Mechanisms of ATM Activation

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Abstract

The ataxia-telangiectasia mutated (ATM) protein kinase is a master regulator of the DNA damage response, and it coordinates checkpoint activation, DNA repair, and metabolic changes in eukaryotic cells in response to DNA double-strand breaks and oxidative stress. Loss of ATM activity in humans results in the pleiotropic neurodegeneration disorder ataxia-telangiectasia. ATM exists in an inactive state in resting cells but can be activated by the Mre11–Rad50–Nbs1 (MRN) complex and other factors at sites of DNA breaks. In addition, oxidation of ATM activates the kinase independently of the MRN complex. This review discusses these mechanisms of activation, as well as the posttranslational modifications that affect this process and the cellular factors that affect the efficiency and specificity of ATM activation and substrate phosphorylation. I highlight functional similarities between the activation mechanisms of ATM, phosphatidylinositol 3-kinases (PI3Ks), and the other PI3K-like kinases, as well as recent structural insights into their regulation.

Contents

INTRODUCTION

The protein kinase ataxia-telangiectasia mutated (ATM) is named for the autosomal recessive disorder ataxia-telangiectasia (A-T), caused by loss of the activity of this kinase and characterized by a progressive loss of cerebellar neuron function resulting in debilitating ataxia, as well as dilation of blood vessels (telangiectasia). Patients also exhibit immunodeficiency, extreme sensitivity to ionizing radiation, and a high rate of malignancies, primarily lymphoreticular (1–4). The disorder is observed worldwide at a frequency ranging from 1 in 40,000 to 1 in 100,000 live births, although the estimated frequency of nonfunctional alleles in the population is much higher than would be expected: Approximately 1 in 100 individuals are thought to be heterozygous for an A-T allele in certain human populations (5). The *ATM* gene was cloned in 1995 by Shiloh and colleagues (6) after linkage studies localized the gene to a region on chromosome 11 (7), showing that the 12-kb transcript encodes a very large protein of 3,056 amino acids (8). The kinase domain is located at the C terminus and exhibits homology to the phosphatidylinositol 3 (PI3) family of lipid kinases (PI3Ks), which phosphorylate inositol phosphate molecules. However, ATM, like the other PI3K-like protein kinases (PIKKs), does not phosphorylate phosphatidylinositol (9).

The family of PIKKs (**Figure 1**) includes several enzymes involved in DNA repair and damage signaling—ATM, ataxia-telangiectasia and Rad3–related (ATR), and DNA-dependent protein kinase catalytic subunit (DNA-PKcs)—all of which associate directly with sites of DNA lesions through DNA-binding cofactor complexes and are thought to be activated primarily at these sites (10). Like ATR and DNA-PKcs, ATM is a serine/threonine-directed kinase and has a preference for SQ/TQ motifs (11). In addition to these, members of this family include the mammalian target of rapamycin (mTor) protein kinase, which is involved in nutrient sensing and the regulation of metabolism, as well as suppressor of morphogenesis in genitalia 1 (SMG-1), which controls nonsense-mediated messenger RNA (mRNA) decay, and the kinase-dead adaptor protein TRRAP (transformation/transcription domain–associated protein), which regulates transcription (12).

All of the PIKKs share a limited homology with the PI3K kinase domain, specifically in the catalytic loop that contains residues essential for coordinating ATP and stabilizing the transition state of the phosphorylation reaction (13). Domains surrounding the kinase homology region also

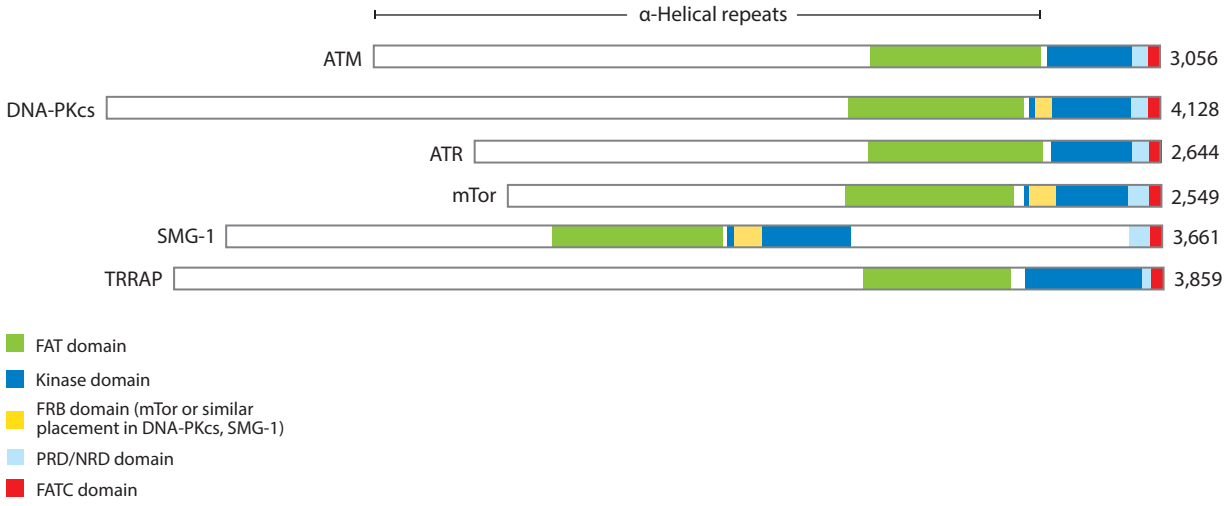

Figure 1

PIKK enzymes. Numerals refer to amino acids. ATM: FAT domain, ~1,966–2,566; kinase domain, 2,614–2,960; FATC domain, 3,025–3,056. DNA-PKcs: FAT domain, ~2,884–3,538; kinase domain, 3,565–4,013; FATC domain, 4,097–4,128. ATR: FAT domain, ~1,640–2,185; kinase domain, 2,209–2,565; FATC domain, 2,613–2,644. mTor: FAT domain, ~1,385–2,002; FRB domain, 2,022–2,118; kinase domain, 2,003–2,450; FATC domain, 2,518–2,549. SMG-1: FAT domain, ~1,271–1,941; kinase domain, 1,942–2,425; FATC domain, 3,630–3,661. TRRAP: FAT domain, ~2,704–3,275; kinase domain, 3,312–3,781; FATC domain, 3,828–3,859. Green represents the FAT domain (approximate) (188); blue, the kinase domain with N-terminal boundaries based on Reference 132; yellow, insertions in kinase domain (FRB in mTor; unknown function in DNA-PKcs and SMG-1) based on the structure of FRB in Reference 132; and red, the FATC domain (189). Light blue represents the sequence between the kinase domain and the FATC domain, also known as the PIKK regulatory domain (PRD), from studies of ATR (133) or the negative regulatory domain (NRD) from studies of mTor (134). Abbreviations: ATM, ataxia-telangiectasia mutated; ATR, ataxia-telangiectasia and Rad3–related; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; FAT, FRAP–ATM–TRRAP (transformation/transcription domain–associated protein); FRB, FKBP12/rapamycin-binding; mTor, mammalian target of rapamycin; PIKK, phosphatidylinositol 3-kinase–like protein kinase; SMG-1, suppressor of morphogenesis in genitalia 1.

show conservation within the PIKK family, including the FRAP–ATM–TRRAP (FAT) domain that is N-terminal to the catalytic domain and the FATC domain [sometimes shown as a bipartite region consisting of the PIKK regulatory domain (PRD) and the FATC domain at the C terminus] (14). PI3Ks also contain helical domains that bracket the catalytic N- and C-terminal lobes, although these are shorter than the equivalent regions in the PIKK enzymes. Most of the length of these PIKK proteins, including the entire N terminus, consists of repeated units of the ~50–amino acid HEAT (Huntingtin, elongation factor 3, protein phosphatase 2A, TOR1) motif (15), as well as tetratricopeptide motifs, which are α-helical domains found in many proteins. Whereas the functions of the large HEAT repeat domains in PIKK enzymes are still not very clear, in other proteins these regions mediate protein–protein interactions (16). The large HEAT repeat structure likely acts as a scaffold upon which the kinase domain and the FAT and FATC domains can interact with each other as well as with other molecules, including proteins bound to DNA and the DNA lesion itself.

This review focuses on mechanisms of ATM activation and regulation of its activity by other proteins, by posttranslational modifications, and by oxidation. Although we do not yet have an atomic-level structure for ATM, it is informative to consider recent structural insights into other PIKK enzymes, including mTor and DNA-PKcs, as well as PI3K itself, for similarities in structure and regulation.

