

ATM protein kinase: the linchpin of cellular defenses to stress

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Abstract ATM is the most significant molecule involved in monitoring the genomic integrity of the cell. Any damage done to DNA relentlessly challenges the cellular machinery involved in recognition, processing and repair of these insults. ATM kinase is activated early to detect and signal lesions in DNA, arrest the cell cycle, establish DNA repair signaling and faithfully restore the damaged chromatin. ATM activation plays an important role as a barrier to tumorigenesis, metabolic syndrome and neurodegeneration. Therefore, studies of ATM-dependent DNA damage signaling pathways hold promise for treatment of a variety of debilitating diseases through the development of new therapeutics capable of modulating cellular responses to stress. In this review, we have tried to untangle the complex web of ATM signaling pathways with the purpose of pinpointing multiple roles of ATM underlying the complex phenotypes observed in AT patients.

Keywords ATM kinase · DNA damage response · Signaling · Carcinogenesis

Abbreviations

A-T	Ataxia-telangiectasia
ATM	Ataxia-telangiectasia, mutated protein
DNA Dsb	DNA double-strand breaks
MRN	Mre11/Rad50/Nbs1 complex
HR	Homologous recombination
NHEJ	Non-homologous end-joining
ROS	Reactive oxygen species
HAT	Histone acetyltransferase

Introduction

Ataxia Telangiectasia (A-T) is a rare autosomal-recessive neurological disorder associated with progressive degeneration in the cerebellum. A-T is a multisystem syndrome that is characterized by immunodeficiency, cancer predisposition, radiosensitivity, insulin-resistant diabetes and premature aging [1, 2]. About 20% patients with A-T develop cancer, especially acute lymphocytic leukemia (ALL) or lymphoma. The defective gene in A-T was identified in 1995 by positional cloning strategy [3] and designated *ATM* (Ataxia telangiectasia mutated). The *ATM* gene encodes the ATM protein, a protein kinase that has a fundamental role in regulating cellular responses to DNA damage through the phosphorylation of proteins involved in the maintenance of cell cycle checkpoints, DNA damage repair and telomere maintenance [2].

Numerous recent reviews e.g., [2, 4–6] cover different aspects of A-T, ATM biochemistry and the role of ATM in the DNA damage response. We have attempted in this review to summarize recent findings on various signaling pathways and stimuli leading to ATM kinase activation. In particular, we have given special emphasis to how ATM

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activation is related to the pathophysiology of disease and carcinogenesis.

ATM, ATR, and DNA-PK: the PIKK family of protein kinases

ATM belongs to a superfamily of phosphatidylinositol (PI) 3-kinase-like kinases (PIKK), which are grouped together on the basis of similarities of a conserved kinase domain in their C-terminus [7]. This group includes ATM, ATR (ataxia-telangiectasia and Rad3-related), DNA-PKcs (DNA-dependent protein kinase catalytic subunit), SMG-1 (Suppressor of mutagenesis in genitalia), mTOR (mammalian target of rapamycin) and TRRAP (transformation/transcription domain-associated protein; contains a divergent, inactive kinase domain) proteins.

ATM, ATR and DNA-PKcs share a high degree of sequence similarity in their C-terminal region, containing kinase, FAT (conserved sequence in FRAP, ATM and TRRAP) and FATC (FAT-C-terminal) domains, with the FAT and FATC domains separated by a kinase domain [8]. FATC is a unique sequence region, spanning approximately 33 aa in the extreme C-terminus of PIKK family proteins and is only found in combination with the FAT domain (Fig. 1). The tandem occurrence of the FATC and FAT domains in the area surrounding the kinase domain suggests functional significance for the regulation of the protein kinase activity.

Structure and activity

Rivera-Calzada et al. (2005) [9] used cryoelectron microscopy to generate a 3-D model showing the overall architecture of DNA-PKcs. The proposed structure showed the kinase domain located in a “head-like” compact region from which the FATC domain projected as a narrow “stalk-like” structure. There was an obvious similarity between this predicted DNA-PKcs structure and that of ATM, which had earlier been reported by the same authors with the catalytic site of the kinase located on the outside surface of the “head” structure [10]. In this model, a sterically hindered catalytic site is restricted to autophosphorylation in “trans” and regulated by inter- and intra-molecular interactions with FAT and FATC domains. Interestingly, the FATC and FAT domains were predicted to work as a “sensor”, which directly activates the catalytic kinase domain upon conformational changes induced during DNA binding [9].

However, the recent determination of 6.6 Å X-ray structure of DNA-PKcs can only support some of the conclusions reached by analysis of low resolution cryoelectron microscopy data [11]. HEAT repeats in the N-terminus of DNA-PKcs form a ring-like structure with a “head or crown-like” structure on top of it. In this model, the “crown” contains the FAT, kinase and FATC domains whereas the ring-like structure has a cavity that can accommodate the ends of a DNA molecule. Autophosphorylation upon DNA binding is predicted to elicit large

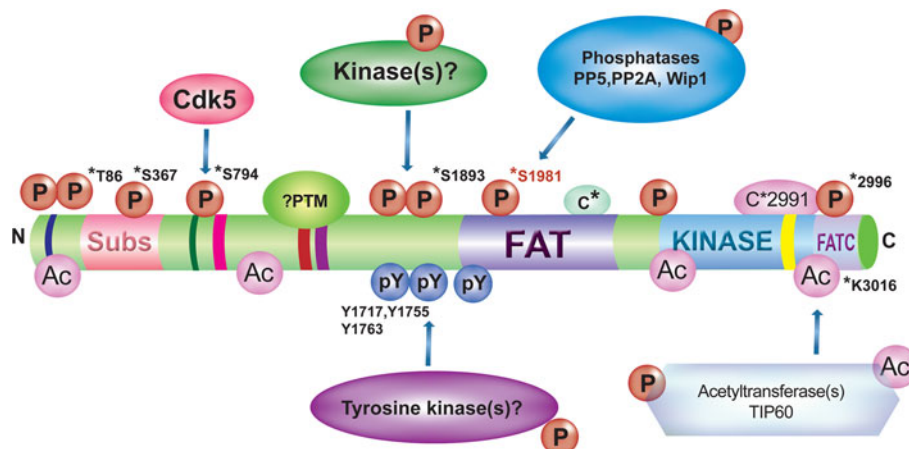


Fig. 1 ATM kinase structure and post-translational modifications. A schematic representation of ATM protein structure. Kinase, FAT, FATC, and PRD (yellow) domains are shown in the C-terminus; HEAT (Huntingtin, Elongation factor 3, alpha subunit of PP2A and TOR1), TPR (tetratricopeptide), ARM (Armadillo), and PFT (protein farnesyl transferase) repeats are shown in the N-terminus schematically in dark green and magenta. Protein interaction motifs of ATM are also shown: TAN (Tel1/ATM N-terminal) motif (dark blue), N-terminal substrate-binding site (Subs); putative leucine zipper (red)

and proline-rich motif (dark purple) (see text for details); An emerging complexity in post-translational modifications of ATM is demonstrated. ATM autophosphorylation and phosphorylation (P), Acetylation (Ac), and cysteine modification (C*) sites are shown. All these sites play a role in ATM kinase activation. The exact number of phosphotyrosine (pY) sites or identities of tyrosine kinases capable to phosphorylate these sites in ATM remain elusive at present (see text). Possible, but yet unidentified post-translational modifications (?PTM) of ATM kinase are also shown

conformational changes in the DNA-PK molecule and eventually release DNA-PK from DNA double-strand breaks, where it is recruited through interaction with Ku70/80. It remains to be seen if the DNA-PK structure can be used in modeling experiments to gain insights into the regulation of ATM kinase activity by phosphorylation. Recent preliminary high-resolution cryoelectron microscopy structure of ATM reported by the Gatti group (Ataxia-Telangiectasia workshop 2010) will help to compare it with previously predicted 3-D structure of ATM [10] and with the DNA-PKcs crystal structure. Resolving a detailed X-ray structure of ATM kinase still presents an enormous technical challenge but undoubtedly will advance our understanding of the precise mechanism of ATM kinase regulation.

