Identification of Domains of Ataxia-telangiectasia Mutated Required for Nuclear Localization and Chromatin Association

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Ataxia-telangiectasia mutated (ATM) is essential for rapid induction of cellular responses to DNA double strand breaks (DSBs). In this study, we mapped a nuclear localization signal (NLS), KKKKKKKKK, within the amino terminus of ATM and demonstrate its recognition by the conventional nuclear import receptor, the importin α1/β1 heterodimer. Although mutation of this NLS resulted in green fluorescent protein (GFP)-ATM(NLSm) localizing predominantly within the cytoplasm, small amounts of nuclear GFP-ATM(NLSm) were still sufficient to elicit a DNA damage response. Insertion of an heterologous nuclear export signal between GFP and ATM(NLSm) resulted in complete cytoplasmic localization of ATM, concomitantly reducing the level of substrate phosphorylation and increasing radiosensitivity, which indicates a functional requirement for ATM nuclear localization. Interestingly, the carboxyl-terminal half of ATM, containing the kinase domain, which localizes to the cytoplasm, could not autophosphorylate itself or phosphorylate substrates, nor could it correct radiosensitivity in response to DSBs even when targeted to the nucleus by insertion of an exogenous NLS, demonstrating that the ATM amino terminus is required for optimal ATM function. Moreover, we have shown that the recruitment/retention of ATM at DSBs requires its kinase activity because a kinase-dead mutant of GFP-ATM failed to form damage-induced foci. Using deletion mutation analysis we mapped a domain in ATM (amino acids 5–224) required for its association with chromatin, which may target ATM to sites of DNA damage. Combined, these data indicate that the amino terminus of ATM is crucial not only for nuclear localization but also for chromatin association, thereby facilitating the kinase activity of ATM in vivo.

ATM is homozygously mutated in the germ line of patients with the neurodegenerative and cancer predisposition syndrome, ataxia-telangiectasia (A-T). Cells derived from A-T patients are hypersensitive to agents that cause double strand breaks (DSBs) in DNA, such as ionizing radiation (IR), but retain normal resistance to UV irradiation and other damaging agents. The role of ATM in the DNA damage response is well documented (see Ref. 1). Loss of ATM function in human and mouse cells cause defects in molecular pathways that are normally activated after DNA DSBs. ATM is reported to be present as inactive dimers in human cells, and exposure to IR induces its autophosphorylation at serine 1981, dimer dissociation, and activation as a kinase (2). Following DNA damage, ATM accumulates at sites of DNA DSBs as marked by phosphorylation of H2A.X at serine 139. The subsequent signaling cascade that results from ATM activation transduces signals to downstream targets such as p53, MDM2, CHK1, CHK2, BRC1, and NBS1, which instigate cell cycle arrest and DNA repair.

ATM is reported to localize predominantly within the nucleus of most proliferating cells, with small amounts residing in the cytoplasm (3, 4), consistent with its role in the DNA DSB response pathway. However, ATM has been reported to localize mainly within the cytoplasm of mouse Purkinje cells, in cells of the human cerebellum, and in a subset of cells in the dorsal root ganglia of mouse (5, 6). Although it is not clear what function ATM performs within the cytoplasm, it has been demonstrated in mouse that ATM deficiency results in abnormalities of organelles. Many mutations identified to date involve truncation of ATM that results in deletion of the carboxy-terminal region containing the kinase domain, thereby eliminating its kinase activity.

The transport of large proteins such as ATM into the nucleus is a complex process that involves active transport from the cytoplasm to the nucleus in signal-dependent fashion through the action of nuclear localization sequences (NLSs), which are recognized by members of the cellular importin superfamily of transport proteins (7, 8). The best understood pathways involve those in which cargoes containing lysine-arginine-rich NLSs are recognized by either importin β1 or the importin α1/β1 heterodimer (7, 8). Subsequent to import into the nucleus through the nuclear envelope-localized nuclear pore complex structures mediated by the importins, release into the nucleoplasm is effected by binding of the monomeric guanine nucleotide-binding protein Ran in activated GTP-bound form to importin β (7, 8).