LESSONS FROM PI3K

PI3K enzymes phosphorylate the 3′ OH on the inositol ring in phosphatidylinositol, or its phosphorylated derivatives, to generate phospholipid second messengers. Although this process is clearly different from the protein phosphorylation observed with ATM and the other PIKKs, at least two general attributes of PI3K enzymes are shared among these families (**Figure 2**). First, PI3K enzymes in a resting cellular state are strongly and redundantly autoinhibited for kinase

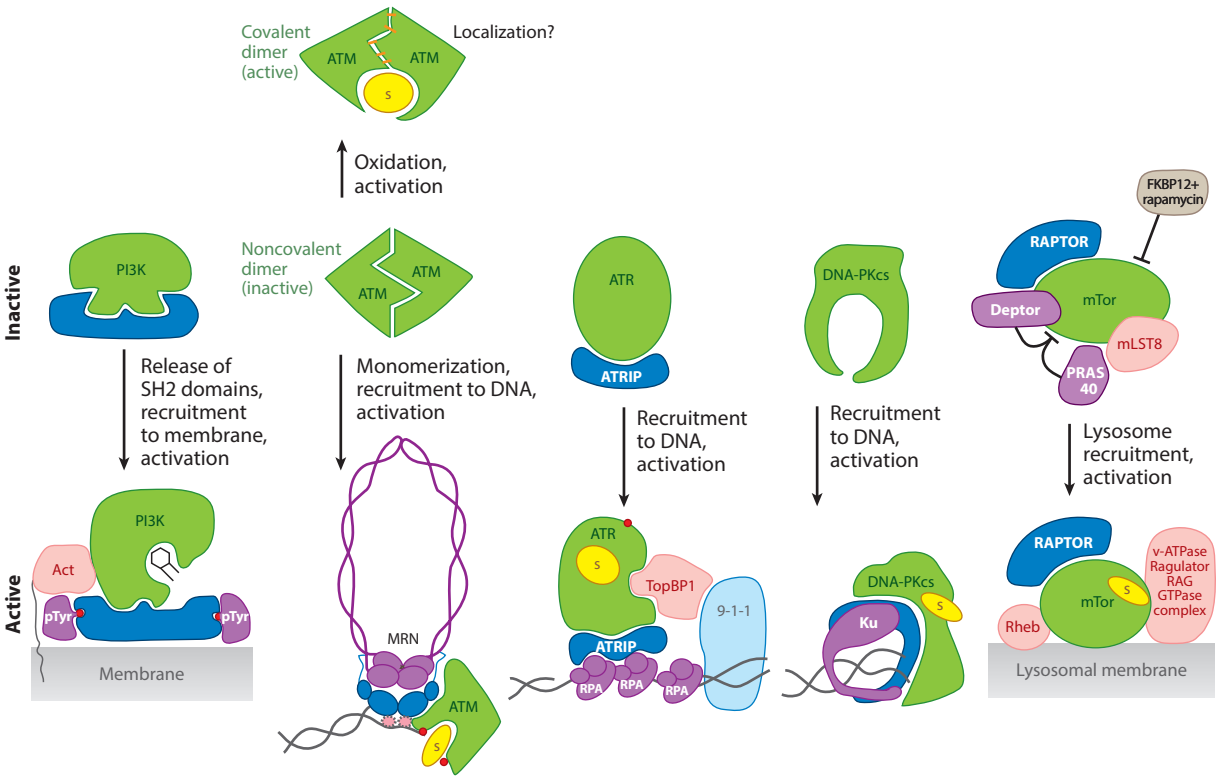

Figure 2

PI3K and PIKK enzymes in inactive and active states. PI3K is shown with p110 (*green*) and p85 (*blue*) subunits in the inactive state, and after conversion to an active state with a combination of positive-acting factors, including binding to phosphotyrosine (pTyr)-containing proteins and recruitment to the membrane by activator(s) (Ras, Rab5, Gβγ) to phosphorylate phosphatidylinositol (PI) or PI derivatives. ATM (*green*) is shown as a noncovalent dimer in the inactive state, converting to an active, covalent dimer with disulfide bonds with oxidation (*top*) or an active monomer bound to DNA ends with the MRN complex (Rad50 in purple, Mre11 in blue, approximate Nbs1 position in pink) (*bottom*). Many other proteins affect activation (see text for details). ATR (*green*) is shown with the cofactor ATRIP in the inactive state and after localization and activation by single-stranded DNA bound by RPA (*purple*), 9-1-1 complex (*light blue*), and TopBP1 (*pink*) (133). DNA-PKcs (*green*) is shown in the inactive state and after localization and activation at DNA ends in complex with the Ku heterodimer (*blue/purple*) (131). mTor (*green*) is shown in the cytosolic inactive state (here modeled after TORC1) with cofactors RAPTOR (*blue*) and mLST8 (*pink*) and inhibitors (*purple*) Deptor and PRAS40 (FKBP12/rapamycin also has inhibitory effects). In response to increases in amino acid levels, mTor is recruited and activated at the lysosomal membrane by Rheb and the v-ATPase–Ragulator–GTPase complex (190). Protein substrates are shown in yellow, and autophosphorylation is shown as red circles. Autophosphorylation is not shown on DNA-PKcs because it induces release from DNA (191). The shapes do not represent actual configurations. Abbreviations: ATM, ataxia-telangiectasia mutated; ATR, ataxia-telangiectasia and Rad3–related; ATRIP, ATR-interacting protein; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; mLST8, mammalian lethal with sec-thirteen 8; mTor, mammalian target of rapamycin; PI3K, phosphatidylinositol 3-kinase; PIKK, PI3K-like protein kinase; RPA, replication protein A; TopBP1, topoisomerase II–binding protein 1.

activity. In the best-characterized group of class I PI3Ks, each enzyme consists of a catalytic subunit (p110) bound to a regulatory subunit (p85, p50, or p55) (13). Src homology 2 (SH2) domains and an intervening coiled-coil domain in p85 bind to the catalytic domain via at least three interfaces, physically blocking the substrate-binding site and inhibiting association of the enzyme with membranes, the site of activation. Thus, in the absence of a stimulus, class I enzymes are inactive and located in the cytoplasm. Release of the linker and SH2 domains of the regulatory subunit from the p110 catalytic domain occurs with the binding of p85 by phosphorylated tyrosine-containing adaptor proteins (formed as a result of receptor tyrosine kinase activation).

The second general feature shared between PI3K and PIKK enzymes is that they require relocalization for full activity. With PI3K, inactive enzymes in the cytoplasm are recruited to the membrane by membrane-bound phosphotyrosine-containing proteins as well as the binding of activated Ras, Rab5, or $G\beta\gamma$ through additional binding domains (13). Only through a combination of positive stimuli (release of SH2 inhibition by phosphotyrosine binding plus binding of Ras, for instance) is the PI3K enzyme fully activated at the membrane to phosphorylate phosphatidylinositol or PIP_2 (phosphatidylinositol 4,5-bisphosphate). Overall, activation of this class of enzymes involves multiple inputs in the form of protein–protein and protein–lipid interactions that act together to release the autoinhibition and promote binding of the inositol substrate (13). The diversity of the binding partners of PI3K also illustrates how multiple regulators can simultaneously influence the activation process. As detailed in the following section, ATM activation at DNA break sites can be considered analogous to this process: ATM is inactive in the resting state but is rapidly activated and localized with the onset of DNA damage.

ACTIVATION OF ATM BY THE Mre11–Rad50–Nbs1 COMPLEX

Mre11, Rad50, and Nbs1 (Nibrin) form the MRN complex, which is highly conserved and plays critical roles in DNA double-strand break repair in all organisms (17, 18). Mre11 is an exo- and endonuclease in the λ phosphatase family of phosphoesterases. It forms a complex with Rad50, an ATPase in the ABC transporter family, which also contains long, intramolecular coiled coils that are similar to cohesins and condensins; these are also in the structural maintenance of chromosomes (SMC) family of enzymes. MRN-deficient cells are impaired in DNA end processing, which is required for homologous recombination, including the processing of Spo11-generated breaks during meiosis, and affects repair of DNA breaks by nonhomologous end joining in some organisms (19, 20). The Nbs1 protein regulates the activities of Mre11 and Rad50 and is found only in eukaryotes (21).

