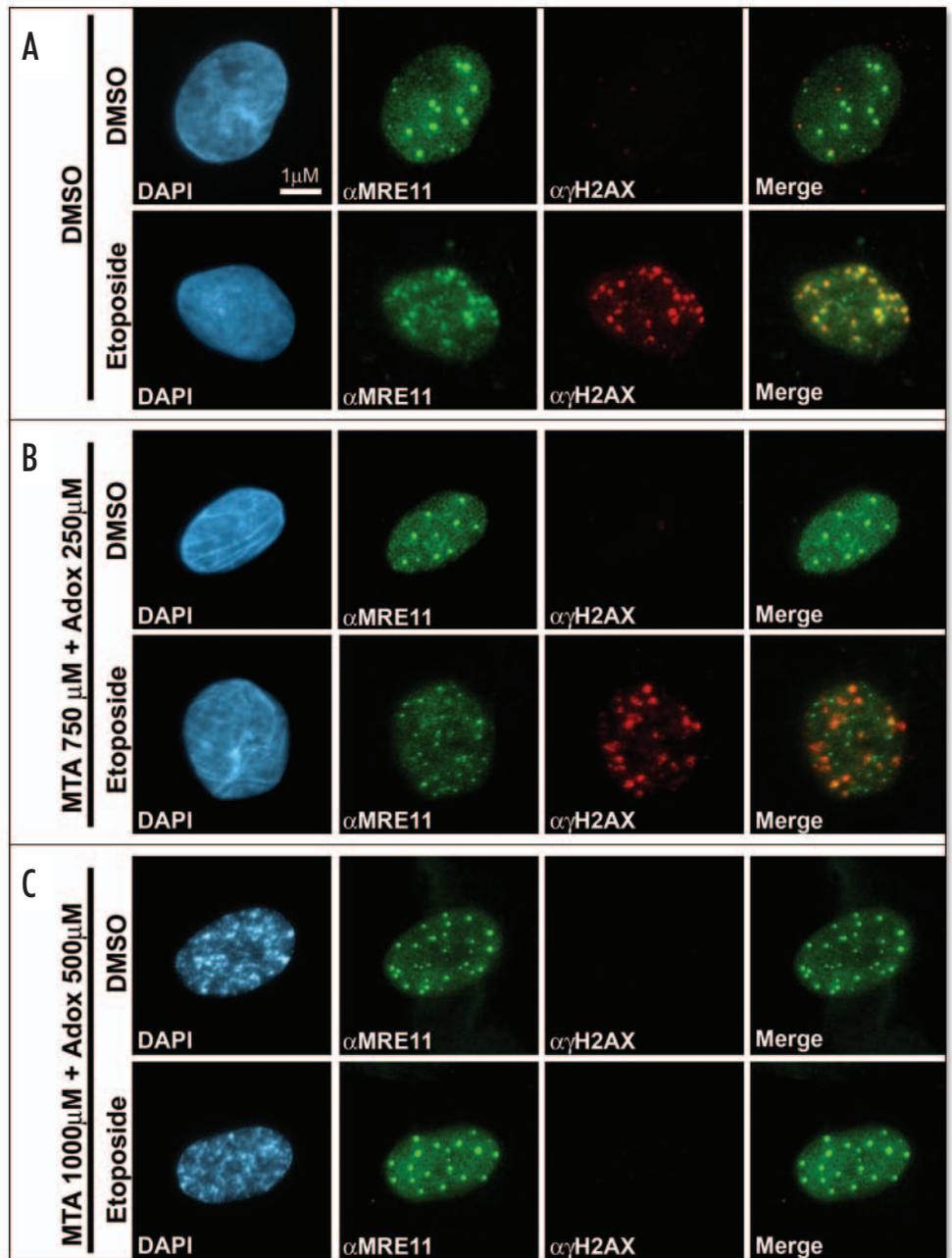


Figure 5. Methylase inhibitors prevent the localization of MRE11 at sites of DNA damage. The human primary fibroblasts CRL2097 were mock treated with DMSO (A), pretreated for 24 hr with the methylase inhibitors MTA at 750 μ M and Adox at 250 μ M (B) or pretreated for 24 hr with the methylase inhibitors MTA at 1000 μ M and Adox at 500 μ M (C) Cells were then treated with etoposide at 50 μ M (A, B and C, second row) for 1 hour, allowed to recover for another hour, fixed and labeled for immunofluorescence with anti-MRE11 and anti- γ -H2AX antibodies and the nuclei stained with DAPI.