Sequences reminiscent of known NLSs have been identified within ATM. As a first step to identifying targeting signals within ATM that regulate its localization and activity, we tagged full-length and various subfragments of ATM with green fluorescent protein (GFP) and analyzed their subcellular localization and function. We characterized an importin α1/β1-recognized NLS in the amino terminus of ATM, mutation of
which in the context of full-length ATM results in predominantly cytoplasmic localization and inhibits interaction with importins. Importantly, we show that the amino-terminal region of ATM confers association with chromatin and is required for its efficient kinase activity in vivo; only the nuclear fraction of ATM is autophosphorylated in response to IR induced DNA damage. Overall, our results imply that the amino terminus of ATM is crucial for both nuclear localization and chromatin association, thereby facilitating the kinase activity of ATM in vivo.

EXPERIMENTAL PROCEDURES

Plasmid Construction—pEGFP-ATM (amino acids 5–1303), encoding the amino-terminal half of the ATM protein fused with GFP, was created by subcloning ATM-(5–1303) as a XhoI/KpnI fragment from pMAT1, which lacks the coding sequence for the first five amino acids (9), into pEGFP-C2 (Clontech). pEGFP-ATM-(1303–3056), encoding the carboxyl-terminal half of ATM fused with GFP, was created by subcloning ATM-(1303–3056) as a KpnI fragment from pMAT1 into pEGFP-C2. pSG5GFP-ATM, encoding full-length ATM fused with GFP, was created by subcloning ATM-(5–1303) as an NheI (end-filled) BamHI fragment from pMAT1 into pSG5 (Stratagene, with an expanded multiple cloning site). Deletion mutants pEGFP-ATM-(5–568), (677–1303), (5–224), and (227–568) were prepared by restriction digestion, end-filling with T4 DNA polymerase, and religation. The NLSm (R336K/R337A) and KD (D287A/N287SK) mutants of ATM were prepared by site-directed mutagenesis using the QuikChange kit (Stratagene) and were verified by DNA sequencing. The separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes and probed with monoclonal anti-FLAG antibody (Sigma, F3165) and were detected using chemiluminescence (Pierce). The separated proteins were visualized by autoradiography and quantified by densitometry.

ATM Is Distributed between the Nuclear Matrix and the Cytoplasm—For quantification of the relative amount of nuclear and cytoplasmic protein, samples were analyzed by Immunoblot assays. The separated proteins were transferred to nitrocellulose membranes and probed with monoclonal anti-FLAG antibody (Sigma, F3165), Alexa Fluor 546 or 488 (Molecular Probes, cat. no. A-11010/003/001). Immunoblot Assays—Cells were pelleted and then washed with PBS prior to being lysed directly in sample buffer (5% glycerol, 1.7% SDS, 100 mM dithiothreitol, 0.2% bromphenol blue, 60 mM Tris, pH 6.8) with sonication and boiling. Protein concentrations were determined by Bradford assay. Proteins were subjected to SDS-PAGE, 4% for full-length ATM or 8% for other proteins and some ATM deletion mutants. The separated proteins were transferred to polyvinylidene difluoride membranes by semi-dry transfer (Bio-Rad), and the proteins were detected with antibodies in Blotto and visualized by chemiluminescence (Pierce). All antibodies used were: anti-ATM (9), into pEGFP-C2 (Clontech). pEGFP