There is accumulating evidence that both FAT and FATC domains are involved in the regulation of the kinase activity of PIKKs (for review see [5]). FATC domains are sensitive to mutagenesis and contain conserved residues critical for kinase activity. Mutation of the conserved W2545 residue in the mammalian TOR (mTOR/RAFT1/FRAP) FATC domain abolishes its kinase activity, suggesting that a functional FATC domain is required for kinase activation [12]. Curiously, however, exchanging the FATC domain of mTOR to that of ATM still resulted in inactive kinase [12]. Similar results were obtained with the replacement of ATR FATC domain with the ATM FATC sequence [13]. On the other hand, replacing ATM FATC domain with FATC domains of ATR, DNA-PKcs or TRRAP did not interfere with its function [14].

In addition, the extreme C-terminus of ATM is also targeted by post-translational modifications (Fig. 1). DNA damage induces the rapid binding of the Tip60 histone acetyltransferase (HAT) to the ATM FATC domain. Tip60 HAT causes the acetylation of the K3016 residue in ATM, which precedes its autophosphorylation [15]. An ATM mutant that cannot be acetylated on K3016 was defective in activation, subsequent autophosphorylation and dimer-monomer transition, implying that acetylation has a predominant role in activating ATM in response to DNA damage. However, it is not yet entirely clear how C-terminal ATM phosphorylation sites influence the regulation of ATM activation [16, 17].

It should be noted that regulation of “classical” PI-3 kinase activities involves a phosphoinositol substrates interaction segment located between conserved kinase subdomains VII (DFG) and VIII (APE) [18]. This area in PI-3 kinases serves as a functional analog of the activation loop of protein kinases. The activation loop (T-loop) in classical protein kinases is a flexible region located between conserved DFG and APE residues. Phosphorylation of conserved S/T/Y residues in this loop leads to kinase activation. Interestingly, DNA damage signaling

PIKKs do not exhibit any homology to the polybasic stretch found in the activation loop of PI-3 kinases. Substitution of this segment with corresponding sequences from mTOR or FRAP kinases completely eliminates lipid kinase activity, while retaining protein kinase activity of PI-3 kinase [5, 18]. Interestingly, all mutated proteins demonstrated autophosphorylation activity. The radiation-induced autophosphorylation site T3950 found in DNA-PK is located in the area functionally resembling the activation loop of typical protein kinases and is important for the regulation of DNA-PK activity [19]. So far, no similar (auto)phosphorylation site has been found in ATM kinase. However, using the conserved phosphorylation motif S/TQ preferred by ATM, it is possible to predict interesting potential sites including S2761 (VPLSQRSGV), located within the ATM kinase domain between two conserved kinase signatures.

PIKK at the DNA damage sites—interacting partners

It seems that all PIKKs utilize conserved modes of interaction and recruitment to the sites of DNA damage via corresponding adaptors [20, 21]. Therefore, a common theme emerging for members of the PIKK family is the requirement for an activator protein(s) or nucleic acid/chromatin complexes for regulation. Indeed, ATM, ATR, and DNA-PKcs are recruited to sites of DNA damage via their respective “targeting” subunits NBS1, ATRIP (ATR-interacting protein), and Ku 70/80, respectively [20].

In addition to the requirement of ATRIP for ATR activation, a secondary activator protein Topoisomerase DNA II binding protein 1 (TOPBP1) is also required. Following DNA damage, TOPBP1 protein localizes at IR-induced nuclear foci and is phosphorylated by ATM and ATR. In order to facilitate full activation of ATR, TOPBP1 binds to the conserved PRD (PIKK Regulatory Domain) domain, a motif located in the ATR C-terminal region between the kinase and FATC domains [13, 22]. Unlike the kinase and FATC domains, this region is not conserved between different PIKKs and varies in length from 16 to 82 aa. It was hypothesized that the PRD domain serves as a binding platform for secondary activators in all PIKK members, including ATM [13, 23]. Such activator protein(s) would help to reconcile current discrepancies in ATM kinase activation models as discussed later in the “ATM activation” section. A number of ATM-interacting proteins can modulate kinase activity in addition to members of the MRN complex, e.g., ATMIN/ASCIZ [24, 25], Aven [26], hSSB1 [27], PP2A [28], HINT1 [29] and Tel2-regulator of PIKK stability [30, 31]. It is likely that many more ATM modulators have already been indirectly identified in massive siRNA screens using ATM-mediated phosphorylation of H2AX [32] or 53BP1 [33] as readouts.

The interaction between Tel2 and ATM has been the focus of recent research, with the mechanism of its influence on the stability of PIKKs clarified [34–36]. Constitutive phosphorylation of Tel2 by casein kinase-2 is important for its interaction with the R2TP/prefoldin-like complex [35]. The Tel2-R2TP/prefoldin-like complex has chaperon/prefoldin activities important for PIKK protein complex assembly. Tel2 acts together with Hsp90 chaperone to bind newly synthesized PIKKs [34], where Hsp90 modulates the ability of Tel2 to bind PIKKs. In addition to its interaction with Hsp90, Tel2 is also able to bind with the Tti1-Tti2 (Tel2-interacting proteins) complex [34, 36, 37]. Several distinct Tti1-Tti2 complexes have been identified, including the large 2MDa “Triple-T” complex with Tel2 [36]. The Tti1-Tti2 complex plays a role in the regulation of ATM/ATR-dependent S- and G2/M cell cycle checkpoints [34, 36]. Tti1 was found to interact with all members of the PIKK family and to influence the assembly of mTOR kinase complexes [37].

ATR has other modulators of its activity, including Cdc5L (cell division cycle 5-like protein), a subunit of the putative E3 ubiquitin ligase complex containing Prp19/Pso4, Plrg1 and Spf27 [38]. Cdc5L was found to regulate the activation of downstream effectors in the ATR signaling pathway, CHK1, RAD17 and FANCD2. Another component of chromatin remodeling complexes, MTA1 (metastasis-associated protein 1), was recently shown to modulate ATR signaling [39] since depletion of MTA1 attenuated ATR mediated activation of CHK1. Earlier studies demonstrated the isolation of complexes containing ATR and other components of the nucleosome remodeling and deacetylase complex (NuRD) containing CHD4 (chromodomain-helicase-DNA-binding protein 4), HDAC2, HDAC1, MTA1, MTA2 [40].

Yet another unexpected level of ATM kinase activity regulation has been discovered recently [41]. ATM has been implicated in the cellular response to oxidative stress for some time (see review [42, 43]), however, the exact mechanism of the ATM response to redox changes remained elusive. Interestingly, the PI-3 kinase family member TOR1 (Target of rapamycin 1) was shown to be regulated via reactive cysteine residues forming a disulphide bond in a highly conserved FATC domain [44]. However, these cysteines are not conserved in other PIKK family members. Recently, the Paull lab used an advanced mass-spectrometry screen to identify modified cysteine residues in ATM after treatment with hydrogen peroxide [41], which resulted in the identification of a functionally important cysteine residue in the FATC domain of ATM (Fig. 1). Importantly, this study has suggested a new mechanism of ATM activity regulation, independent of its DNA-damage signaling role (see “Oxidative stress and ATM cytoplasmic signaling” section for further discussion).

