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REVIEW

Mechanisms of Non-canonical Activation of Ataxia Telangiectasia Mutated

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Abstract—ATM is a master regulator of the cellular response to DNA damage. The classical mechanism of ATM activation involves its monomerization in response to DNA double-strand breaks, resulting in ATM-dependent phosphorylation of more than a thousand substrates required for cell cycle progression, DNA repair, and apoptosis. Here, new experimental evidence for non-canonical mechanisms of ATM activation in response to stimuli distinct from DNA double-strand breaks is discussed. It includes cytoskeletal changes, chromatin modifications, RNA–DNA hybrids, and DNA single-strand breaks. Noncanonical ATM activation may be important for the pathology of the multisystemic disease Ataxia Telangiectasia.

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ATM (Ataxia Telangiectasia mutated, EC 2.7.11.1) is a serine/threonine protein kinase that belongs to a family of phosphatidylinositol 3-kinase related kinases (PIKK). The *ATM* gene encodes a 350-kDa protein consisting of 3056 amino acids. The domain structure of ATM includes HEAT repeats, FAT (FRAM/ATM/TRRAP), C-terminal FATC and kinase domains as described elsewhere [1]. ATR (ATM- and Rad3-related kinase) and DNA-PKcs (catalytic subunit of DNA-dependent protein kinase) display significant homology with ATM. ATM, ATR, and DNA-PKcs are all important for the cellular response to DNA damage, although specific functions of these kinases differ significantly [2].

The primary function of ATM is coordination of the cellular response to DNA damage caused by ionizing radiation. The mechanism of ATM kinase activation in response to ionizing radiation, initially proposed by Kastan's laboratory, involves intramolecular autophosphorylation of ATM at serine 1981 followed by its monomerization [3]. However, subsequent studies showed that many other amino acid residues undergo posttranslational modification. These include acetylation of lysine 3016 by the Tip60 histone acetyltransferase required for induction of kinase activity followed by autophosphorylation [4, 5], as well as autophosphorylation of serines 367, 1893, and 2996 and tyrosine 1885 [6-8]. These modifications are functionally important in human cell lines, but their relevance in mouse models and *Xenopus* extracts remains controversial [9, 10]. The canonical model for activation invokes a specific role for DNA double-strand breaks (DSBs), which are highly mutagenic DNA lesions induced by ionizing radiation. ATM activation requires the presence of the MRN (Mre11–Rad50–Nbs1) complex that ensures the initial localization of ATM in a complex with Tip60 at sites of DSB formation [4, 5, 11-14]. In response to ATM activation, a cascade of kinase activities leads to the phosphorylation of over a thousand substrates that are required for the coordination of various cellular processes such as chromatin remodeling, transcription and splicing, cell cycle progression, DNA repair, and apoptosis (for reviews see [15-17]).

It has also been proposed that ATM is activated following oxidative stress by a fundamentally different mechanism [18]. In this case, an active ATM dimer contains a disulfide bond formed upon oxidation of two cys-

Abbreviations: A-T, Ataxia Telangiectasia; ATM, Ataxia Telangiectasia mutated; ATR, ATM- and Rad3-related kinase; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; DSB, DNA double-strand break; MRN, Mre11-Rad50-Nbs1 complex; R-loop, RNA–DNA hybrid; SSB, DNA single-strand break; Top1, DNA topoisomerase I; Top1cc, Top1–DNA intermediate.

teine 2991 residues. In contrast to DSB-dependent activation of ATM in the nucleus, this oxidative-dependent ATM activation mechanism also occurs in the cytoplasm and is important for the coordination of insulin signaling and mitochondrial and peroxisome functions [19-21]. Consistent with these proposals, several cytoplasmic substrates of ATM have been identified by quantitative proteomics [22]. The mechanisms of ATM activation in response to DSBs and oxidative stress are discussed in detail in several comprehensive reviews [17, 23-26].

Given the functional importance of ATM in the cellular landscape, it is not surprising that inactivation of its function underpins the disease Ataxia Telangiectasia (A-T), also referred to as Louis–Bar syndrome [27-29]. A-T is a rare autosomal recessive multisystemic disorder (one case per 40,000-100,000) that develops in early childhood [30, 31]. A-T is characterized by immunodeficiency, progressive neurodegeneration, and increased predisposition to cancer. There are currently no treatments for A-T [32].