RESULTS

GFP-ATM Is Distributed between the Nucleus and Cytoplasm—To initiate our studies of ATM localization, we created a plasmid for expression of GFP-tagged ATM in mammalian
cells. Localization of the GFP-ATM fusion protein was determined by fluorescence microscopy after transient expression into various cell types including HEK293T, COS-7, and AT5BIVA (an ATM-deficient cell line) cells. The majority of GFP-ATM protein was localized within the nucleus, with variable levels of cytoplasmic localization being observed. A minority of cells displayed a predominantly cytoplasmic accumulation around the nuclear periphery (Fig. 1A). The distribution of GFP-ATM within cells was determined statistically over a larger sample, with at least 200 transfected cells, repeated over three independent experiments in three cell lines. In 60–70% of log phase cells GFP-ATM localized predominantly nuclear; in the remaining 5% of the cells GFP-ATM localized predominantly around the cytoplasmic periphery (Fig. 1B). A similar distribution of GFP-ATM was seen in all three cell lines. The observed nuclear/cytoplasmic ratio was independent of the amount of DNA transfected, indicating that protein expression levels did not impact the observed intracellular distribution of GFP-ATM (data not shown). In addition, GFP-ATM was excluded from the nucleoli.

Next we wished to determine whether GFP-ATM was able to form foci and phosphorylate substrate in response to DNA DSBs, as has been reported for the endogenous wild type protein. When A-T fibroblast cells (AT5BIVA) expressing GFP-ATM were exposed to IR (30 min after 4 Gy IR), GFP-ATM was seen to change localization from being evenly distributed throughout the nucleus to forming distinct foci that co-localized with phospho-H2A.X (S139) as a marker for sites of DNA DSBs (Fig. 1C). A small number of ATM foci were sometimes present in nuclei in the absence of DNA damage. Interestingly, GFP-ATM(KD) could not form foci following induction of DNA DSBs. As expected, GFP-ATM(KD) did not phosphorylate H2A.X (Ser-139). This experiment was repeated using FLAG-tagged ATM. Once again foci formation occurred only for wild type ATM and not for the KD mutant. These data indicate that ATM kinase activity is required for ATM foci formation.

**Identifying Nuclear Localization Signals within ATM**—To map functional NLSs within ATM, a series of deletion mutation constructs was prepared in the pEGFP-C2 vector (Clontech) (Fig. 2A). Expression of these GFP-tagged deletion mutants was confirmed by immunoblot analysis (Fig. 2B), with all proteins being of the predicted size. Ectopically expressed GFP-ATM-(5–1303) exhibited a subcellular distribution similar to that of the full-length protein, with mixed nuclear and cytoplasmic localization in the majority of cells (Fig. 2C). However, the carboxyl-terminal half of ATM-(1303–3056) was localized exclusively within the cytoplasm. This implied the existence of
one or more nuclear localization signals within the amino-terminal half of ATM. To narrow down the region containing the NLS, we performed deletion mutation analysis of the amino-terminal half of ATM. This led to the identification of two non-overlapping amino-terminal fragments, ATM-(5–224) and ATM-(227–568), which were localized predominantly within the nucleus (Fig. 2C).

Both these deletion mutants contained a short motif of basic amino acids reminiscent of the SV40 large T-antigen NLS. Deletion mutant ATM-(5–224) contained a cluster of three (+) charged basic residues (23RKK25), whereas ATM-(227–568) contained a cluster of four (+) charged basic residues (385KKK388). To determine whether these amino acid sequences form functional NLSs, site-directed mutagenesis of these motifs was performed, changing core lysine or arginine residues to alanine. Amino acids 23RKK25 were mutated to 23RAA25 in the construct GFP-ATM-(5–224), and amino acids 385KKK388 were mutated to 385KAAK388 in the construct GFP-ATM-(227–568). When expressed in cells the 23RKK25 mutant protein remained localized predominantly within the nucleus of transfected cells, indicating that 23RKK25 is not important for nuclear transport of the GFP-ATM-(5–224) construct (Fig. 2D). In contrast, the 385KAAK388 mutant localized predominantly within the cytoplasm, indicating that 385KKK388 is critical for nuclear import of the GFP-ATM-(227–568) construct. Subsequently, the 385KAAK388 mutation was introduced into the full-length ATM construct, GFP-ATM(NLSm), which also localized predominantly within the cytoplasm of transfected cells (Fig. 2D), indicating that this is the dominant NLS within full-length ATM. The distribution of GFP-ATM(NLSm) within cells was determined statistically over a larger sample, at least 200 transfected cells, repeated over three independent experiments in AT5BIVA cells (Fig. 2B). The results indicate that the mutation of this NLS sequence in ATM does not result in complete absence of ATM from the nucleus, as GFP-ATM(NLSm) was still faintly nuclear in about 18–25% of cells.