Role of the N-terminus

Moving away from the C-terminus of PIKK, the N-terminal regions of these kinases are less conserved and have similarity signatures of interspersed HEAT repeats (from Huntingtin, Elongation factor 3, alpha subunit of PP2A and TOR1) [45]. Intriguingly, when HEAT repeats within ATM, ATR and DNA-PK were subjected to detailed bioinformatics analysis by the Blundell group [46], several repeats were re-classified as TPR (tetratricopeptide), ARM (Armadillo) and PFT (protein farnesyl transferase) repeats. It is interesting to note that protein phosphatase 5 (PP5), one of the phosphatases involved in the regulation of ATM, ATR and DNA-PKs kinase activities contains a TPR regulatory domain that can be activated by lipids [47]. Moreover, the region of DNA-PKs containing the T2609 autophosphorylation site interacts with the PP5 TPR domain, suggesting that TPR domain interactions might be regulated by phosphorylation. T2609 in DNAPKs can also be targeted by the ATM and ATR kinases [48, 49]. It would be of considerable interest to look at the possible role of TPR repeats in the inter- and intramolecular interactions with ATM kinase (auto)phosphorylation sites.

Important interaction surfaces are located in the N-terminus of ATM: these are the NBS1 interaction motif [20, 21], N-terminal substrate-binding site [50, 51], “chromatin-association” domain and nuclear localization signal [52] and TAN (Tel1/ATM N-terminal) motif [53] (Fig. 1). In the *Xenopus* system, the NBS1-ATM interaction is essential for ATM activation. ATM autophosphorylation is secondary to the recruitment of unphosphorylated ATM to the sites of DNA DSBs [21]. You et al. (2005) suggested a mechanism where MRN complexes in the vicinity of DNA DSB support signal amplification (i.e., phosphorylation of ATM substrates) by successive recruitment, activation and release of ATM kinase, where the release rate of active ATM could be influenced by autophosphorylation. Data on the dynamics of multiple ATM autophosphorylation sites [54] and behavior of active forms of ATM kinase on chromatin breaks support this idea [55]. Interestingly, Tel2 (hCLK2), a regulator of ATM stability also binds to the HEAT repeat area in ATM (aa 830-1290) as well as the C-terminus of ATM (2,680–3,056) [30].

As shown by the Price group [51], the N-terminal substrate interaction domain resides within amino acids 81–106 of ATM, with important functional residues located between amino acids 91–97. A similar area of interaction (amino acids 82–89) in the ATM N-terminus was also mapped earlier [56]. Interestingly, ATM with mutations between residues 91–97 (“ATM90”) had normal kinase activity and was autophosphorylated on S1981 after DNA damage [51]. It is not clear at the moment what the relationship is between the “chromatin-association” domain of

ATM (amino acids 5–224) and its N-terminal substrate-binding domain [52]. It is also puzzling how ATM kinase devoid of N-terminal domains (1,300–3,060) can initiate DNA damage signaling when artificially tethered to chromatin [57] (see section “ATM and chromatin”).

Another motif, designated as the TAN motif is located in the first 30 amino acids of Tel1/ATM proteins and contains a conserved (L/V/I)XXX(R/K)XX(E/D)RXXX(L/V/I) signature [53]. The TAN motif plays a role in telomere length maintenance and in the recruitment of Tel1 (and presumably ATM) to the DNA DSBs but the exact mechanism remains unknown since deletion of the motif does not reduce Tel1 expression or nuclear localization, neither does it affect Tel2-Tel1 or Xrs2 (homologue of Nbs1 in yeast)-Tel1 interactions.

Interestingly, manipulation of the ATM N-terminus by addition of the ATRIP-binding domain from ATR creates a “chimeric” ATM kinase able to bind ATRIP and localize to ssDNA structures containing RPA, including stalled replication forks. This “chimeric” ATM protein retained autophosphorylation activity, but displayed defects in checkpoint signaling and correction of the A-T cellular phenotype [58]. In addition to the presence of a number of conserved regulatory domains, the N-terminal area of ATM is subjected to a number of post-translational modifications, including an autophosphorylation site (S367), a possible acetylation site as well as a site for non-covalent interaction with poly(ADP)ribose (PAR) (Fig. 1). It is possible that the putative S367 autophosphorylation site is directly involved in PP2A binding regulation, given that phosphatase PP2A was shown to interact with several regions of ATM including aa 250–522 [28]. Haince et al. (2007) [59] demonstrated the existence of a specific PAR-binding domain spanning amino acids 99–120 of ATM. They noted that this domain overlaps with the N-terminal substrate-binding site of ATM [51] as well as the putative “chromatin-binding” domain [52] suggesting that PAR modification of ATM plays a role in the recruitment and optimal activation of ATM kinase at the sites of DNA DSBs. Consistent with this, PAR was shown to activate ATM in vitro [60]. Recently, the role of poly(ADP)ribose polymerase (PARP) in the recognition and signaling of DNA breaks was strengthened by the observation that PAR synthesis facilitated formation of dynamic signaling complexes of ATM, IKK γ (NEMO) and PIASy-, SUMO-1 ligase [61]. Both PIASy and ATM act in a concerted fashion to SUMOylate and phosphorylate NEMO (IKK γ), which leads to the activation of I κ B kinase complexes and NF- κ B apoptotic response [62, 63]. Direct interaction of ATM and PARP-1 has also been demonstrated [64] and covalent modification of ATM by poly (ADP) ribose was suggested to be necessary for optimal kinase activation.

In summary, complex arrays of post-translational modifications, targeting subunits, activator proteins and dynamic protein complexes are able to regulate the initial response of PIKKs to DNA damage and stress.

MRN complex: the intimate link to PIKKs

The MRN complex is an evolutionarily conserved protein complex composed of MRE11, RAD50, and NBS1 (XRS2 in yeast). Mutations in NBS1 and MRE11 lead to Nijmegen breakage syndrome (NBS) and Ataxia-Telangiectasia-Like Disorder (A-TLD), respectively [65, 66]. Recently, a single patient with mutated unstable Rad50 protein was described and classified as having NBS-like disorder (NBSLD) [67]. NBS and A-TLD have related symptoms to A-T and show defective DNA damage-induced activation of ATM [65, 66, 68] (discussed further in the following section).

The MRN complex

MRE11 is a 708-amino acid DNA-binding protein with single-strand endonuclease and double-strand-specific 3′–5′ exonuclease activities [69]. RAD50 is a 1,312-amino acid protein that contains a bipartite ATP-binding cassette (ABC) ATPase domain characteristic of the ABC-ATPase superfamily of transporters [70] (Fig. 2a). Like other ABC transporters, RAD50 exhibits adenylate kinase activity [71]. The two ABC segments are located at the amino- and carboxy-terminal ends of the protein and contain Walker A and B nucleotide (NTP)-binding motifs, respectively. The N- and C-terminal ABC segments are separated by two long heptad-repeat regions, which fold into an antiparallel, intramolecular coiled-coil structure. The hinge region at the apex of the coiled-coil includes a highly conserved Cys-X-X-Cys (CXXC) “hook” motif that functions as a dimerization domain to allow interactions between MRN complexes bound to different DNA molecules [70]. NBS1 is a 754-amino acid protein containing the FHA and BRCT phosphopeptide interacting domains at its N-terminus and non-overlapping MRE11- and ATM-binding domains at its C-terminus [20, 72] (Fig. 2a). The MRN complex is a key regulator of the response to DSBs and is involved in many aspects of DNA end metabolism, including DSB sensing, processing and repair by homologous recombination as well as the activation of ATM [73, 74], but also involved in telomere maintenance and meiotic recombination.