In recent years, the exclusivity of DNA double-strand breaks and oxidative stress as inducers of ATM activation has been questioned. In this review, new experimental data are discussed that indicate a wider cellular role for ATM as a sensor and regulator of the cellular response to DNA single-strand breaks, RNA–DNA hybrids, as well as changes in the structure of chromatin and the cytoskeleton.

NON-CANONICAL ACTIVATORS OF ATM

DNA lesions (except for DNA double-strand breaks). *DNA single-strand breaks*. More than 20 years ago, Tomas Lindahl (2015 Nobel Laureate in Chemistry) proposed that the number of DNA double-strand breaks arising due to the inherent instability of DNA is significantly lower (10-20 lesions per cell per day) than that of endogenous DNA single-strand breaks (15,000-20,000 lesions/cell/day) [33]. In addition, DNA single-strand breaks (SSBs) form as intermediates during the repair of damaged DNA bases (the so-called base excision repair pathway) [34]. The repair of SSBs is crucial for a cell as the replication of SSB-containing DNA leads to the formation of highly mutagenic DSBs [35]. Moreover, the transcription of SSB-containing DNA is inefficient and can be blocked [36, 37]. Defects in SSB repair have been linked to various diseases, including neurodegeneration and cancer [38-42].

Unrepaired SSBs activate ATM in the absence of DSBs [43]. This activation is important for promoting a G_1 cell cycle delay, thus providing time for the controlled repair of SSBs prior to the replication of DNA and therefore preventing the formation of replication-associated DSBs. In addition, ATM-dependent signaling is important for regulating the capacity of DNA base damage and SSB repair [44, 45]. Consequently, inadequate signaling of unrepaired SSBs in the absence of ATM leads to replication of the damaged DNA followed by accumulation of mutagenic DSBs, thus contributing to the genetic instability phenotype characteristic of A-T (Fig. 1).

A role for ATM in SSB signaling is consistent with the sensitivity of A-T cells to DNA-damaging agents that cause DNA base damage and SSB formation [46, 47]. However, the mechanism of ATM activation in response to SSBs – its dependence on the presence of MRN complex components or the oxidation of disulfide bonds, or the existence of an independent mechanism – is presently unknown. It also remains unclear whether SSB-dependent ATM activation contributes to ATM activation in response to ionizing radiation, a treatment that induces a significant number of SSBs and DNA base damage in addition to DSBs [48].

Covalent DNA topoisomerase I–DNA adducts. DNA topoisomerase I (Top1) catalyzes the relaxation of DNA supercoiling that is produced during DNA replication and transcription. The mechanism of relaxation involves formation of a Top1–DNA intermediate (Top1cc), cleavage of one DNA strand of DNA, followed by ligation [49]. Top1 inhibitors, such as camptothecin, stabilize Top1cc adducts and thus prevent DNA ligation, inducing transcription defects [50]. ATM is activated in response to treatment of quiescent (nonreplicating) human cells and post-mitotic mouse cortical neurons with camptothecin [51]. SSBs with a covalent link between the 3′-phosphoryl end and a tyrosine residue of the Top1 active site peptide have been suggested as possible inducers of ATM activity. Such unconventional SSBs form during partial proteasomal degradation of Top1 within camptothecin-stabilized Top1cc adducts. Consequently, the accumulation of Top1cc adducts and ATM autophosphorylation can be rescued upon inhibition of transcription and proteasomal degradation using DRB (5,6-dichloro-1-β-D-ribofuranosylbenzimidazole) and MG-132 proteasome, respectively. The formation of DSBs that could contribute to ATM activation was excluded using DNA Comet assays.

The mechanism of activation and signaling for activation will require further investigation, but it is possible that activation results from local changes in chromatin structure at sites of arrested transcription complexes [51]. It is also possible that ATM activation involves DNA single-strand breaks with a 3′-modification. Interestingly, endogenous Top1cc adducts aberrantly accumulate in the brain cells of $Atm^{-/-}$ mice and in human A-T cells [52]. This effect is independent of ATM kinase activity, and it is related to abrogated proteasomal degradation of Top1 adducts in the absence of ATM.