Nuclear Import of ATM Is Mediated by Importin α1 and Importin β1—To identify proteins involved in transporting ATM into the nucleus, we assayed for interaction between ATM and members of the importin family of proteins by co-immunoprecipitation with ATM. We were able to detect interaction between ATM and importin β1 in whole cell extracts prepared from an A-T lymphoblastoid cell line stably expressing ATM-(227–568), which were immunostained with anti-GFP polyclonal antibody (Molecular Probes). All deletion mutant proteins were expressed in yeast in fusion with the GAL4 binding domain. Yeast were co-transformed with plasmids expressing importins 1, 5, and 7, or their GAL4 activation domain. We were able to detect interaction of ATM-(227–568) with importins 1 and 5 (Fig. 3A). Attempts to detect importin α by this technique were inconclusive because of close migration of importin α (58 KDa) with immunoglobulin. As an alternative strategy we used a yeast two-hybrid assay to assess whether ATM interacts directly with importin α1, α5, or α7 and β1. The fragment of ATM that contains the NLS (ATM-(227–568)), was expressed in yeast in fusion with the GAL4 binding domain. Yeast were co-transformed with plasmids expressing importins in fusion with the GAL4 activation domain. We were able to show interaction of ATM-(227–568) with importins α1 and, to a lesser extent, α5 (Fig. 3B). To establish positive controls we assayed for interaction of p53 with the SV40 T-antigen (Clontech, yeast two-hybrid controls), which are known to interact very strongly, and of BRCA1 with importin α (11). The extent of interaction between ATM-(227–568) and importin α1 was comparable with that seen between BRCA1 and importin α1. The interaction with importin β1 was negligible compared with
survive DNA damage induced by IR at doses of 1, 2, or 4 Gy. The above mentioned mutants were assayed for their ability to transfected A-T fibroblast cells (AT5BIVA), which are ex-
insertion of an exogenous NLS), and GFP (1303–3056) (which is artificially targeted to the nucleus by
activation domain. One-way analysis of variance indicates that the means are significantly different; the activity of the positive controls and results would be expected, as GFP
rection of radiosensitivity at the higher dose of 4 Gy. These
GFP signal that allows these cells to survive. Cells expressing
Even this low level of GFP
GFP that localizes exclusively within the cytoplasm, had a substantially
importin only after DNA damage (13). We wished to determine
Amino-terminal Sequences of ATM Are Required for Survival of Complemented A-T Cells in Response to IR-induced DNA Damage—We chose to compare the protective function of GFP-ATM with that of GFP-ATM(NLSm) (which localizes pre-
dominantly with the cytoplasm), GFP-ATM-(1303–3056) (which localizes within the cytoplasm), GFP-NLS-ATM-(1303–3056) (which is artificially targeted to the nucleus by
insertion of an exogenous NLS), and GFP-ATM(KD) in stably transfected A-T fibroblast cells (AT5BIVA), which are ex-
quently sensitive to IR. Cells complemented with GFP-ATM or the above mentioned mutants were assayed for their ability to survive DNA damage induced by IR at doses of 1, 2, or 4 Gy. Cells expressing GFP-ATM demonstrated a similar, although slightly higher, level of survival compared with that of cells expressing GFP-ATM(NLSm) (Fig. 4A). An explanation for this observation may be that low, but sufficient, amounts of GFP-ATM(NLSm) are present within the nucleus of these cells. Even this low level of GFP-ATM(NLSm) may provide a damage signal that allows these cells to survive. Cells expressing GFP-ATM(KD) and GFP-ATM-(1303–3056) exhibited no cor-ection of radiosensitivity at the higher dose of 4 Gy. These results would be expected, as GFP-ATM(KD) cannot phosphorylate substrate, whereas GFP-ATM-(1303–3056) localizes exclusively within the cytoplasm and may therefore not be able to be activated or to phosphorylate substrate in response to a damage signal. Interestingly, cells expressing GFP-NLS-ATM-(1303–3056) were also impaired in their ability to correct radiosensitivity. This mutant of ATM contains a functional kinase domain and is artificially targeted to the nucleus, indicating that it is not sufficient to simply target the isolated kinase domain of ATM to the nucleus of cells to correct radiosensitivity. From these data we infer that amino-terminal sequences of ATM are required for its biological activity.