Architecture of the MRN complex at the damage site

The MRN complex binds to the DNA DSB via MRE11 and RAD50 [74]. In particular, the conserved ATPase domain of RAD50 is important for enzymatic activities and dimer

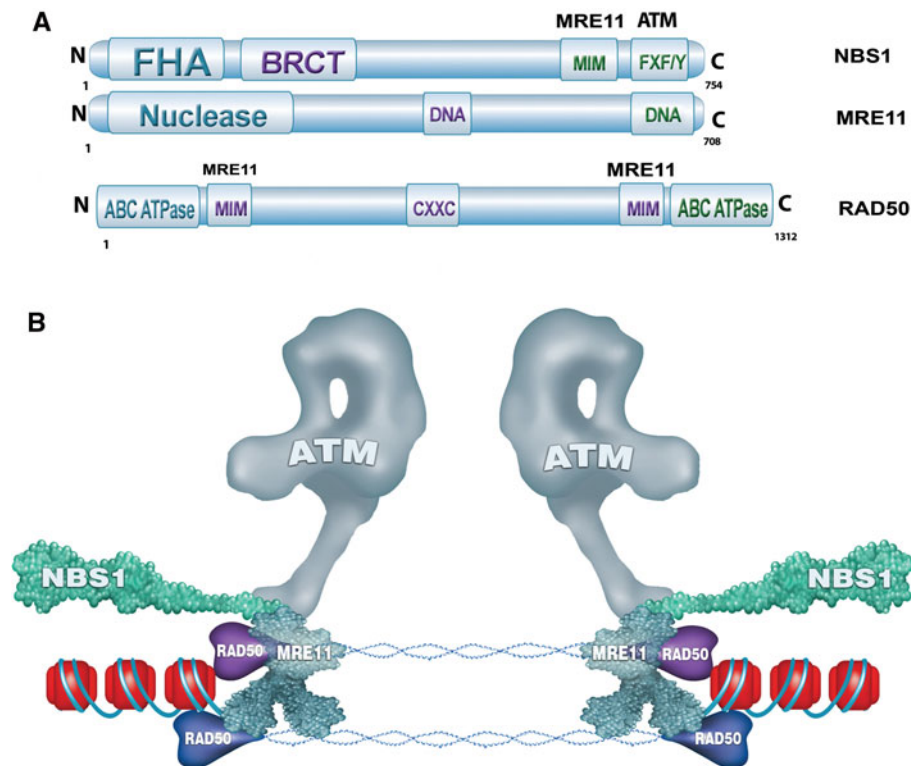


Fig. 2 MRN complex and recognition of DNA double-strand break. **a** Schematic representation of the domain structures of MRE11, RAD50 and NBS1. FHA (fork-head associated) and BRCT (BRCA1 C-terminal) domains are shown in the Nbs1 N-terminus; MRE11-interacting motifs (MIM) and ATM-interacting domains (FXY/Y) are located at the C-terminus. MRE11 has N-terminal nuclease domain and two DNA-binding domains. NBS1-interacting motif is also located in the N-terminus. In RAD50, two Walker A and B motifs (ABC ATPase) are shown at the N- and C-termini. Adjacent MRE11-interacting motifs (MIM) are also depicted. The rest of the molecule is long coiled-coil domains containing zinc-hook motif (CXXC) in

the middle. **b** Positioning of MRN–ATM complex at the site of DNA break. MRN complex recognizes DNA breaks in chromatin via DNA-binding motifs in MRE11 and RAD50. DNA-end tethering activity of MRN complex is mediated by RAD50 coiled coil “hooks” (see text). NBS1 is important for recruitment of ATM kinase to the site of the break through its C-terminal ATM-interacting motif. In the absence of determined ATM X-ray structure, the overall geometry of the complex at the site of the break remains hypothetical. ATM activation is possible in the absence of MRN complex (see text), however, molecular mechanisms of this process remain to be elucidated

formation as shown by studies of the RAD50 protein with a mutation (S793R) in the conserved domain signature [75]. Importantly, the recently discovered adenylate kinase activity of RAD50 is also important for MRE11/RAD50-dependent tethering of DNA ends [71]. It seems that at least in vitro stable complexes of RAD50 and NBS1 can be formed that retain DNA-binding and DNA bridging activities [76]. Scanning force microscopy and electron microscopy studies have shown that the MRN complex has a DNA-binding globular head and two long protruding arms [73, 77]. The globular head consists of an MRE11 dimer and the ABC-ATPase domain of RAD50 (Fig. 2b). Each 60-nm-long arm that protrudes from the globular head is constituted by an antiparallel coiled-coil formed by the intramolecular folding of the two long heptad-repeat regions of a RAD50 molecule. The Cys-X-X-Cys motif in the hinge region at the apex of each coiled-coil domain forms a two-cysteine molecular hook, which requires the binding of Zn²⁺ ions [73, 78, 79]. The flexibility of the

coiled-coils allows the hook-containing apices to adopt different orientations, providing the basis for MRN DNA-end tethering activity.

Recent studies revealing the structure of NBS1 and MRE11 domains have shed light at the organization of the MRN complex at the DNA DSBs [80, 81]. NBS1 adopts an elongated conformation where the N-terminal FHA-BRCT tandem domains are linked to the C-terminal motifs interacting with MRE11 and ATM (Fig. 2b). Binding of phosphorylated CtIP to the FHA domain of NBS1 links it to the MRE11/RAD50 heterotetramer, juxtaposing DNA DSBs. Therefore, the MRN complex emerges as a central and initial coordinator of DNA repair and checkpoint signaling activities at the sites of DNA breaks.

ATM–MRN pathway

In vitro experiments performed with purified components (ATM, MRN and linear dsDNA molecules) suggest that

MRN recognizes DSBs in an ATM-independent manner. Formation of the MRN–DNA complex is responsible for the recruitment of ATM to the break site and for its subsequent activation (see the section on “[Role of adaptors in ATM activation](#)” for further discussion). Elucidation of the contributions of MRN complex components toward ATM signaling is complicated by the fact that probably all members of the MRN complex serve as substrates for ATM or other PIKKs (Fig. 3). These phosphorylations are important for downstream signaling. ATM-dependent phosphorylation of NBS1 at S343 activates the S-phase cell cycle checkpoint and increases cell survival post-irradiation [82]. The ATM-dependent phosphorylation of S278 and S343 on NBS1 has been shown to facilitate the phosphorylation of SMC1 [83]. The NBS1 phosphorylation by ATM is important for CHK2 activation and for establishing the S-phase checkpoint [84, 85]. It was shown that RAD50 and MRE11 are also substrates for ATM kinase [86–91]. However, there is no conclusive evidence that MRE11 and RAD50 phosphorylations are functionally significant. It has been hypothesized that the phosphorylation of MRE11 and RAD50 may also play adaptor roles in ATM mediated phosphorylation of downstream substrates [2].

As is the case with ATM, multiple post-translational modifications are involved in the regulation of MRN activities. NBS1 was found to be acetylated by p300 and CBP HATs [92]. Importantly, SIRT1 deacetylase interacts with the complex and keeps NBS1 in a hypoacetylated state, which is a prerequisite for ATM-dependent phosphorylation of S343 on NBS1. MRE11 was also found to be acetylated [92]. This acetylation can be mediated by TIP60 [93], since a complex of MRN with another member of PIKK family—TRRAP—is devoid of any histone acetyltransferase (HAT) activity [94]. The MRN–TRRAP complex plays a role in non-homologous end-joining (NHEJ), while TRRAP–TIP60 is important for homologous recombination (HR) and also gets recruited to chromatin surrounding DNA DSBs [95]. A recent study has shed light on the mechanism by which the MRN complex mediates activation of TIP60, a regulator of ATM kinase, in response to DNA damage [96]. The MRN complex was found to target TIP60 to histone H3 trimethylated on K9 (H3K9me3) at sites of DNA breaks. The TIP60–H3K9me3 interaction serves as a signal for activation of acetyltransferase activity of TIP60. Curiously, accumulation of H3K9me3 in heterochromatin is controlled by SUV39H1 (suppressor of variegation 3–9