R-loops. More recently, R-loops, or RNA–DNA heteroduplexes [53], that form upon inhibition of Top1 activity during transcription [54], have been implicated in ATM activation. However, it remains controversial whether R-loops can activate ATM directly.

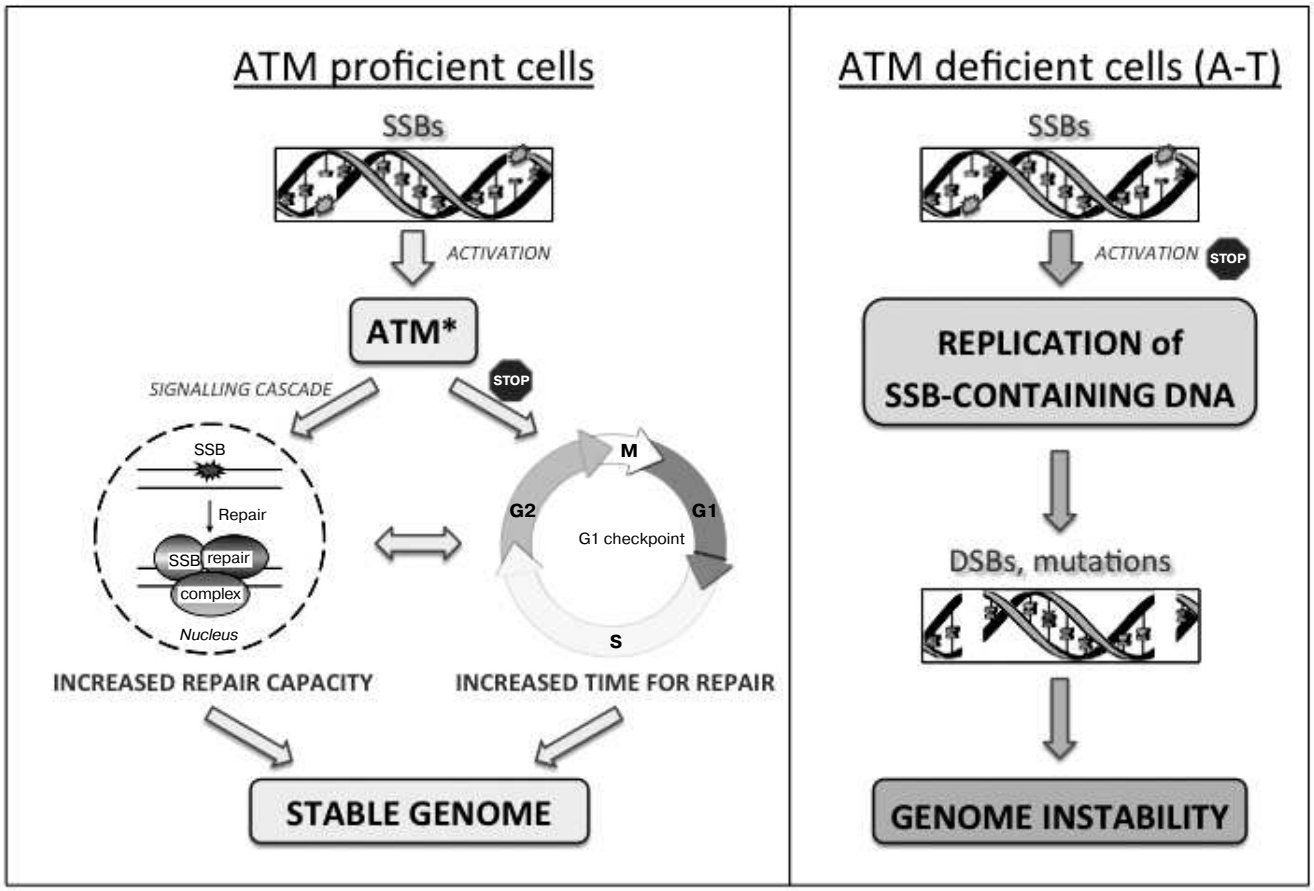

Fig. 1. ATM-dependent coordination of SSB repair. Activation of ATM in response to unrepaired SSBs coordinates their repair by increasing the efficiency of DNA repair and promoting a G_1 cell cycle delay that provides additional time for multiple rounds of repair. This results in coordinated and timely repair of SSBs prior to DNA replication, thus supporting the stability of the genome (left panel). In the absence of ATM (A-T), the detection of SSBs is abrogated, leading to replication of damaged DNA followed by the formation of DSBs and accumulation of mutations (right panel).