Amino-terminal Sequences of ATM Are Required for Substrate Phosphorylation in Vivo—To assess whether the differences in survival of complemented A-T fibroblast cells correlate with restoration of the kinase activity of ATM, we examined the ability of A-T lymphoblastoid cells stably expressing GFP-ATM, GFP-ATM(KD), GFP-ATM(NLSm), and GFP-NLS-ATM-(1303–3056) to phosphorylate Ser-15 of p53 after exposure to IR (30 min after 4 Gy). GFP-ATM and GFP-ATM(NLSm) were able to phosphorylate p53 (Fig. 4B), whereas GFP-ATM(KD) and GFP-NLS-ATM-(1303–3056) could not phosphorylate p53. The residual level of signal seen with these mutants was comparable with GFP-vector only-
expression cells, suggesting that the amino terminus of ATM is required for optimal phosphorylation of substrates in vivo. Furthermore, when the above mentioned constructs were tested for ATM activation using phospho-Ser-1981 antibody, the GFP-ATM(KD) and GFP-NLS-ATM-(1303–3056) mutants did not show any detectable autophosphorylation relative to GFP-ATM. The autophosphorylation of GFP-ATM(NLSm) was consider-
ably weaker than that seen with GFP-ATM. To find an explanation for our observation concerning the reduced auto-
phosphorylation of GFP-ATM(NLSm), we performed immuno-
fluorescence assays on A-T fibroblasts expressing these fusion proteins. We found that only the nuclear fraction of GFP-ATM or GFP-ATM(NLSm) was autophosphorylated (Fig. 4C). Con-
sidering the relatively small amount of GFP-ATM(NLSm) protein within the nucleus of these cells, this observation would explain why an apparent substantial reduction in the amount of autophosphorylated GFP-ATM(NLSm) mutant was seen by immuno-
blot analysis. To determine whether GFP-ATM(NLSm) is able to confer radioresistance and normal phosphorylation of p53, perhaps because of the small amount of ATM that is localized in the nucleus, we introduced the HIV-Rev nuclear export signal (NES) between GFP and ATM(NLSm), GFP-
NES-ATM(NLSm), in order to remove ATM from the nucleus. A-T lymphoblastoid cells stably expressing GFP-ATM (wild type), GFP-ATM(KD), GFP-ATM(NLSm), and GFP-NES-ATM-
(NLSm) were compared for their ability to correct radiosensi-
tivity and to phosphorylate p53 on Ser-15 after IR-induced DNA damage. We found that GFP-NES-ATM(NLSm), which localized exclusively within the cytoplasm, had a substantially reduced ability to phosphorylate p53 on Ser-15 (Fig. 4D) and increased radiosensitivity, compared with GFP-ATM(NLSm) (Fig. 4E), suggesting that nuclear localization is required for ATM activity. Taken together, these results suggest that the amino terminus of ATM is required for optimal ATM activation and the subsequent ATM activity.
ATM Associates with Chromatin before and after IR-induced DNA Damage through Its Amino-terminal region—Previously ATM has been reported either to associate with chromatin before and after DNA damage (12) or to associate with chromatin only after DNA damage (13). We wished to determine
the strength of this association and to map the domain of ATM that is required for association with chromatin. To access the strength of binding we employed an assay used to examine the interaction of ORC (origin recognition complex) and MCM (mini-chromosome maintenance) proteins with chromatin (10). Using this assay we found that endogenous ATM in HeLa cells and GFP