The discovery of the functional relationship between ATM and the MRN complex arose from the finding that patients with a rare clinical phenotype similar to that observed with A-T patients [termed A-T-like disorder (ATLD)] have mutations in the *MRE11* gene (22). Because loss of any of the individual MRN components generates early embryonic lethality in mice (24–26), and these rare ATLD patients do express low levels of mutant MRN protein, ATLD probably represents MRN hypomorphism rather than complete loss of the complex. ATLD is nearly indistinguishable from A-T in clinical presentation; patients exhibit later onset and slower progression of ataxia compared with A-T patients (with no telangiectasia), and the cellular phenotype is similar to that of A-T. Importantly, ATLD cells with mutant Mre11 exhibit markedly reduced activation of ATM by DNA double-strand breaks and, consequently, reduced ATM-dependent phosphorylation of substrates (27, 28). This observation demonstrates that MRN is required for optimal ATM activation following double-strand break induction (28) and shows that the effects of MRN loss on ATM function are most acute with low levels of DNA damage, whereas high levels of radiation damage can induce ATM even in cells expressing the ATLD hypomorphic alleles. These

experiments can be complicated to interpret, however, because MRN is essential for cell viability in dividing vertebrate cells (29, 30) and the low levels of mutant complex expressed in ATLD patient cells definitely provide a basal level of activity that supports essential functions of the complex in DNA repair.

The cellular characteristics of A-T and ATLD, namely radiation sensitivity, radioresistant DNA synthesis, and chromosomal instability, are similar to those of patients with the Nijmegen breakage syndrome (NBS), caused by a rare hypomorphic allele of *NBN* that contains an internal deletion and truncation of the C terminus of Nbs1 (657del5) (27, 31–33). However, the clinical phenotypes of NBS are not identical to those of A-T and ATLD, as NBS patients show microcephaly and growth retardation but not the ataxia that is diagnostic of A-T. *MRE11* and *RAD50* mutations have also been identified in patients with NBS-like microcephaly (34, 35), showing that the phenotype is not specific to *NBN* mutation but is rather a consequence of a specific class of MRN mutations that dramatically destabilize the MRN complex. The Nbs1 protein regulates the enzymatic activities of Mre11 and Rad50 in vitro (36, 37) but is also required for the nuclear localization of the other components of the complex (38), and the 657del5 mutation in *NBN* that is responsible for essentially all NBS patients generates a mutant that fails to promote nuclear localization of Mre11 and Rad50 (39).

In general, observations of MRN mutant function in cells (40) and in vitro (37) are consistent with the idea that mutations that retain the integrity of the MRN complex but reduce the overall levels give rise to an A-T-like phenotype, whereas mutations that compromise complex formation are correlated with the earlier-onset microcephaly phenotype observed in NBS patients. In addition, whereas cells from both ATLD and NBS patients are deficient in ATM signaling, there are clearly neuron-specific differences in apoptotic signaling between these different classes of mutations that may determine the outcome of MRN loss or destabilization in the brain (41).

Lastly, there is also a group of MRN mutations that lead to a gain-in-function phenotype for ATM, as elucidated in mouse models studied by Petrini and colleagues (42, 43). These *RAD50* mutations, some modeled after mutations isolated in budding yeast (44), cause cellular attrition of hematopoietic cells and other development defects, which can be partially suppressed by deletion of ATM. These results demonstrate that the MRN complex regulates ATM function both positively and negatively, with extreme consequences for the maintenance of diverse tissues during mammalian development.

ATM ACTIVATION BY DNA DAMAGE: MONOMERIZATION AND AUTOPHOSPHORYLATION

The ATM protein is predominantly nuclear in most cell types, where it exists as a noncovalent homodimer (45). This form of the protein is catalytically inactive, but with DNA damage it converts to an active monomer form that phosphorylates a large number of substrates (estimated in the hundreds) involved in cell-cycle checkpoints, DNA repair, and a diverse array of cellular processes (46–48). The discovery of the inactive dimer form of ATM (45) highlights the similarity to PI3K in its autoinhibited basal state, and suggests that each subunit of the dimer likely participates in blocking the activity of the other subunit until activation occurs.

In addition to observing inactive ATM dimers in resting cells, Bakkenist & Kastan (45) documented ATM autophosphorylation at serine (S)1981 (a site in the FAT domain), which occurs at the same time as monomerization and activation of ATM in response to DNA damage. Autophosphorylation of this residue occurs in *trans*, and phosphomimic forms of the kinase containing an S1981D mutation failed to form dimers, suggesting that S1981 phosphorylation disrupts the inactive ATM dimer. In addition, an S1981A nonphosphorylatable mutant form of the kinase

acts as a dominant negative allele for autophosphorylation, localization to sites of DNA breaks, and regulation of the S-phase checkpoint, consistent with the idea that autophosphorylation and thus monomerization are essential for ATM function in the DNA damage response (45, 49). Subsequently, additional ATM autophosphorylation sites were identified, and their role in optimal ATM-mediated responses to DNA damage in human cells was also demonstrated (47, 50).

Despite these compelling results, later studies showed that in mice, mutations that abolish the autophosphorylation site on murine Atm (which corresponds to S1981 in human ATM), alone or in combination with two other identified autophosphorylation sites, yields an essentially wild-type phenotype (51, 52), as well as normal activation in an in vitro reconstituted system (53). Studies using *Xenopus* egg extracts also indicated that high concentrations of DNA can promote ATM activation in the absence of autophosphorylation and that ATM recruitment to DNA can precede autophosphorylation (54, 55). The difference between human cells and mouse models has not yet been explained, although a subsequent study found that the S1981A mutation in ATM affects primarily the sustained retention of ATM at double-strand break sites, not the initial recruitment, and that a failure to remain at the break site affects association with the mediator protein Mdc1 (also discussed in the section titled ATM Recruitment to Sites of DNA Breaks, below), as well as the phosphorylation of downstream substrates (56). S1981 autophosphorylation remains a valuable and sensitive marker for ATM activation in human cells, irrespective of the controversy surrounding its functional importance.

ATM autophosphorylation with DNA damage also depends on Tip60/KAT5-dependent acetylation of a lysine (K) in the FATC domain of ATM, K3016, that occurs immediately upon DNA damage (57, 58). Price and colleagues (58) found that blocking this acetylation with a K3016 mutation or Tip60 depletion results in loss of ATM autophosphorylation and phosphorylation of substrates including p53 and Chk2; these results showed that ATM autophosphorylation is one of a progression of events starting with DNA damage recognition and resulting in stable association of acetylated, autophosphorylated monomers at sites of DNA damage. Tip60 itself is regulated by phosphorylation on tyrosine that is catalyzed by the protein kinase c-Abl and promotes the binding of Tip60 to H3K9me3 and subsequent acetylation of ATM (59).

Similar to ATM autophosphorylation at S1981, the related PIKK enzyme ATR also undergoes autophosphorylation on a threonine residue in the FAT domain (T1989), which depends on its cofactor, ATR-interacting protein (ATRIP), as well as on RPA-bound single-stranded DNA (60). This modification is essential for binding of topoisomerase II–binding protein 1 (TopBP1), which facilitates ATR activation and substrate binding. Thus, modifications within this part of the FAT domain clearly play important roles in PIKK regulation. Autophosphorylation of DNA-PKcs also regulates the association of this kinase with DNA, although the primary sites identified are N-terminal to the FAT domain (sites are in two regions, spanning amino acids 2,023–2,056 and amino acids 2,609–2,647) (61).

ACTIVATION OF ATM BY THE Mre11–Rad50–Nbs1 COMPLEX AND DNA

The transformation of inactive ATM into an active kinase has also been observed with purified proteins (53, 62), as well as with *Xenopus* egg extracts and human cell extracts (54, 55, 63) in vitro. These experiments focused initially on the role of the MRN complex, which has several distinct roles in ATM activation. First, the MRN complex promotes monomerization of inactive dimeric ATM into active monomers: Conversion of dimers to monomers occurs with the addition of the MRN complex to purified dimeric kinase but does not appear to require DNA (53). In contrast, experiments in *Xenopus* egg extracts showed that MRN tethering of DNA is required

for this monomerization and that DNA by itself, at high concentrations, could even perform this function (54). Why these differences exist between purified proteins and extracts is unknown, but a general conclusion from these experiments is that monomerization is an initial step in the activation process and is not sufficient for complete activation of the kinase.

In the purified kinase reaction and in *Xenopus* egg extracts with low levels of DNA double-strand breaks, it is clear that DNA by itself does not activate ATM. The fact that ATM does possess a weak DNA end-binding ability (64) and the observation that high levels of breaks in extracts can monomerize ATM in the absence of the MRN complex (54) suggest that this complex may help unmask a normally inaccessible DNA-binding surface on ATM. The MRN complex binds to DNA through multiple interfaces and can bind to DNA without ends, but it forms stable complexes with ends in the presence of ATP or ATP analogs (37, 65–67). Under these conditions, ATM is recruited to the DNA in a manner that requires an open double-strand break end. A later study further clarified that the MRN complex requires ATP for ATM activation but that this process does not require ATP hydrolysis (68). Similar to the regulation of PI3K, ATM autoinhibition is relieved by the MRN complex through monomerization, but an additional input is required: localization to the site of activation, which in this case is a double-strand break in chromatin.