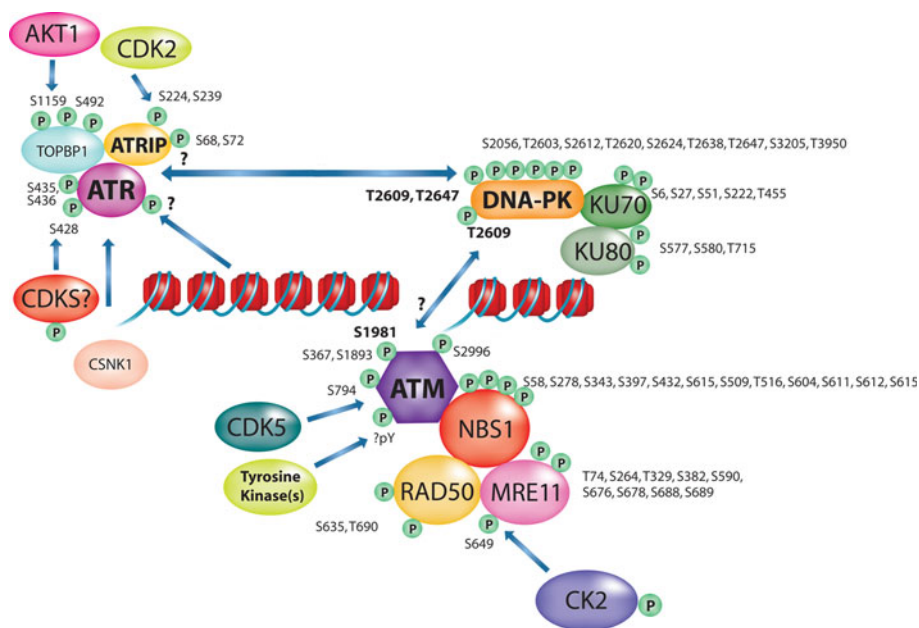


Fig. 3 Cross-talk between ATM, ATR, and DNA-PK kinases at the sites of DNA double-strand breaks. Identified phosphorylation sites of phosphatidylinositol (PI) 3-kinase-like kinases (PIKKs) and their targeting subunits are shown. Functional significance of many phosphorylation sites is not known. It is not yet clear if all trans-phosphorylation signaling events exist between ATM, ATR, and DNA-PK kinases [49, 152] (see text). Activity of individual PIKK

complexes can be modulated by signaling inputs from other kinases [155, 157] (e.g., CDKs and CK2). While ATM-dependent phosphorylation of MRN complex occurs in vivo [82, 87], phosphorylation of Ku70/80 by DNA-PK can be demonstrated in vitro, but does not have functional significance in vivo. ATR-dependent ATRIP phosphorylation was demonstrated both in vitro and in vivo, however, it does not play a role in DNA damage signaling

homologue 1) methyltransferase, which in turn is modulated by SIRT1-dependent deacetylation [97]. MRE11 can also be methylated at arginines by protein arginine methyltransferase 1 (PRMT1), which impacts its DNA repair ability [98].

Others join the ATM–MRN party

The interaction of ATM with the MRN complex initiates a highly coordinated program of further recruitment of DNA damage response proteins, such as MDC1 (Mediator of DNA Damage Checkpoint Protein 1), 53BP1, RNF8, RNF168, BRCA1, CtIP and BRIT1/MCPH1 to sites of DSB. MDC1 forms an important protein interaction platform by binding γ H2AX via its BRCT domain and ATM via its FHA domain [99]. MDC1 facilitates the continuing accumulation of active ATM in the areas of chromatin surrounding DNA DSBs and sustains amplification of DNA damage signaling. In addition, MDC1 also interacts with NBS1 and targets NBS1 to DNA DSBs [100]. Constitutive phosphorylation of MDC1 by Casein kinase-2 mediates interaction with the FHA and BRCT domains of NBS1 in undamaged cells. This phosphorylation event is important for retention of NBS1 at chromatin areas surrounding DNA DSBs [101, 102].

Additional proteins, including BRIT1, which contains three BRCT domains, are involved in facilitating the recruitment of NBS1 to the DSBs. Loss of heterozygosity in BRIT1 is involved in cancer development and decreased BRIT1 levels were observed in various cancers [103]. BRIT1 also plays a major role in the regulation of SWI-SNF, an ATP-dependent chromatin remodeling complex [104]. DNA damage causes ATM/ATR-dependent phosphorylation of the BAF170 subunit in SWI-SNF, which in turn promotes its interaction with BRIT1. Therefore, BRIT1 serves as recruiting module for SWI-SNF complex to sites of DNA damage. Unexpectedly, another PI3 kinase family member, phosphoinositide 3-kinase β (PI3K β) was recently shown to participate in the sensing of DNA damage [105]. The recruitment of NBS1 to damaged DNA is facilitated by PI3K β , and PI3K β -deficient cells show defects in ATM/ATR activation and signaling.

ATR and MRN

Like ATM, the MRN complex also plays a role “upstream” and “downstream” of ATR. Resection of DSBs to generate single-stranded tails is a critical step in DNA damage response that is required for activation of ATR kinase. MRE11 together with CtIP (Sae2 in yeast) carries out limited resection of DSBs, which is necessary for extensive resection by the concerted action of nucleases and helicases such as EXO1, BLM, and Dna2 to create single-stranded DNA [80, 106]. ATM is also involved in

the creation of RPA-coated ssDNA structures [107, 108]. Single-stranded DNA coated with RPA is an essential structure required for recruitment of ATR to DSBs [109, 110]. As an upstream player, the MRN complex interacts with ATRIP through the FHA/BRCT domain of NBS1 to recruit ATR to sites of damage and as a downstream target NBS1 is phosphorylated by ATR on S343 in response to a stalled replication fork. The MRN complex is also required for ATR-directed phosphorylation of RPA2 [111]. The MRN complex prevents rereplication of DNA in the presence of overexpression of the licensing factor CDT1 [112]. MRN modulates ATR phosphorylation of SMC1 [113], and mediates ATR-dependent but replication-independent signaling via interaction with N-terminal domains of NBS1 [114]. However, the study of ATR-dependent signaling in ATM-deficient cells showed that ATR is also capable of phosphorylating p53, CHK1, CHK2, SMC1 and 53BP1 in an MRE11-independent fashion [115]. ATR can also be recruited to DNA DSBs independently of MRE11, and this process is mediated by EXO1. A highly coordinated biphasic DNA damage mechanism involving regulated an ATM-to-ATR pathway switch was recently described [116], where resection of blunt DNA ends is essential to pass an activation signal from ATM to ATR. Curiously, this process also leads to ATM inactivation suggesting that the length of single-stranded overhangs directly modulates ATM activity. It is not entirely clear how these processes operate in vivo within the chromatin environment. It is interesting to note that active (phosphorylated) forms of ATM kinase persist in cells for an extended period of time following DNA damage [54].

MRN is a vital DNA damage complex

Studies of cells from ATLD and NBS-like disorder [67] patients demonstrated the tight inter-dependence of levels of MRN complex members on each other. Moreover, deficiency in MRE11 was reported for several colon cancer cell lines [117]. MRE11 was found to be necessary for the stability of the MRN complex and downregulation of MRE11 by siRNA resulted in downregulation of NBS1 and RAD50 levels. It seems that components of the MRN complex can also influence the stability of other proteins involved in DNA damage response. For example, MRN is an indispensable for stabilization of FANCD2 during DNA repair as silencing of MRN by the specific inhibitor Mirin influenced accumulation of FANCD2 at the sites of DNA DSBs [118].