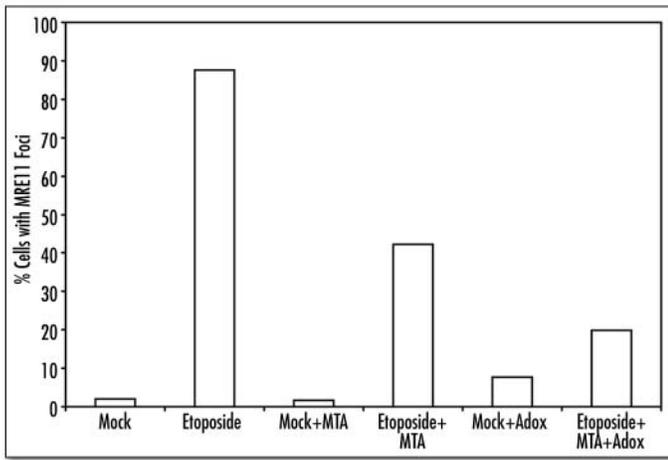
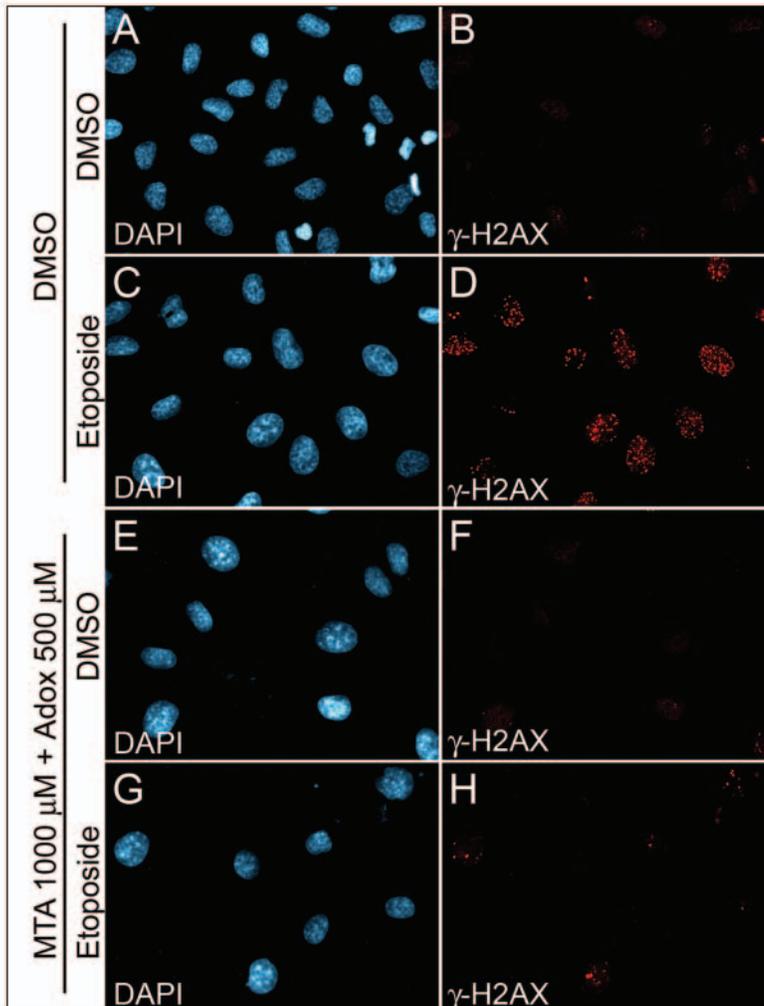


Figure 6. Methylase inhibitors prevent the localization of MRE11 at sites of DNA damage. The human primary fibroblasts CRL2097 were mock treated with DMSO or pretreated for 24 hr with the methylase inhibitors MTA at 750 μ M or pretreated for 24 hr with the methylase inhibitors MTA at 750 μ M and Adox at 250 μ M. Cells were then treated with etoposide at 50 μ M for 1 hour, allowed to recover for another hour, fixed and labeled for immunofluorescence with anti-MRE11 and anti- γ -H2AX antibodies. The number of cells with DNA damage foci was quantified and expressed as a percentage of cells with MRE11 colocalizing with γ -H2AX foci. 500 cells for each condition were counted.