The structure and length of DNA are important for ATM activation. In the presence of MRN, a DNA end is essential for activation of ATM, consistent with original observations that A-T patient–derived cells are sensitive only to DNA damaging agents that induce double-strand breaks (69) and that ATM localizes at sites of DNA breaks (70). Optimal activation of ATM occurs with long (≥1-kb) DNA (53, 71), a requirement that is observed even with purified proteins in which nucleosomes and chromatin are not relevant. The reason for this requirement is not clear but is likely specific to MRN-DNA-binding preferences, which strongly favor long DNA (T. Paull, unpublished observations). ATM activation occurs with blunt ends or with short overhangs (53) but is inhibited by long (>25-nt) overhangs of 3′ or 5′ single-stranded DNA in human cell extracts (63). MRN binding to longer overhangs is inhibited in the extracts but not in the purified form, suggesting that other proteins compete effectively for binding. A likely candidate for this inhibitory factor is replication protein A (RPA), which has high affinity for single-stranded DNA and coordinates a switch from ATM activation at sites of double-strand breaks to ATR activation after break resection and formation of RPA-coated single-stranded DNA (63).

Whereas ATM is specifically activated by double-strand breaks, there are strong indications that the form of DNA presented to ATM when bound by MRN has single-stranded character, even in the absence of DNA end resection. A single-molecule fluorescence resonance energy transfer (FRET) study recently showed that, in the presence of ATP, DNA ends bound to MRN are significantly unwound, equivalent to a branch of ~15 nt (66). MRN is not a translocating helicase; rather, it binds DNA in a manner that destabilizes the double-helical structure of DNA at the end. In addition, a mutant form of Rad50 that is deficient in unwinding also fails to activate ATM and can be complemented by the use of unwound DNA ends (53). This may be one reason that Mre11–Rad50 is essential for ATM activation in addition to Nbs1: The complex holds the strands open in a way that can be achieved only at the site of a DNA break. In addition to the direct contact between the Nbs1 C terminus and ATM, Mre11–Rad50 also binds to ATM through an interface located in the Rad50 protein (62).

A second phase of activation requires MRN to act as a cofactor for ATM catalytic activity, according to experiments with monomeric ATM showing that the presence of the MRN complex promotes the activity of ATM in physiological conditions in which magnesium is the primary cation (62). Previously, most activity assays with ATM in the absence of the MRN complex used manganese, which allows for ATM activity in the absence of that complex but does so through an oxidation-specific mechanism (see the section titled ATM Activation by Oxidative Stress, below)

(53). The Nbs1 component of the MRN complex plays an important role in this step, particularly the last 20 amino acids at the C terminus. Jackson and colleagues (72) showed that these 20 amino acids contact ATM in a manner conserved with the protein–protein interactions between the C termini of Ku and ATRIP with DNA-PKcs and ATR, respectively. These short interaction motifs are essential for ATM, DNA-PKcs, and ATR activation (72, 73), and in the case of fission yeast, this section of Nbs1 interacts with a segment of HEAT repeat in the N terminus of Tel1 (ATM ortholog; repeats 17–18 and 21–22) (55). In this second step, ATM in extracts can be activated with the C-terminal peptide of Nbs1 in the absence of Mre11–Rad50, but only if the extracts have previously been incubated with the MRN complex and DNA (54), suggesting that the Nbs1 C terminus–dependent process is distinct from the initial monomerization step. Thus, there are probably at least three interaction surfaces between MRN and ATM—one through the Nbs1 C terminus, one through Mre11–Rad50, and one in contact with the unwound DNA end—that collectively release autoinhibition of the dimer, recruit ATM to the activating molecule (DNA), and promote activation.

The MRN complex exhibits exo- and endonuclease activity on DNA double-strand break ends and hairpin structures through the Mre11 component, a member of the λ phosphatase family of phosphoesterases (36, 74–77). The physiological activities of the nuclease have been a topic of much debate, but it is possible that both exo- and endonuclease activities occur in cells (78). Several studies have addressed the potential role of Mre11 nuclease activity in regulating ATM activation using both in vivo and in vitro approaches. A mouse model was created to investigate the role of the nuclease activity in vertebrates, which demonstrated, surprisingly, that mutation of the Mre11 nuclease active site blocks embryonic development (77a). A conditional allele was used to investigate the functions of the H129N nuclease–deficient allele in cells, which demonstrated that the mutant complex causes poor growth, premature senescence, and chromosomal instability, but normal ATM activation. In vitro ATM kinase assays with recombinant MRN and dimeric ATM also failed to show any defect in activation with a nuclease-deficient MRN complex (53). Lastly, as mentioned above, MRN that is bound to ATP in the absence of ATP hydrolysis is competent to promote ATM activation (68), indicating that nuclease activity is not essential for ATM activation because the nuclease activity of Mre11 requires a conformation change in Rad50 that is driven by ATP hydrolysis (65). An alternative conclusion was proposed by a study using complemented ATLD fibroblasts (28), and another with *Xenopus* egg extracts, which showed that small oligonucleotides generated by Mre11 nuclease activity are essential for ATM kinase activity (79). These discrepancies have not been fully resolved; however, the bulk of the evidence shows that the nuclease function of MRN is not absolutely essential for ATM activation in mammalian cells.

Although not catalyzed by MRN, the endonucleolytic removal of a short part of the 5′ strand at a DNA break by the nuclease Sae2 (also known as Ctp1 or CtIP) does regulate ATM activity, on the basis of evidence from budding and fission yeasts (80, 81). In *Saccharomyces cerevisiae*, the ATM ortholog Tel1 functions redundantly with the ATR ortholog Mec1 in most cellular contexts and can be unambiguously monitored only in a *mec1* deletion strain (82). In this background, Tel1 signaling to downstream substrates is significantly improved with the deletion of Sae2, an endonuclease that participates with MRX (MRN ortholog) in the initial stage of DNA end processing (80). A similar situation has been observed in fission yeast, wherein Ctp1 negatively regulates Tel1, which in this case can be monitored even in the presence of functional Rad3 (ATR ortholog); however, in fission yeast Mre11 nuclease activity is also required for a step downstream of ATM activation (81). A likely explanation for this phenomenon is that DNA end processing by Ctp1 changes the DNA substrate in a way that can no longer activate Tel1 (ATM); thus, removal of Ctp1 in a *mec1* strain promotes Tel1 activity. This explanation is consistent with Shiotani & Zou (63), who initially proposed that the structure of the DNA break gradually changes over the

course of the DNA damage response from a blunt end to a 3′ single-stranded DNA due to end processing, and that this process drives a transition from ATM to ATR signaling. These insights illustrate the complex interplay between the structure of the DNA end and the occupancy and activity of proteins bound to the ends, most significantly the PIKK enzymes.

The Rad50 protein contains long, antiparallel coiled-coil domains that link two Rad50 monomers together through a zinc-mediated "hook" connection (21). The role of the coiled-coil domains is, on at least in part, to link the Rad50 catalytic domains and facilitate ATP binding between the monomers. Consistent with this hypothesis, removal of part or all of this domain blocks Rad50 function in DNA repair in vivo in budding and in mammalian cells (43, 83). With respect to ATM, Rad50 hook mutations or coil deletions impair ATM activation in vitro (68), although a hypomorphic hook mutant expressed in mice appears to show a hyperactive ATM phenotype (43), similar to *RAD50*(*S*) mutations that exhibit a separation of function between meiotic and mitotic phenotypes in budding yeast (42, 44). Why these mutations hyperactivate ATM is unknown, but this is not a general characteristic of MRN mutations, given that mutations in the mouse that mimic human ATLD or NBS do not have this effect and can actually rescue phenotypes of the hyperactive mutants (84). A DNA lesion stabilized by these mutations might overactivate ATM, or perhaps hyperstimulation of ATR may play a role.

Mre11–Rad50–Nbs1 POSTTRANSLATIONAL MODIFICATIONS

Components of the MRN complex are also phosphorylated by PIKK enzymes, with varying effects on DNA repair as well as on ATM signaling. Mre11 is phosphorylated on at least five C-terminal SQ/TQ sites by ATM and ATR following DNA damage, and these modifications reduce the affinity of MRN for DNA, resulting in lower levels of ATM recruited to damage sites (85). The purpose of this process is unknown, although phosphorylation mimic mutations block ATM signaling, suggesting that Mre11 phosphorylation could be a mechanism for turning off the DNA damage signal. Rad50 is also phosphorylated by ATM, although not hyperphosphorylated. S635 in the coiled-coil domain is the target of ATM, and Lavin and colleagues (86) showed that it specifically modulates the activity of ATM toward one downstream substrate, SMC1. ATM-dependent phosphorylation of SMC1 on S957 is absent in Rad50-deficient cells expressing the Rad50 S635G mutant allele, whereas ATM autophosphorylation, phosphorylation of other targets, and activity in vitro all appear similar to the wild-type enzyme. SMC1 is important for checkpoint control and DNA repair, both of which are deficient in the Rad50 mutant–expressing cells; thus, this Rad50 modification is functionally important and may allow the MRN complex to serve as an adaptor for specific downstream substrates. The same serine is also phosphorylated by ATR during replication stress and is required in this context for ATR-dependent signaling through Chk1, as well as for replication restart (87).