In addition to the possible involvement in ATM and ATR signaling pathway and processing of DSBs, MRN complex proteins also play multiple distinct roles at the telomeres [119]. Telomere ends are protected from recognition by the DNA damage signaling machinery by the

specialized protein complex called Shelterin, consisting of the TRF1, TRF2, TIN1, Rap1, TPP1, and POT1 proteins [120]. NBS1 interaction with TRF2 was shown to be specific for the S-phase of cell cycle. However, ATM, phosphorylated forms of NBS1 and MRE11 were also found to be associated with telomeres in G2 phase cells and it was proposed that the MRN complex recruits activities necessary for the formation of a telomere end protection complex [121]. Studies in mouse models with deficient MRE11/TRF2 [122] and NBS1/TRF2 proteins [123] demonstrated an exclusive role of the MRN complex in the initiation of ATM-dependent signaling at damaged telomeres. TRF2 was proposed earlier to act as inhibitor of ATM activation at telomeres [124]. Indeed, the role of TRF2 in suppressing ATM signaling at damaged telomeres was demonstrated, as well as a similar role for POT1 in controlling ATR kinase activity [125]. In addition, the MRN complex is also required for telomere maintenance through a telomerase-independent mechanism, referred to as alternative lengthening of telomeres (ALT) pathway [126].

In summary, the MRN complex has a complex intimate link to the ATM, ATR and DNA-PK kinases and orchestrates their activation and fine-tuning of DNA damage responses. Not unlike air-traffic controllers, the MRN complex skillfully communicates with “fast flying” kinases, responds to incoming signals and passes them through “stations” of DNA damage response proteins while guarding against any mistakes.

ATM activation in a nutshell

The ATM protein kinase is a nuclear phosphoprotein [127] activated by DNA damage [128, 129]. Current models of ATM activation involve autophosphorylation events, the MRN complex and chromatin alterations. Evidence for and against each of these models are presented below.

Autophosphorylation

Autophosphorylation of ATM was observed [130, 131] and suggested as an important step in the activation process [132, 133]. A hypothesis explaining initial steps in the ATM kinase activation can be summarized as follows: as soon as there is DNA double-strand break (DSB), di/tetramer of ATM undergoes monomerization by autophosphorylation at the critical S1981 residue [133]. Consequent detachment of Protein phosphatase 2 A (PP2A) is a requisite for the activation of ATM [28]. ATM also undergoes acetylation by the TIP60 histone acetyltransferase at the K3016 residue, which is essential for the activation [15, 134]. TIP60 is constitutively associated with

ATM and DNA damage increases its acetylase activity. Recruitment/retention of ATM at DSBs requires its kinase activity because a kinase-dead mutant of ATM failed to form damage-induced foci [52]. Autophosphorylation is required for chromatin retention of ATM kinase after the introduction of DNA DSBs in cells by the expression of endonuclease I-PpoI [135]. The importance of autophosphorylation on S1981 for stabilization of ATM at the sites of DNA DSBs was recently elegantly demonstrated using fluorescently tagged ATM kinase [136]. However, these authors found that the initial recruitment of ATM to laser-generated DNA DSB does not require autophosphorylation sites, since “kinase-dead” (KD) ATM localizes to DNA DSBs. Furthermore, the kinetics of wild-type (wt), S1981A, and KD YFP-ATM recruitment to breaks were similar within the first 10 min after laser micro-irradiation. However, thereafter both kinase-dead and S1981A mutated ATM proteins rapidly dissociated from damaged chromatin. At 2 h post laser micro-irradiation, 20% of S1981A and KD ATM were present at the breaks in comparison to 65% of wt ATM kinase. It was also observed that S1981A mutant ATM had a weaker interaction with MDC1, supporting the active role of ATM autophosphorylation for its retention at sites of DNA DSBs through MDC1 interaction.

Other (auto) phosphorylation sites (see Fig. 1) also play a role in the ATM activation process [16, 54]. Kozlov et al. (2006) used mass spectrometry to identify additional phosphorylation sites on ATM isolated from radiation-exposed cells and showed functional significance of three autophosphorylation sites (pS367, pS1893, and pS1981) in the activation process. Massive phosphoproteomic screens of DNA damage signaling networks also revealed these and other ATM phosphorylation sites [17, 89, 137]. A comprehensive list of ATM phosphorylation sites is maintained at PhosphoSite database <http://www.phosphosite.org/proteinAction.do?id=1393&showAllSites=true>). Therefore, ATM activation is controlled by interplay of complex post-translational events in human cells.

Role of adaptors in ATM activation

Similar to other members of PIKK family, ATM kinase requires “targeting subunits” or “adaptor proteins” for full activation including MRN complex [20, 21]. The similarity of clinical symptoms and cellular phenotypes of ATLD (Mre11 deficiency), NBS (NBS1-deficient), and AT patients suggested early on that the MRN complex was involved in signaling pathways governed by ATM. Indeed, the work of several laboratories using mouse models, cultured cells, cell-free extracts and *in vitro* biochemistry has established that MRN is required for optimal induction of ATM enzymatic activity [88, 138, 139]. Seminal work from Paull’s lab showed that the MRN complex modulates

the activation of ATM kinase, by increasing its affinity towards substrates [85]. In the absence of MRN, dimeric ATM kinase did not respond to DNA DSBs, suggesting that MRN is required for the monomerization and activation of ATM [140].

Studies in *Xenopus* system showed that autophosphorylation is dispensable for ATM monomerization in MRE11-depleted *Xenopus* extracts in the presence of a high concentration of DNA ends [141]. These authors suggested a two-step model of ATM activation, first ATM dimers are dissociated into inactive monomers in a DNA-dependent fashion, and the second step is DNA-independent conversion of inactive ATM monomers into catalytically active monomeric forms. Notably, this second step can be supported by a small peptide derived from the NBS1 C-terminus. Studying the requirement for DNA fragments for ATM activation in *Xenopus* system in vitro, You et al. (2007) [142] found evidence that the binding of ATM to DNA areas flanking DNA DSBs is required for complete ATM activation. In another study by Costanzo group, a novel role of MRN in ATM activation was documented when it was demonstrated that that MRN caused the generation of short single-stranded DNA oligonucleotides, which stimulated the activity of ATM [143]. Indeed, in *Xenopus* extracts MRN was found to be complexed with single-stranded oligos, and importantly presence of these oligos can be identified in human cells after irradiation. Interestingly, ss/ds DNA junctions are also involved in ATM activation, suggesting important roles for the RAD9-RAD1-HUS1 clamp loader complex, which binds to these structures.

While it is clear that ATM is recruited to sites of DNA DSBs through its interaction with the C-terminal domain of NBS1 [20, 140] (Fig. 2b), however, the MRN complex does not play an absolute role in activation of ATM kinase. It was shown that expression of NBS1 protein lacking the ATM targeting domain in cells derived from NBS patients reduced but did not eliminate ATM-activation and activity. Furthermore, high doses of radiation or later time points after damage can bypass the requirement for the MRN complex in ATM activation [139]. It is also clear that active (or “partially active”) ATM kinase can initiate at least a subset of DNA damage signaling events in the absence of NBS1. Notably however, in NBS1-deficient cells activated ATM is not localized at irradiation-induced foci (IRIF), but forms a diffuse staining pattern in the nucleus and is capable of phosphorylating its nucleoplasmic substrate, p53 [144]. However, ATM-dependent phosphorylation of chromatin bound substrates including SMC1 and BRCA1 was affected by the absence of functional MRN.