ATM is activated in non-replicating cells (primary lymphocytes, rat cortical neurons, and synchronized human primary fibroblasts) treated with camptothecin [55, 56]. However, in contrast to some observations [51], DSBs were detected by the accumulation of γH2AX and 53BP1 foci, which serve as DSB markers, and by neutral Comet assays. Both ATM activation and DSB formation were rescued upon inhibition of transcription or expression of RNase H1 that cleaves RNA within R-loops, indicating an indirect role for R-loops in this activation. It has been proposed that transcription-blocking Top1cc lesions lead to R-loop formation, which are then processed into DSBs that activate ATM [55, 57]. The mechanism by which R-loops can be converted into DSBs is unknown. However, it is thought that the transcription-coupled nucleotide excision repair endonucleases XPG and XPF-ERCC1 can cleave both DNA strands during resolution of R-loops, resulting in replication-independent DSBs [58]. Alternatively, SSBs with partially cleaved Top1 peptide at the 3′-end might act as precursors of replication-independent DSBs. These DSBs could result from a proximal SSB formed by repair of another Top1cc adduct, an endogenous DNA lesion, or an R-loop (cleavage of a single DNA strand during classical transcription-coupled nucleotide excision repair) [56]. It therefore appears that R-loops can mediate ATM activation indirectly, through the formation of replication-independent DSBs.

More recently, R-loops were proposed to be the primary inducers of ATM activity, which regulates alternative pre-mRNA splicing [59]. R-loop formation and ATM activation have been observed in UV-treated non-replicating human skin fibroblasts, and ATM activation can be rescued by the treatment of cells with inhibitors of transcription elongation and overexpression of RNase H1. Importantly and in contrast to [55, 56], ATM activation occurs in the absence of DSBs, as determined by the absence of γH2AX and 53BP1 focus formation. The role for R-loops in ATM activation and its mechanism require further investigation [59].

Structural changes. *Changes in chromatin structure.* DSBs are known to initiate significant changes in the

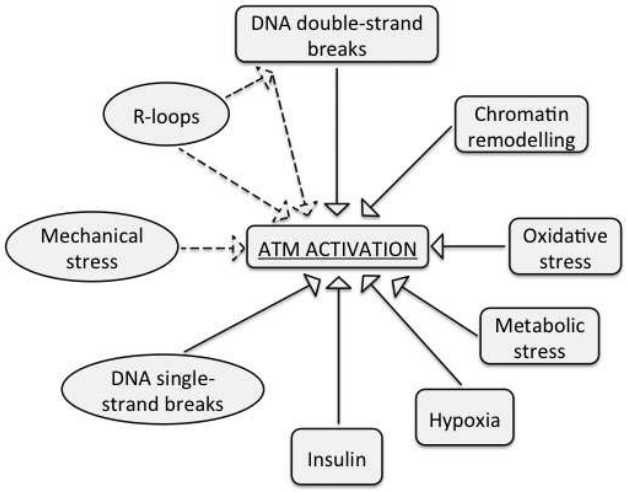

Fig. 2. Inducers of ATM activity. Non-canonical activators that are discussed in this review are designated with ovals.

structure of chromatin (see [60] for review), and it is possible that chromatin remodeling is a direct inducer of ATM activation in response to ionizing radiation [3]. Indeed, the importance of chromatin remodeling has been demonstrated by the activation of ATM in the absence of DSBs. This was shown by treatment of human cells in hypotonic solution with chloroquine, using inhibitors of histone deacetylases that promote chromatin decompaction and a siRNA-mediated knockdown of the heterochromatin protein 1α [3, 61]. MRN-independent activation of ATM under hypotonic stress conditions was found to be dependent on the interaction of ATM with the ATMIN protein, whereas ATM activation in response to irradiation is ATMIN-independent [62]. Further details of a role for chromatin remodeling in ATM activation is discussed elsewhere [63].