Nbs1 is also phosphorylated by ATM on S278 and S343, modifications required for S-phase checkpoint control and complementation of radiosensitivity in NBS cells (88–91), as well as for radiation-induced DNA damage responses in vivo in mice (92). The S343A mutant form of Nbs1 is deficient in supporting both the ATM phosphorylation of Chk2 (93), an important regulator of checkpoint activation because of its regulation of cell division cycle 25c (cdc25c), and the phosphorylation of SMC1 (94). Loss of S278 and S343 phosphorylation also leads to increased rates of telomere loss (95). Similar to Rad50 S635 phosphorylation, Nbs1 S343 modification can also be catalyzed by ATR during replication stress, in which it is important for the regulation of DNA synthesis (96). The mechanistic role of these phosphorylation events in MRN function are not yet understood, but clearly they can regulate several different downstream functions of the DNA damage response through both ATM and ATR.

A recent study demonstrated that Nbs1 is a target of K63-linked ubiquitination by the E3 ligase Skp2 and that this modification affects ATM autophosphorylation, recruitment to chromatin via MRN interaction, and ATM substrate phosphorylation (97). Ubiquitination of Nbs1 appears to be responsible for the effects of Skp2 on ATM function, and the target of ubiquitination is K735 in the Nbs1 C-terminal motif that binds directly to ATM. Interestingly, a region of the ATM HEAT repeats (amino acids 1,245–1,770) binds specifically to K63-linked ubiquitin chains but not to K48-linked chains, indicating an additional component of binding specificity.

Although the MRN complex is clearly important for ATM activation, it is not absolutely required. This issue has been difficult to address because MRN is essential for cell viability in vertebrates (29), at least in cell populations that are not postmitotic (30). Conditional deletion of Nbs1 in neural tissue yields chromosomal DNA damage, proliferation defects, and loss of ATR-dependent phosphorylation events, but increased ATM-mediated p53 activation, suggesting MRN-independent control of ATM in this context (98, 99). In addition, recent evidence has elucidated a new pathway for MRN-independent ATM activation that operates through changes in redox state, as described in the following section.

ATM ACTIVATION BY OXIDATIVE STRESS

A-T is pleiotropic, generating cerebellar atrophy, telangiectasia, premature aging, and many symptoms specific to lymphoid cells, including immunodeficiency and lymphoid cancers. Arguably the most debilitating aspect of the disorder, however, is the neuronal cell loss that occurs progressively during childhood, leading to cerebellar degeneration and severe neuromotor dysfunction. The neuronal component has been difficult to understand, in part because mouse models of A-T do not exhibit cerebellar dysfunction, although these mice do show some indications of motor function disability as well as immune system defects, infertility, checkpoint activation deficiency, and radiation sensitivity (100–104). During the past 10 to 15 years of research, many investigators have become interested in the idea that oxidative stress may be important for the neuronal dysfunction observed in A-T patients (105, 106). This interest was initially based on observations that cells from A-T patients, as well as from mouse A-T models, exhibit higher levels of reactive oxygen species (ROS) compared with normal cells (107, 108), as well as increased sensitivity to ROS-generating agents (109–111). Purkinje neurons, a cell type that is significantly affected in A-T patients, have a high rate of energy and oxygen metabolism, similar to that of other neuronal cell types (112). In addition, ATM responds to increased levels of ROS with phosphorylation of known downstream targets, and checkpoint activation in response to ROS is absent in A-T cells (111, 113). Interestingly, some of the adverse effects of ATM loss in A-T cells and mouse models can be reduced or delayed in onset with antioxidant treatment, including the growth defect, loss of hematopoietic stem cells, and T cell lymphoma incidence (114–117).

Coincident with this observed relationship between ATM function and redox state, Paull and colleagues (118) found evidence for ATM activation in both primary and transformed human cells with hydrogen peroxide exposure under conditions in which DNA damage was not observed by S139-phosphorylated H2AX (γH2AX) staining. Under these conditions, ATM autophosphorylation and phosphorylation of downstream targets p53 and Chk2 were observed, but the heterochromatin protein KAP-1 was not phosphorylated. Similar results were obtained in an MRN-deficient cell line derived from an ATLD patient. These results suggest that ATM can be activated by low levels of ROS independently of MRN and that a full DNA damage response does not occur. Similar observations were made with ATM inactivation during hypoxia, a low-oxygen state in which ATM autophosphorylates and phosphorylates downstream targets in an MRN-independent manner (119). In this study, ATM activation was also blocked by the

antioxidant *N*-acetylcysteine. The phosphorylated ATM that accumulates under these conditions is diffuse throughout the nucleus and does not form discrete foci as it does following the induction of DNA damage—similar to previous observations of ATM behavior during high-salt and chloroquine treatments (45). In theory, the activating agent in the case of hypoxia could be mitochondria-generated ROS species that form during hypoxia (120), although experiments with mitochondria-depleted cells argue against this interpretation (119). Hyperthermia also induces ATM autophosphorylation in the apparent absence of a full DNA damage response and in an ATLD patient cell line (121), suggesting that this response may also be a manifestation of MRN-independent activation.

In vitro, purified recombinant ATM can be activated 10- to 100-fold by the addition of hydrogen peroxide in the absence of either the MRN complex or DNA (118), indicating that these components are indeed unnecessary for the ROS stimulation observed in human cells. ATM activated in this way is not a monomer, as in the case with MRN–DNA stimulation, but rather is found in a disulfide-linked, covalent dimer. Several disulfide bonds were mapped in this dimer form, one of which was found to involve cysteine (C)2991 in the PRD/NRD (negative regulatory domain) region between the kinase domain and the FATC domain. Mutation of this cysteine (but not any of the other cysteines involved in disulfide bond formation) eliminated the oxidation-induced activation of ATM while preserving the MRN–DNA stimulation at normal efficiency, indicating that the two activation pathways can be separated. A similar effect was observed with a C-terminal deletion mutant of ATM, R3047X, which lacks the last 10 amino acids of the FATC domain. Even though this deletion does not overlap with the C2991 residue, the mutant R3047X ATM protein also fails to be activated by oxidation but still exhibits normal MRN-dependent activity. The R3047X mutation is significant in that it is an A-T patient mutation, found in several cases of "A-T variants" who were diagnosed with A-T and exhibited ataxia, similar to the other A-T patients, but lacked either the radiosensitivity or the immunodeficiency that is generally coincident with A-T pathology (122–124). In retrospect, this finding is logical because the R3047X ATM protein exhibits normal MRN-dependent responses to DNA damage, which is likely the source of the lymphoid-related deficiencies and the radiation sensitivity. R3047X A-T patient lymphoblast cells (as well as A-T lymphoblasts complemented with the C2991L allele) showed normal autophosphorylation and apoptosis in response to DNA damage (induced by camptothecin) but no response to low levels of hydrogen peroxide (118), consistent with this interpretation.

Activation of ATM in the form of a covalent dimer prompts many new questions for both the biology of the activation and its mechanism. How does disulfide bond formation eliminate the autoinhibition that seems to be inherent to the noncovalent dimer? Without an atomic structure of the two states, this question is difficult to answer, although experiments using varying substrate concentrations (62, 118) demonstrate that both the MRN–DNA pathway and oxidation strongly increase the affinity of ATM for its substrates. Reorganization of the kinase domain may occur with disulfide bond formation that, in some way, also involves the C terminus of the FATC domain. We also know that an ATM heterodimer containing one wild-type subunit and one C2991L subunit is functionally inactive under oxidizing conditions, consistent with a requirement for disulfide bond formation engaging the C2991 residue in both monomer subunits (118).

When purified recombinant ATM is activated with peroxide in vitro, very little if any autophosphorylation is observed, although the kinase is fully activated (Z. Guo & J.-H. Lee, unpublished observations). Autophosphorylation in vitro appears to depend entirely on the MRN complex. Yet in cells, ATM autophosphorylation certainly accompanies activation by ROS (118), which could be interpreted to mean that at least a subset of the ATM molecules activated via oxidation in vivo are also bound to MRN, or that other factors stimulate ATM autophosphorylation with oxidation in cells.