\text{ATM} \quad \text{expressed in AT5BIVA cells bound chromatin before and after IR-induced (30 min after 4 Gy) DNA damage with equal strength (Fig. 5A). When probed with antibody specific for ATM autophosphorylation (pS1981), only ATM protein from cells exposed to IR showed evidence of autophosphorylation of ATM (pS1981, 40 μg/lane) in AT1ABR cells (an A-T-lymphoblastoid cell line) stably expressing GFP

\text{ATM} \text{ and mutants. Whole cell extracts were prepared from cells 30 min after exposure to 4 Gy IR. C, fluorescent microscopy (×100, then digitally enlarged and cropped) for autophosphorylation of GFP

\text{ATM} \text{ or GFP

\text{ATM}(\text{NLSm}) on Ser-1981 with (+) or without (−) exposure to IR (30 min after 4 Gy). Cells were fixed with paraformaldehyde and then permeabilized with 0.2% Triton X-100 prior to staining. DNA was visualized by staining with DAPI. D, immunoblot showing in vivo phosphorylation of pS15 p53 and autophosphorylation of ATM (pS1981) in AT1ABR cells stably expressing GFP

\text{ATM} \text{ and mutants including GFP

\text{NES-ATM}(\text{NLSm}), to demonstrate that rescue of p53 phosphorylation by NLSm is dependent on small amount of ATM present in the nucleus. Whole cell extracts were prepared from cells 30 min after exposure to 4 Gy IR. E, clonogenic survival of A-T fibroblast cells (AT5BIVA) stably expressing GFP

\text{ATM}, GFP

\text{ATM}(\text{KD}), GFP

\text{ATM}(\text{NLSm}), and GFP

\text{NES-ATM}(\text{NLSm}), exposed to 1, 2, or 4 Gy of IR. Colonies of 50 or more cells were counted and plotted on a graph (mean ± S.E.).}

**DISCUSSION**

**GFP\text{ATM} Localization and Identification of an NLS—ATM** is most often described as a nuclear phosphoprotein and is believed to be involved in initiating signal transduction pathways in response to DNA DSBs. There is strong evidence that ATM is localized predominantly in the nucleus of proliferating cells (3, 4). Here we provide evidence for the first time that ATM gains nuclear entry through an NLS that resembles the monopartite SV40 NLS. AtM NLS was found to be necessary for efficient transport of the full-length protein into the nucleus, as mutation of this NLS leads to predominantly cytoplasmic localization of ATM in a majority of cells. Our data suggest that ATM is imported into the nucleus in a classical manner through the importin α/β heterodimer.