Recently, it was shown that the interaction between ATM and MRN can be modulated by other proteins, including 53BP1 and BRCA1 [145]. Addition of 53BP1

and BRCA1 proteins to in vitro kinase reactions containing ATM and suboptimal amount of the MRN complex had a three-fold stimulatory effect on ATM kinase activity. This effect was even more pronounced in the presence of a defective MRN complex, containing mutant NBS1(Δ C) protein with deletion of 20 amino acids at C-terminus, a region required for the recruitment of ATM to the sites of DNA DSBs. However, 53BP1 and BRCA1 failed to stimulate ATM activity in the presence of the MR (S1202R) N complex, defective in all ATP-dependent activities due to catalytic mutation in RAD50 protein, suggesting that 53BP1 and BRCA1 cannot generally compensate for all deficiencies in MRN. 53BP1 was found to directly interact with ATM and RAD50 protein in the MRN complex. Direct association of 53BP1 and MRN produced optimal phosphorylation of NBS1 and 53BP1 [145].

Notably, additional factors have been implicated in the activation of ATM including phosphatases PP5 [47], PP2A, and WIP1, which negatively regulate ATM activity [28, 146]. Another class of proteins is also required for sustained ATM activity (MDC1, BRIT1, BAAT1, and hSSB1).

Influence of chromatin structure

In the original model of ATM kinase regulation by autophosphorylation, it was shown that changes in chromatin structure can lead to ATM autophosphorylation and activation, suggesting that the actual trigger for ATM activation might be modifications in the higher order chromatin structure surrounding the DNA DSBs [133]. These results also implied that a “partially” active pool of ATM molecules can exist before localization and retention at the sites of DNA. Indeed, ATM kinase found at regions adjacent to the break might represent this “partially active” fraction [135]. Notably, tethering of ATM to chromatin activates the DNA damage response demonstrating the importance of chromatin as a scaffold in the activation and amplification of ATM signaling [57]. By fusing portion of ATM protein kinase (1,300–3,060) to the Lac repressor, Misteli lab demonstrated targeting of recombinant protein to LacO array in chromatin. Amazingly, recruitment of ATM kinase activity, as well as NBS1, MRE11 or MDC1 was sufficient to initiate checkpoint signaling, suggesting that localization and accumulation of these proteins to chromatin can initiate DNA damage signaling without actual DNA damage. Study of ATM activation in lymphoblastoid cells derived from patients with various chromatin-related disorders found that ATM can be constitutively phosphorylated on S1981 in cells of immunodeficiency, centromeric region instability, facial anomalies (ICF) patients [147]. ICF syndrome is a rare autosomal recessive disorder often caused by mutations in

DNA methyltransferase DNMT3B [148]. Interestingly, ATM was not capable of phosphorylating downstream substrates in ICF cells, suggesting that autophosphorylation on S1981 cannot serve as a sole marker of ATM activation. While an involvement of DNMT3B in the DNA damage response was suggested, it is not clear how chromatin perturbations caused by absence of DNMT3B can lead to defective ATM activation. It is also unknown at present what structural determinant(s) in chromatin could be a candidate for an ATM activation signal, neither it is defined what motifs/domains of ATM could mediate the detection of changes in the chromatin environment.

Debate on the current models of ATM activation

In spite of the rapid progress made in our understanding of the initial steps of DNA damage response, a number of logical contradictions exist in the current model of ATM activation, especially in the role of autophosphorylation events and MRN complex [4]. Experiments *in vitro* suggest that ATM autophosphorylation can be separated from ATM monomerization and activation events. Mutant ATM protein (S1981A) in dimeric form is capable to monomerize and be activated *in vitro* [140]. Goodarzi and Lees-Miller (2004) [60] investigated the behavior of purified human ATM by gel filtration chromatography and found that phosphorylation and/or dephosphorylation of S1981 did not affect the apparent native molecular weight of the ATM protein. They suggested that the changes in the quaternary structure of ATM may not be directly influenced by autophosphorylation of S1981.

Even more puzzling are mouse models where one [149] or more [150] of ATM autophosphorylation sites have been mutated to alanine and mutant ATM proteins expressed on ATM-null background. It seems that ATM-dependent DNA damage signaling and ATM localization at sites of DNA DSBs are normal in these mouse models. It is not entirely clear why such differences exist between ATM activation events observed in human cells, mouse models and *in vitro* experimental systems. Several explanations have been suggested e.g., [151], including the existence of specific activators/inhibitors of ATM activation in human cells [4] (see section “ATM, ATR, DNA-PK”).

Under specific conditions ATM, ATR, and DNA-PK can interact and phosphorylate each other (see Fig. 3). While not all possible cross-phosphorylation events have yet been demonstrated, it seems that the S1981 autophosphorylation site in ATM can be a target of ATR kinase after UV or replication fork arrest [152], as well as DNA-PK can be phosphorylated by ATR in response to UV [49]. Furthermore, DNA-PK can phosphorylate histone H2AX in an overlapping manner with ATM. It was also shown that in the absence of ATM, ATR and DNA-PK can phosphorylate

a subset of ATM substrates, triggering cell cycle checkpoint responses [115]. Initial phases of DNA damage response are dependent on ATM activation, while a switch to full ATR activation is regulated both by processing of DNA single-stranded ends [116] and by MRN-dependent phosphorylation of TOPBP1 by ATM, which serves as activator of ATR kinase activity [153].

Existence of kinases capable of phosphorylating ATM has also been suggested [54, 154], but only recently it was shown that CDK5 can phosphorylate ATM at serine 794, which results in the activation of ATM [155]. However, this signaling pathway may be restricted to specific cell types, primarily neurons. It has been demonstrated that CDK5 activity increases after treatment of primary striatal neurons with camptothecin [156] however, it is not clear how activation of CDK5 can be regulated in the DNA damage response. Tyrosine phosphorylation of ATM and ATR by *c*-ABL was recently suggested as an additional step in the activation of these kinases on chromatin [157]. The interaction of ATM with *c*-ABL was initially thought to follow a linear signaling pathway where *c*-ABL serves as an ATM substrate in the apoptotic pathway [158, 159]. The discovery of feedback loops in ATM/ATR signaling pathways makes it possible to speculate that other kinases/enzymes being identified as ATM/ATR substrates could also be involved in modulating ATM activity (Fig. 1). As ATM serves as a central integrator of many stress-related signaling circuits, it should be expected that other post-translational modifications will be discovered that play a role in the ATM activation mechanism. All of these signaling events are unfolding within the intricately organized nucleus, and therefore chromatin structure and organization dramatically influence ATM-mediated DNA damage response.

It is also hard to accommodate the existence of massive protein complexes involving ATM into current models of ATM activation. The BRCA1-associated genome surveillance complex (BASC), which contains ATM, NBS1, MRE11, BLM, MSH2-MSH6, and MLH1 (with molecular weight around 2 MDa), has been described [160]. In addition, there are three other BLM complexes (~600 kDa) that can be isolated apart from BASC (see [161]). They contain BLM, TOPOIII, MLH1, RPA, and the Fanconi anemia proteins. These complexes do not contain BRCA1 and eight of other known-components of BASC except MLH1. Interestingly, the majority of the proteins found in these complexes can also be found in chromatin complexes containing PARP, one of the earliest enzymes appearing at the sites of DNA breaks. The large PARP-containing complex-DNA synthesome, was initially purified in the Smulson lab [162] and consists of PARP, DNA polymerases, RFC, primase, RPA, TOPOI, TOPOII, DMT, Ligase, PCNA interacting with hMLH1, hMSH2, hMSH6,

hPMS1/2 and other proteins. DNA synthesis might play an active signaling role in the DNA damage checkpoint operating in S-phase [163]. It is not clear how to reconcile the simultaneous existence of all these complexes with nuclear structures observed after DNA damage by immunofluorescence microscopy—IRIF “IR-induced nuclear foci”. Many DNA damage associated proteins, including ATM, the MRN complex, phosphorylated histone H2AX (γ H2AX), MDC1 and 53BP1 can be visualized in irradiation-induced foci (IRIF), and presumably IRIF serve as “chromatin status” marks and sites of accumulation of proteins necessary for processing and repair of DNA damage.