Along the same lines, it is important to note that under physiological stress conditions, ROS accumulation and DNA damage likely occur at the same time. In vitro kinase assays testing the effects of simultaneous double-strand breaks (in the presence of the MRN complex) and ROS show robust ATM activity at all ROS levels (125). However, the C2991L mutant ATM protein fails to exhibit kinase activity in the presence of both the MRN complex and hydrogen peroxide, because the former is inactivated and unable to bind DNA after hydrogen peroxide treatment (125). Thus, the existence of the oxidation pathway has a useful role in preserving ATM activity even under high-ROS conditions, in which the MRN complex is nonfunctional.

The ability of ATM to be activated independently of DNA also suggests that the kinase could function outside of the nuclear compartment. This suggestion has been widely debated, but it now seems clear that in some cell types, extranuclear ATM does exist. For example, in neuronal cells the cytoplasmic pool of the kinase is substantial (102, 126). These observations suggest further questions about the identity of cytoplasmic ATM targets; many ATM and ATR targets have been identified (46, 47), but for the vast majority of these we do not know their function. In addition, we do not know whether there are any ATM substrates that are specifically phosphorylated in response to oxidative stress. A recent report demonstrated that extranuclear ATM localizes to mitochondria (127), and earlier studies suggested microsome and peroxisome localization (128, 129). These findings may help explain the alterations in redox control observed in ATM-deficient cells, as well as altered mitochondrial homeostasis (127).

INSIGHTS INTO ATM FUNCTION FROM PIKK STRUCTURES

The lack of a crystal structure of ATM limits our ability to fully understand its catalytic mechanism, although recently solved structures of other PIKK enzymes have illuminated some of the structural features of this kinase family. For DNA-PKcs, a 6.6-Å structure shows the overall ring-shaped outline of the HEAT repeats in this enzyme that surrounds a central open space, which is hypothesized to bind DNA (130). In this case, the kinase domain is surrounded by a clamp of α-helical domains that are likely to be the HEAT repeats adjacent to the kinase domain in the primary structure; this observation is consistent with small-angle X-ray scattering measurements of the protein in solution (131). The crystal structure of mTor, recently solved at 3.2-Å resolution with the regulatory protein mLST8 (mammalian lethal with sec-thirteen 8), shows more clearly how the kinase domain in this enzyme is cradled by a ring of tetratricopeptide repeat (TPR) domains and HEAT motifs that are part of the FAT domain (**Figure 3**) (132). Because the FAT domain is conserved throughout the PIKK family (not necessarily in primary sequence but in predicted secondary structure), the FAT domain of ATM probably assumes a similar configuration. The mTor kinase domain itself shares many characteristics with PI3K enzymes and canonical protein kinases, whose similarities are not apparent from the sequence but which are clearly structurally similar in function. Like many kinases, the mTor catalytic domain is separated into N-terminal and C-terminal lobes that hold the ATP molecule between them. The C-terminal lobe contains the catalytic loop that shows most of the discernible homology between ATM and mTor, as well as the activation loop in the catalytic cleft. Importantly, key residues responsible for coordinating the γ phosphate and stabilizing the substrate hydroxyl group are conserved between mTor and canonical kinases despite little obvious sequence homology in the activation loop or other helices, even to the point where the mTor and CDK2 active sites are superimposable (132). This conservation of secondary structure also suggests that ATM may be similar in organization to PI3K and other protein kinases in its catalytic mechanism and the overall arrangement of the active site.

The mTor structure also shows that the FATC domain is an integral part of the kinase domain, which was not previously known. The N-terminal part of the FATC domain of mTor forms a helix

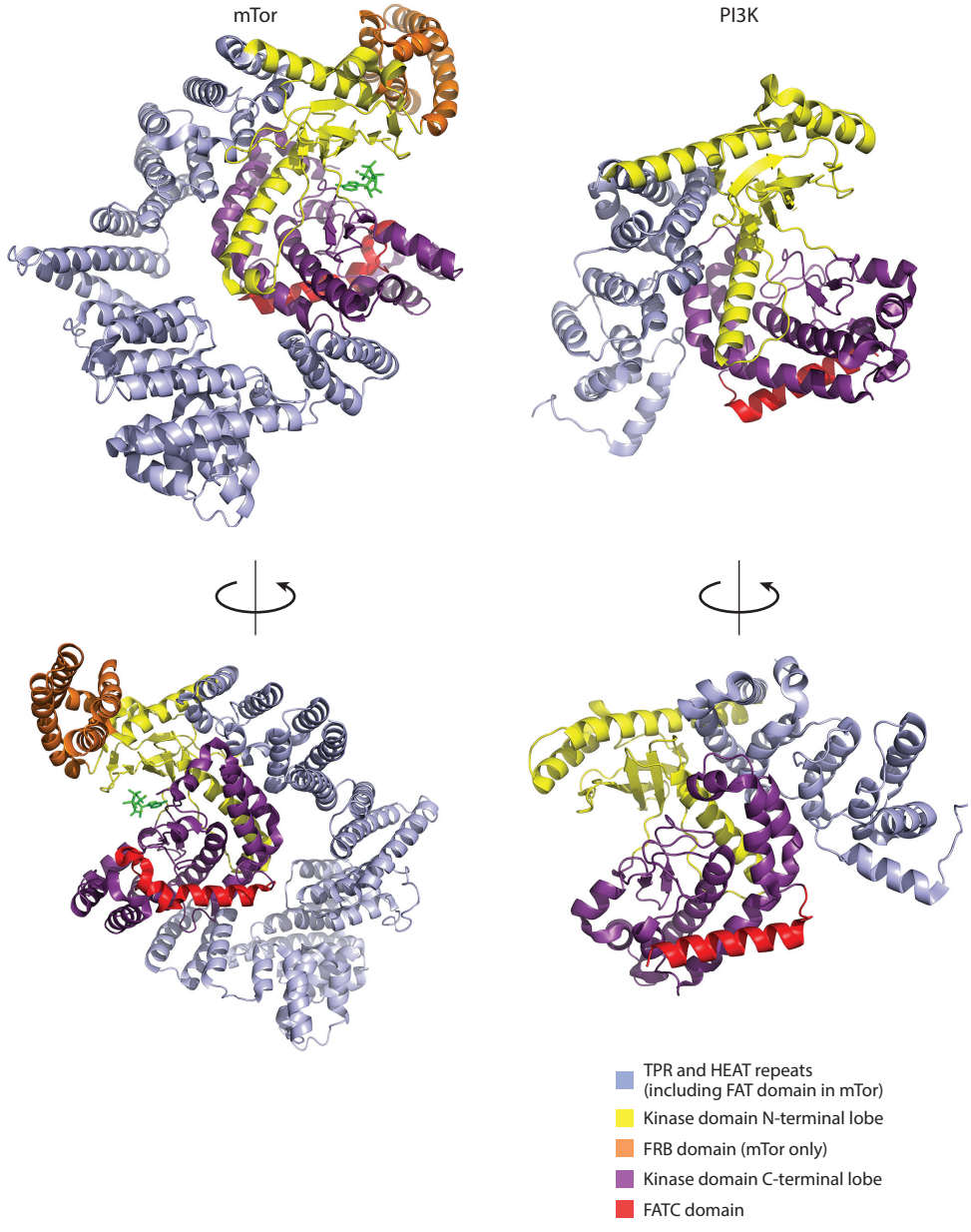

Figure 3

Structural similarity between mTor and PI3K enzymes. The crystal structures of mTor [Protein Data Bank (PDB) identifier 4JSP] (132) and PI3K3C3 (PDB 3IHY) are shown with similar domains colored. mTor is bound to magnesium and ATP-γ-S. Numerals refer to amino acids. mTor: HEAT/TPR repeats in the FAT domain, 1,385–2,002; kinase domain N-terminal lobe, 2,003–2,241 with FRB motif 2,022–2,118; kinase domain C-terminal lobe, 2,242–2,450; FATC domain, 2,518–2,549. PI3KC3: α-helical repeats, 282–530; kinase domain N-terminal lobe, 531–686; kinase domain C-terminal lobe, 687–848; FATC domain, 849–887. Abbreviations: FAT, FRAP–ATM–TRRAP (transformation/transcription domain–associated protein); FRB, FKBP12/rapamycin-binding; HEAT, Huntingtin, elongation factor 3, protein phosphatase 2A, TOR1; mTor, mammalian target of rapamycin; PI3K, phosphatidylinositol 3-kinase; PIKK, PI3K-like protein kinase; TPR, tetratricopeptide repeat.

in the kinase domain that is also present in PI3K, whereas the last nine C-terminal residues are aligned next to and in contact with the activation loop. Interestingly, if ATM is similar to mTor in this regard, these nine amino acids are essential only for oxidation-mediated but not MRN-DNA-mediated activation (118), which suggests that they might directly affect the structure of the activation loop.