**Nuclear Localization Is Required for ATM Activity—Surprisingly we found that ATM(NLSm) could complement the radiosensitivity of A-T cells, albeit at a slightly reduced level compared with wild type ATM. Furthermore, it retained the
Intrinsic kinase activity of wild type ATM. In most cells (75–82%), ATM(NLSm) was localized predominantly within the cytoplasm. However, in about 18–25% of cells it did display some nuclear staining, suggesting that there may be an additional, although less efficient, NLS within the amino acid sequence of ATM. These data would suggest that even relatively low levels of nuclear ATM may be sufficient to elicit a response to DNA damage. By introducing the HIV-Rev NES between GFP and ATM(NLSm) to remove ATM from the nucleus, our data confirm that rescue of radiosensitivity and p53 phosphorylation by NLSm is dependent on a small amount of ATM present in the nucleus. Consistent with this finding, many A-T carriers (ATM heterozygotes) show normal phosphorlylation of p53 and CHK2 despite the reduced ATM expression (about 50% lower than normal) (14, 15). Furthermore, it has been demonstrated that ATM protein levels in a range of cell lines vary considerably and that the steady state levels of ATM assessed by chromatin association. The assay we employed in vivo associates with chromatin before and after induction of DNA damage. In contrast, the assay used by Andegeko et al. (13) reports that ATM associates with chromatin before exposure of cells to DNA-damaging agents. In a report by Andegeko et al. (13), a fraction of ATM was reported to become resistant to detergent extraction following induction of DNA damage with DSB-inducing agents. However, Gately et al. (12) also report that ATM associates with chromatin before and after induction of DNA damage. These reports and our own differ in the methods employed in assessing chromatin association. The assay we employed involves releasing nucleoplasmic proteins under hypotonic (20 mM NaCl) conditions in the presence of detergent (0.5% IGE-PAL). Proteins associated with chromatin are then released with increasing salt concentrations (0.1–0.4 mM NaCl) but in the absence of detergent. Considering that detergents disrupt hydrophobic interactions and salt disrupts hydrophilic interactions, our data would suggest that ATM associates with chromatin through hydrophilic interactions before and after DNA damage. In contrast, the assay used by Andegeko (13) maintains salt at a constant level (150 mM NaCl) while increasing detergent concentrations (0.2–0.4% Nonidet P-40, then boiled in SDS sample buffer). Under these conditions, ATM associated with chromatin more tightly following DNA damage, suggesting that ATM association with chromatin changes from being more to less hydrophobic following DNA damage. Combined, these data may suggest that ATM associates with chromatin through both hydrophobic and hydrophilic interactions and

![Image](https://example.com/image1.png)

**FIG. 5. ATM associates with chromatin through its amino-terminal region.** Lysates were prepared from cells for cytosolic and nucleosolic proteins, and proteins were eluted from IGEPAL-resistant residual nuclear structure (chromatin and nuclear matrix) by successive washing with 0.1, 0.2, and 0.4 M NaCl. C, cytosolic; N, nucleosolic. A, immunoblot of extracted (endogenous) proteins from HeLa cells was probed initially for phosphorylation on ATM Ser-1981 and then stripped and reprobed for ATM. Immunoblots were repeated and probed for BRCA1, MCM3, or α-tubulin to verify purity of fractions. The assay was repeated using AT5BIVA cells stably expressing GFP-ATM and probed with anti-GFP antibody. B, mapping of a domain within ATM involved in association with chromatin. Lysates were prepared as described for panel A from cells expressing GFP-ATM deletion mutants. Immunoblots were probed with anti-GFP antibody.

![Image](https://example.com/image2.png)

**FIG. 6. Proposed model for ATM function.** A schematic representation of ATM activation and its association with chromatin is shown.
that the nature of ATM association with chromatin changes following DNA damage. We found that the amino terminus of ATM associated with chromatin; this would free the carboxyl terminus, which contains the kinase domain, to interact with substrate and other proteins.

A Proposed Model for ATM Activity—Using our data and the data of others we propose a possible model for ATM activity (Fig. 6) in which ATM is associated with chromatin as an inactive dimer prior to its activation. Upon induction of DNA DSBs, torsional change in the DNA helix activates ATM with input from checkpoint proteins such as MRN complex, which is recruited to DSBs (2, 21–23). Activated ATM is then recruited along chromatin to sites of DNA damage where it is able to phosphorylate substrates also present at sites of DSBs; this explains the rapid and highly localized appearance of phosphorylated ATM substrates such as histone H2AX and MRN complex at discrete foci. Interestingly, the phosphorylated MRN complex in turn acts as a scaffold to bring other substrates in to the vicinity of ATM kinase domain. Our data using KD ATM indicate that the kinase activity is indispensable for ATM foci formation after DNA damage. Kinase activity may be required to release ATM from sites on chromatin so that it can migrate to sites of damage.

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