Mechanical stress. The activity of ATR kinase, a close homolog of ATM, is canonically induced in the presence of nucleofilaments of single-stranded DNA and the replicative protein A induced in response to mechanical stress [64]. The localization of ATR to the nuclear envelope, together with the induction of its kinase activity, was observed in human cells under hypertonic conditions that induce osmotic shock and mechanical (membrane) stress in the absence of DSBs. Similar results were obtained upon mechanical stretching of cells and on cell compression, within the physiological range of mechanical forces, using a compressive-load system. The mechanism of ATR activation in response to mechanical stress requires further investigation, although it appears to be distinct from the canonical mode of activation. The activation of ATR is important for regulation of the plasticity of the nuclear envelope and the association of chromatin with the nuclear envelope [64]. It remains to be established whether mechanical stress plays a role in ATM activation.

Activation of ATM in the absence of DSB sensors. Two independent protein complexes are important for the detection of DSBs in eukaryotes [65]; DSB recognition by the MRN-complex results in the activation of ATM, whereas the Ku complex (Ku70–Ku80) promotes the induction of DNA-PKcs activity, leading to the repair of DSBs via nonhomologous end-joining (NHEJ) [66]. In recent elegant work [67], activated DNA-PKcs was shown to functionally substitute for ATM in the absence of MRN in mouse embryonic fibroblasts, whereas MRN-dependent ATM activation was observed in the absence of Ku. Unexpectedly, ATM-dependent phosphorylation of histone H2AX, as well as a G_2M cell cycle delay in response to ionizing radiation, were observed in cells deficient in both DSB sensors. The mechanism of such MRN-independent ATM activation remains unclear [67].

ETIOLOGY OF A-T

The clinical symptoms of A-T that relate to the functions of ATM in the coordination of the cellular response to DSBs include immunodeficiency, sensitivity to ionizing radiation (radiosensitivity) and other DNA damaging agents, and an increased risk of tumorigenesis [68, 69]. In addition, A-T patients present with progressive neurodegeneration, including atrophy of the spinal cord, cerebellum, and brain stem, coupled with the loss of Purkinje cells, as well as granular neurons and cells of the molecular layer [70-72], and ataxia.

The neurodegenerative phenotype of A-T is likely to be multifactorial in accordance with the variety of cellular functions of ATM, and the reasons for neurodegeneration are yet to be established. Our progress in understanding the molecular basis underlying A-T has been limited due to the absence of a good animal model for the disease – the progressive neurodegeneration phenotype that is observed in $Atm^{-/-}$ mice is rather mild compared to that in humans [73-75]. Interestingly, this mild phenotype is partly rescued using antioxidants, indicating a role for oxidative stress [76, 77]. Increased levels of oxidative stress are also detected in A-T patient cell lines [78, 79]. It is worth noting that no increase in the levels of R-loops has been observed in brain tissues of $Atm^{-/-}$ mice, rejecting a role for RNA–DNA heteroduplexes in the etiology of the disease [80]. The neurodegenerative pathology of A-T can also be linked to defects in the elimination of cells containing unrepaired DSBs during development of the neural system [81]. Moreover, the novel role for ATM in coordination of the repair of SSBs indicates a possible contribution of transcription-inhibiting SSBs to the etiology of ataxia telangiectasia.

The experimental data demonstrate that ATM can be activated by a few non-canonical inducers (i.e. distinct

from DNA double-strand breaks). These include DNA single-strand breaks, RNA–DNA hybrids, and changes in chromatin structure (Fig. 2). However, despite our progress in understanding functions of the ATM kinase, it is yet to be established, whether there exists a universal inducer of ATM activation, such as, for example, DNA lesions or chromatin remodeling. In addition, whether ATM gets activated in response to various discrete types of stress and the mechanisms of its activation are yet to be studied.

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