modified following DNA damage and are recruited to sites of DNA breaks, along with the MRN complex, p53 and other proteins involved in DNA repair.⁴⁵ The function of PML nuclear bodies remains undefined, but the presence of a number of protein modification activities including acetylation,⁴⁶ phosphorylation,⁴⁷ SUMOylation⁴¹ and now arginine methylation, suggests that many proteins localize to PML nuclear bodies to acquire nuclear post-translational modifications. The fact that the majority of the MRN complex was present in the nucleoplasm and only a fraction was localized to PML nuclear bodies, suggests that MRE11 may transit through the PML nuclear bodies to receive methyl groups. This model would suggest that PML nuclear bodies represent a nuclear structure where the maturation of MRE11 takes place. Thus, arginine methylation of MRE11 within PML nuclear bodies could possibly prepare the MRN complex for further activation through phosphorylation and recruitment to sites of DNA damage.

The cellular response to DNA damage includes the initial recognition and recruitment of several protein complexes in an orderly fashion.¹ The localization of the MRN complex to sites of DNA damage is very rapid⁴² and is required for activation of the ensuing intra-S checkpoint.¹⁸ We noticed that replacing the arginines with alanines within the MRE11 GAR domain accelerated the movement of MRE11. Since these amino acid substitutions do not affect the MRN complex this suggests that the GAR domain is required to 'anchor' MRE11 to nuclear structures. One of the roles of MRN complex at sites of DNA damage is to activate the ATM kinase.^{17,18,20} ATM phosphorylates known effectors including H2AX, p53, Chk2 and other substrates and this initiates the DNA damage response.^{48,49} The absence of MRE11 at sites of DNA damage coincides with few γ -H2AX foci with methylase inhibitor treatment is consistent with the requirement for the MRN complex at sites of DNA damage prior to the phosphorylation of H2AX.

We have shown previously that the exonuclease activity of MRE11 is abrogated without arginine methylation. And that cells display intra-S phase defects with methylase inhibitors or in cells treated with PRMT1 siRNA.¹⁴ Our observation that arginine methylation may impair the ability of MRE11 to localize to DNA damage sites suggests that the intra-S phase defects in the absence of methylation may be due to both the abnormal exonuclease activity and the inability of the MRE11 to localize to DNA damaged sites. Since we showed that intra-S phase defects occurred in PRMT1 siRNA treated cells, this confirms the role of arginine methylation within this process. Recently, the methylation of lysine 79 was shown to be required for recruitment of 53BP1 to sites of DNA damage.⁵⁰ Thus, it demonstrates that methylation is an important modification for the recruitment of several DNA repair complexes to sites of DNA damage.

Until recently, methylation of arginines and lysines was presented as an irreversible modification.^{51,52} However,

Figure 7. Inhibition of methylation inhibits H2AX phosphorylation following DNA damage. The human primary fibroblasts CRL2097 were mock treated with DMSO (A–D) or pretreated for 24 hrs with the methyltransferase inhibitors MTA at 1000 μ M and Adox at 500 μ M (E–H). Cells were then treated with etoposide at 50 μ M (C, D, G and H) for 1 hr, allowed to recover for another hour, fixed and labeled for immunofluorescence with the anti- γ -H2AX (Upstate) antibody and the nuclei stained with DAPI.

recent evidences identifying enzymes capable of removing methyl groups on those residues suggested that methylation of lysine and arginine might be a more dynamic process than first anticipated.⁵³⁻⁵⁵ This raises the possibility that the methylation of MRE11 might not be a constitutive and irreversible modification, but that its arginine methylation might be a reversible, regulated process that can switch the active state of the protein to an inactive state. Thus, the role of arginine methylation in DNA repair is the dynamic regulation of both the enzymatic activity of MRE11 as well as its ability to signal DNA damage.

In conclusion, we provide evidence that PRMT1 is localized within PML nuclear bodies and that methylation may regulate the intranuclear trafficking of proteins. More specifically, we show that arginine methylation is required for the mobility of MRE11 and its association with sites of DNA damage. These data suggest that protein methylation regulates protein localization and recruitment during the DNA damage response.

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