C2991, the residue that is essential for ATM oxidative activation, is in a variable loop that is disordered in the mTor structure and not conserved between the PIKKs. This region is important for the regulation of multiple PIKK enzymes, however: Mutation or deletion of residues in this part of ATR (also termed the PIKK regulatory domain) abolishes activation of ATR by TopBP1 (133). In addition, loss of the N-terminal section of this short domain increases the basal kinase activity of mTor; thus, this part of mTor is known as the NRD (134). C2991 is conserved among ATM orthologs in terrestrial vertebrates but is not present in either budding or fission yeasts, suggesting that the kinase may not play a role in redox control in all eukaryotes. However, there is evidence that Tel1 in *S. cerevisiae* responds to mitochondrial ROS in a manner that extends chronological life span (135); therefore, the function of Tel1 in redox sensing may not absolutely require C2991 in all organisms.

mTor is bound by several other proteins that regulate its activity (136). RAPTOR in mTor complex 1 (mTorc1), and RICTOR in mTorc2, binds to a region just N-terminal to the FAT domain, a part of the protein that is not in the structure, whereas FKBP12/rapamycin binds to the FRB (FKBP12/rapamycin-binding) domain, a helical motif that sticks out of the N-terminal lobe of the mTor kinase domain. RAPTOR and RICTOR are important for the kinase activity of mTor in mTorc1 and mTorc2, respectively, and appear to be mutually exclusive for binding in these complexes (137). mTorc2 containing RICTOR is not sensitive to FKBP12/rapamycin; thus, a subset of mTor-dependent phosphorylation events is unaffected by rapamycin treatment.

A model of mTor bound by FKBP12/rapamycin suggests that this binding would greatly restrict access of polypeptides to the mTor active site (132), consistent with the known inhibitory effects of rapamycin on mTor activity in mTorc1. ATM does not have an insertion equivalent to the FRB domain, and no other proteins have been identified that contribute to the autoinhibition of the noncovalent dimer other than the dimer formation itself. However, ATM does have a domain (amino acids 2,791–2,828) in the same location as the mTor mLST8-binding element domain that binds to positive-acting factor mLST8, which was cocrystallized with mTor and is important for its stability (136). This part of the ATM kinase domain is a good candidate for a binding surface with other proteins or within the ATM dimer, and mutations in this region have been identified in at least two A-T patients (129, 138). C2801 in this loop was also identified in a screen for cysteines in disulfide bonds in oxidized ATM (118), although it is not essential for activation.

The idea that the basal state of PIKK enzymes is autoinhibited is borne out by the identification of many activating mutations in mTor that promote kinase activity independently of nutrient levels (139, 140). These gain-of-function (or loss-of-inhibition) mutations map to several helices in the mTor structure that pack against the catalytic residues (132). Interestingly, gain-of-function mutations identified in yeast Tel1 (ATM) promote survival of *mec1* strains in the presence of DNA damaging agents (141). In vitro kinase assays showed that some of these mutant proteins are hyperactive, with a corresponding gain of biological activity in phosphorylation of downstream substrates. At least two of the four hyperactive mutants contain amino acid changes in the same residue as or in a residue directly adjacent to those identified in mTor hyperactive mutants, including Tel1 R2675G and N2692D. This finding suggests that there may be good correspondence between regulation of mTor and regulation of ATM. Also, note that one of the gain-of-function

mutations identified in the yeast screen is K2751R, equivalent to K3016 in human ATM, the residue that is acetylated by Tip60 (58) and is clearly important for its regulation. Together, these observations suggest that there may be structural similarities between all the PIKK kinase members and that the basal inhibited state can be disrupted by a combination of many effects: the binding of specific cofactors or small molecules in response to stimuli or by mutations in conserved, critical regions that normally enforce the inactivity of the kinases under resting conditions. If the atomic structure of ATM is obtained, these predictions could be tested more specifically for this kinase to solidify this hypothesis.

PHOSPHATASES THAT REGULATE ATM ACTIVITY

Many proteins have been reported to modulate ATM signaling in mammalian cells. This section summarizes the best-characterized examples, with an emphasis on factors that specifically affect activation of the kinase. ATM is autophosphorylated during physiological activation, as discussed above, and several groups have observed that depletion or inhibition of phosphatases generates spontaneous autophosphorylation, even in the absence of DNA damage. These include the ubiquitous phosphatases PP2A and PP1 (specifically, PP1γ) as well as the type 2C phosphatase WIP1/PPM1D (wild-type p53-induced phosphatase 1/protein phosphatase Mg^{2+}/Mn^{2+} dependent 1D). In the case of PP2A, inhibition of the phosphatase with okadaic acid generates S1981 phosphorylation but not γH2AX, and association between PP2A and ATM is constitutive but lost after DNA damage (142). A similar phenomenon was observed with PP1γ, which associates with chromatin and ATM through a DNA-binding subunit, Repo-Man (143). In this case, inhibition also leads to ATM autophosphorylation, and simultaneous inhibition of PP1 and PP2A generates what appeared to be a complete DNA damage response, including phosphorylation of downstream substrates. Like PP2A, PP1γ dissociates from ATM after damage (143). Lastly, the phosphatase Wip1 also regulates ATM activation and is induced after DNA damage via p53-dependent expression (144). Upregulation of Wip1 leads to reduced ATM autophosphorylation after DNA damage, a finding that was also confirmed with in vitro assays of ATM protein immunoprecipitated from WIP1-overexpressing cells (145). Although the effects of these phosphatases on ATM appear to be similar, clearly Wip1 plays a unique role in cancer biology, given that mice in which the WIP1/PPM1D gene is deleted are highly resistant to activated oncogene-induced carcinogenesis (146). The antitumorigenic effects of Wip1 require ATM and p53, and the generation of p53 "pulses" (oscillations in ATM signaling and p53 accumulation after DNA damage) is Wip1 dependent (147).

Observations of constitutive association between ATM and phosphatases and spontaneous autophosphorylation with phosphatase inhibition suggest that the autophosphorylation does not depend on stress or stimuli (other than, perhaps, a constitutive association with the MRN complex) and that phosphatases play an important role in ATM regulation. Also, the phosphatase PP5 associates with ATM, but in this case only after DNA damage; its role appears to be in ATM activation rather than in ATM repression (148, 149), although the mechanism underlying this relationship is not yet understood.

ATM RECRUITMENT TO SITES OF DNA DOUBLE-STRAND BREAKS

Localization of ATM to sites of DNA damage is clearly an important component of the activation process. In addition to the contribution of the MRN complex to the recruitment of ATM at sites of double-strand breaks, ATM is tethered to chromatin domains containing breaks via indirect

association with the Mdc1 protein (150). Mdc1 was identified as the primary "reader" of γH2AX; it binds directly to the modified histone that is phosphorylated at sites of double-strand breaks by a combination of ATM and DNA-PKcs activities (151). Binding of γH2AX by the BRCA1 C terminus (BRCT) domain of Mdc1 has no effect on ATM autophosphorylation, but it enhances ATM's association with chromatin and strongly promotes the level of the total γH2AX signal, suggesting that stable binding of the kinase promotes further modifications, leading to an amplification loop (150). Mdc1 binding to γH2AX also protects γH2AX from dephosphorylation, thereby contributing to the maintenance of the histone mark during a sustained DNA damage response (151). Chen and colleagues (150) showed that ATM binds directly to Mdc1 but also can associate indirectly through the MRN complex, which binds to Mdc1 independently via casein kinase 2 (CK2)-phosphorylated motifs that are recognized by the N-terminal FHA and BRCT domains on Nbs1 (152–155). An investigation of the DNA damage response with *Xenopus* egg extracts also showed that recruitment of ATM to undamaged chromatin can play an important role in its activation, particularly when levels of double-strand breaks are below a critical threshold to mount a full response (156).

Because the functions of the MRN complex are intertwined with those of ATM, it is worth noting here the recent observation that the Rad17 protein can also recruit the MRN complex to sites of DNA damage. Rad17 normally acts as part of the replication checkpoint machinery, but it is also phosphorylated by ATM, leading to direct interaction between Rad17 and MRN, more efficient recruitment of both MRN and ATM, and higher levels of ATM autophosphorylation (157). This interaction precedes localization through Mdc1 and is independent from Mdc1-dependent mediator functions.

All of these observations are consistent with the idea that the chromatin context of ATM localization is a very important factor in its activation. The importance of localization is also evident in other chromatin modifications that indirectly affect ATM, including (*a*) histone H3 K9 trimethylation that is catalyzed by the methyltransferase Suv39h1, which recruits Tip60 (158); (*b*) histone H3 K36 trimethylation that affects the level and duration of ATM activation (159); (*c*) BRG1-, SNF5-, and RSC-dependent chromatin remodeling that is required for full ATM activity (160–163); (*d*) MOF-dependent acetylation of histone H4 K16 (164), which is necessary for efficient ATM autophosphorylation and activation; and (*e*) HMGN1 association with chromatin, which is required for ATM localization to DNA break sites (165).

The importance of localization was also clearly shown in a study in which the C-terminal half of ATM or other DNA damage response factors (MRN components or Mdc1) were physically tethered to chromosomal DNA in the absence of breaks. In this case, the increased local concentration of ATM, MRN, or Mdc1 in a single chromosomal domain (256 repeats of the lac operator sequence) was sufficiently high to drive phosphorylation of H2AX at the site, as well as Chk2-dependent checkpoint arrest (166). Thus, the requirement for a DNA double-strand break, while normally an important component in ATM activation, can be bypassed with very high local concentrations of activating protein–protein interactions.

As discussed above, ATM autophosphorylation promotes binding to Mdc1 and maintenance of ATM activity at sites of DNA breaks (56). This phenomenon of extended association between ATM and chromosome domains containing break sites is distinct from the initial activation process and may have distinct cellular functions. For instance, Bakkenist and colleagues (167) found that inhibition of ATM activity after an initial phase of DNA damage induction blocked subsequent DNA repair and generated chromosomal aberrations. An important point about these experiments and others utilizing small-molecule ATM inhibitors is that the cellular consequences of ATM inhibition versus ATM deletion (100–104); are likely different. Mice engineered to

express kinase-dead ATM show an embryonic lethal phenotype (168, 169), completely different from mice with ATM deletion (100–104); thus, there are apparently functions or binding partners of inactive ATM that strongly affect mammalian development.

MODULATION OF ATM RESPONSES TO DNA DAMAGE AND OXIDATIVE STRESS

The ATMIN protein was initially reported to be an ATM-interacting factor, and it binds to ATM through a C-terminal motif similar to the ATM-interacting sequence in the C terminus of Nbs1 (170). ATMIN is not essential for the DNA damage response but instead affects the activation of ATM by non–DNA damaging agents, such as osmotic shock and chloroquine. The idea that ATMIN and Nbs1 may compete for binding to ATM was borne out in a recent study by Behrens and colleagues (171), who showed that MRN/DSB-induced signaling through ATM is increased in ATMIN-deficient mouse cells, whereas ATMIN-dependent signaling is upregulated in Nbs1-deficient cells. Consistent with this idea, the loss of proliferative capacity and increased senescence observed in NBS fibroblasts depend on ATMIN, and depletion of both factors yields a phenotype similar to loss of ATM, at least with respect to DNA damage sensitivity. Recent studies have also shown that ubiquitination of ATMIN by UBR5 blocks the interaction between ATMIN and ATM, promoting MRN-dependent ATM activation (172). Although the mechanism of ATMIN's effect on ATM signaling is not yet clear, it is apparent that ATMIN plays an important role in modulating the effects of ATM in mammalian cells.

There is a direct and nearly linear relationship between the levels of the MRN complex and the level of ATM phosphorylation of a model substrate in vitro (173), consistent with in vivo evidence for the role of MRN in ATM activation. However, at low levels of MRN, when ATM activation is suboptimal, many other factors appear to affect the process of ATM activation in cells and in purified systems in vitro. One example is the stimulation of ATM signaling by p53-binding protein 1 (53BP1) and the Brca1 protein in vitro, where these mediator proteins increase ATM phosphorylation of a GST–p53 model substrate by as much as 14-fold, but only when MRN is limiting (173). This effect also occurs with an Nbs1 mutant lacking the C-terminal ATM interaction domain, indicating that this motif is not absolutely essential, although these proteins cannot rescue an MRN complex that lacks DNA unwinding activity (Rad50 S1202R). This effect correlates with direct binding between 53BP1 and both the MRN complex and ATM (dependent on the BRCT domains of 53BP1), suggesting that it may bridge these factors to promote complex formation at low levels of MRN.

Several other proteins augment ATM activity in mammalian cells and bind to the kinase directly. The NKX3.1 and homeobox B9 (HOXB9) proteins, both members of the homeodomain family of transcriptional regulators, bind to ATM after DNA damage, increasing the level of autophosphorylation and stimulating substrate phosphorylation in cells, particularly at early times after DNA damage (174, 175). NKX3.1 is a tumor suppressor in prostate cancer, whereas HOXB9 is overexpressed in breast cancer. Thus, the increased DNA damage response induced by these proteins may be protective in one tissue but in another is associated with increased DNA damage resistance in transformed cells.

Interestingly, the effects of HOXB9 on ATM require activation of transforming growth factor (TGF)-β, which is a transcriptional target of HOXB9 (175). Depletion of Smad4, an effector of TGF-β, effectively blocked the DNA damage response observed in HOXB9-overexpressing cells. Other TGF-β-related factors have also been implicated in regulation of the DNA damage response through ATM; these include Smad2 and Smad7, both of which localize to sites of ionizing

radiation–induced double-strand breaks (176). Also, coimmunoprecipitation experiments (177) showed that Smad7 stimulates ATM autophosphorylation and γH2AX phosphorylation and binds to ATM and the MRN complex.

The apoptotic inhibitor Aven has also been implicated in ATM function in *Xenopus* extracts and human cells, in which it associates with ATM after DNA damage, promotes autophosphorylation, and phosphorylates ATM substrates (178). Aven is also a target of ATM, and mutation of the ATM-targeted SQ sites in the protein partially abrogated Aven's activities in controlling the G_2/M checkpoint through ATM. Similarly, the translationally controlled tumor protein (TCTP) in *Drosophila* and in human cells binds directly to ATM after DNA damage and controls ATM affinity for its substrates, a finding that was also confirmed using kinase assays in vitro (179, 180). Ribosomal S6 kinase 2 (Rsk2) is phosphorylated by ATM, and it increases its autophosphorylation and augments its effects on the G_2/M checkpoint (181); however, the opposite effects have been reported in *Xenopus* egg extracts and human cells (182).

Lastly, several groups have shown that the activity of ATM is stimulated by the flavonoid compound resveratrol in human cell lines in culture, augmenting both ATM autophosphorylation and the phosphorylation of downstream targets (183–185). One study demonstrated that this process depends on the MRN complex, according to the lack of stimulation observed in NBS cells (185), although a recent study of resveratrol effects on ATM in both primary and transformed human cells suggests that ROS levels correlate well with the degree of ATM stimulation by resveratrol (186). In a purified protein system, resveratrol increases ATM activity in the presence of either MRN–DNA or hydrogen peroxide; a more dramatic effect (3.5-fold increase) is observed with hydrogen peroxide treatment. Resveratrol has been widely studied because of its effects on life span and metabolism in some organisms, and it clearly has many cellular targets, including sirtuins, cyclooxygenase-1, and AMP kinase (187). The stimulation of ATM by resveratrol under oxidizing conditions may be important for the effects of this molecule, but further study is clearly necessary to define this role.

It is not yet clear how so many factors interact functionally with ATM yet do not appear to share a common sequence or motif. Various sections of the HEAT repeat–containing N terminus have been implicated in some of these interactions; thus, a large protein such as ATM with many HEAT repeats may provide many binding surfaces. Considering that ATM is actively autoinhibited, fortuitous binding of proteins and/or small molecules to different surfaces of the inactive dimer might result in partial loss of inhibition. If these events occur in the presence of low levels of other activators such as the MRN complex and DNA, or in oxidation, they could activate the kinase through a combination of cooperative effects. Thus, as detailed in the many examples discussed above, no single activator of ATM is absolutely essential in all circumstances.

In summary, research during the past two decades has provided a rich set of data that illuminate many aspects of ATM regulation in eukaryotic cells. We now know that the MRN complex plays a central role in ATM activation, but that this role can be modulated by many factors and even replaced altogether through oxidation. Although we do not yet have atomic-level detail of the structure of ATM, information from other PIKK enzymes informs us that these proteins are likely more similar to canonical kinases than was previously thought, but also that they also share unique features with PI3K, including autoinhibition and a requirement for localization. It will be critical for future studies of ATM function to determine whether there are cellular targets of ATM that are specific to oxidation-mediated activation and, if so, what the consequences of these modifications are for the function of both neuronal cells and other cell types. Hopefully, these data and future studies of this enzyme will provide useful ideas for the treatment of the A-T disorder, as well as for cancers in which the *ATM* gene is disrupted.

DISCLOSURE STATEMENT

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