ATM and chromatin remodeling

It is clear that chromatin restructuring in response to DNA damage is essential for the initiation, propagation and termination of DNA repair. This process opens the DNA allowing the recruitment of repair factors and the amplification of the checkpoint and downstream signals [57]. Before the A-T gene, *ATM*, was cloned, it was suggested that the cellular phenotype of A-T cells in particular radiation-resistant DNA synthesis (RDS) and radiosensitivity could in-part be attributed to an anomaly in chromatin structure leading to defective repair in A-T cells [164, 165]. Nevertheless, the chromatin structure controlled by the histone acetylation-deacetylation surveillance system was found to be normal in A-T cells [166]. Later studies provided evidence that the majority of telomeric DNA in A-T cells is associated with the nuclear matrix [167]. Defects in the spacing of telomeric nucleosome arrays suggested altered periodicity of chromatin compaction in A-T cells. Cells of A-T patients displayed chromatin decondensation at the level of nucleosomal and higher order structures [168]. In agreement with this, observations were made on the existence of “nucleomegaly” in the tissues of A-T patients [169]. Curiously, treatment of cells with Wortmannin, a broad range inhibitor of PIKKs, causes an increase in nuclear size over a wide range of concentrations [170].

Currently, it is believed that there is an intimate link between ATM protein distribution in chromatin, ATM kinase activation, and chromatin remodeling complexes involved in different aspects of chromatin dynamics in mammalian cells. Consistent with this, biochemical fractionation showed the presence of ATM in different nuclear compartments including the nuclear matrix [171]. It was also shown that DNA damage causes retention of active ATM kinase in detergent-resistant nuclear fractions [172]. However, the majority of inactive ATM kinase exists in a readily extractable form in the nucleus of cells in the absence of DNA damage, as well as active ATM kinase

after DNA damage [133]. Therefore, dissecting the interplay of ATM kinase and chromatin modifying activities would lead to a better understanding of the DNA damage response pathway.

Histone modifications regulated by ATM

ATM kinase activity is a primary driving force causing rapid changes in chromatin structure and the assembly of multiple protein complexes (Fig. 4). The most prominent chromatin modification is phosphorylation of histone H2AX on its C-terminal tail (γ H2AX) in the chromatin regions surrounding DSBs by ATM, ATR and DNA-PK kinases, which plays a primary role in DNA repair by facilitating the access of repair proteins to sites of DSBs. Historically, observations of H2AX phosphorylation were made in relation to chromatin structure long before the discovery of ATM kinase and the role of DNA damage in histone phosphorylation. Phosphorylation of H2AX was observed in chromatin assembly extracts from *Xenopus laevis* and correlated with assembly of regular nucleosomal arrays and was dependent on the exogenous Mg/ATP [173]. Intriguingly, the H2AX is a major variant of H2A in human sperm and A-T patients show problems in gonadal development. It was demonstrated that physiological spacing of the nucleosomes during Mg/ATP-induced sperm chromatin reorganization was dependent upon phosphorylation of H2AX at the C-terminus [174].

H2AX phosphorylation after DNA damage can be performed by PIKKs, including ATM, ATR or DNA-PK. Detailed mapping of γ H2AX foci has revealed that they are initially present as small compact structures, close to the site of the break and quickly spread along the chromatin, away from the break, encompassing 2 Mb of DNA in higher eukaryotes [175]. These observations led the Bonner group to suggest three possible explanations to account for an apparent limit for foci appearance in relation to ATM kinase activity distribution [175]. The first possibility is that discontinuity in chromatin fiber presents as a physical barrier to phosphorylation; second possibility is tracking of kinases along the chromatin fiber and falling off at a certain point creating a kinetic boundary and a third possibility is coating of chromatin fiber with kinases, which transmit the signal via changes in chromatin structure to kinases further from the site of damage. Indeed, studies of the dynamics of formation of γ H2AX in chromatin led to the surprising observation that IRIF form in euchromatin with a higher efficiency compared to heterochromatin [176, 177]. It is possible that heterochromatin is refractory to the generation of large spreading γ H2AX domains of more than 1 Mb in size surrounding DNA DSB or DNA DSB formation in heterochromatin happens less frequently. In this regard, it is interesting to analyze data on γ H2AX distributions

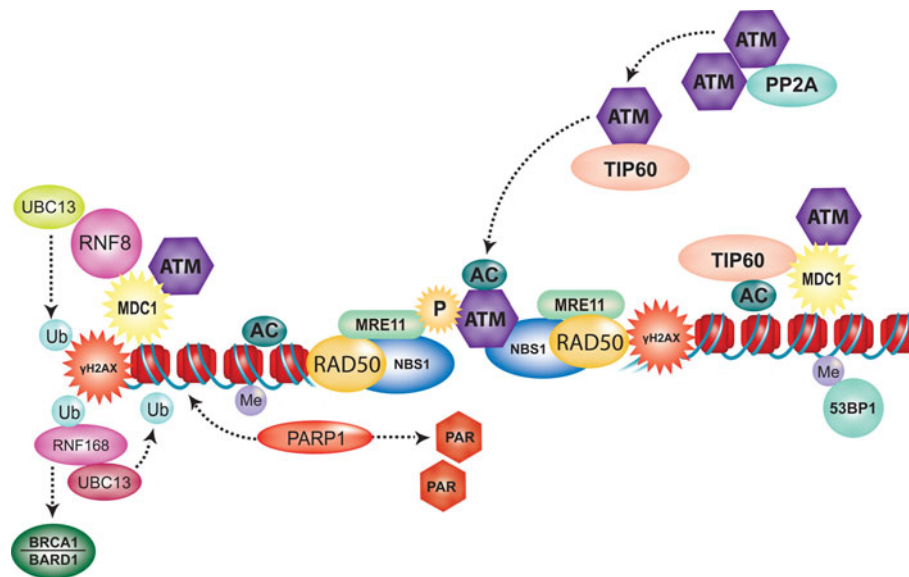


Fig. 4 Events happening at or around DNA double-strand breaks (DSBs) in chromatin. The recruitment of protein complexes and modifications in the chromatin area surrounding DNA breaks are shown. Activation of ATM kinase depends on series of phosphorylation and acetylation events as well as its recruitment to the site of the break via interaction with MRN complex. Non-covalent modification of ATM kinase by poly-ADP-ribose polymers (PAR) also plays a role. Early events include ATP-dependent chromatin relaxation, recruitment of HP1beta, exposure of histone H3 methylation sites and recruitment of histone acetyltransferase TIP60. The hierarchy of events or cross-talk between signaling pathways has not yet been established (see text). Acetylation of ATM by TIP60 is

important for subsequent autophosphorylation and activation steps. Activated ATM phosphorylates histone H2AX and triggers a cascade of further histone modifications and recruitment of DNA damage response proteins. Phosphorylated histone H2AX (γ H2AX) recruits MDC1, which in turn is phosphorylated by ATM. This event leads to recruitment of ubiquitin ligase RNF8, which in cooperation with UBC13 ubiquitinates H2AX. Recruitment of second ubiquitin ligase RNF168 is important for growth of ubiquitin chains. Acetylation of H2AX by TIP60 is important for its ubiquitination by UBC13. Exposure of histone H3/H4 methylation sites also leads to recruitment of 53BP1 to the sites of DNA DSBs and its subsequent phosphorylation by ATM

obtained after irradiation of cells with heavy ions. Traverse of heavy ions across the nucleus creates “tracks” of complex DNA damage, which serve as sites for recruitment of DNA damage response proteins. Costes et al. [178] demonstrated that kinetics of formation of pS1981 ATM foci was different between X-ray and nitrogen ion irradiation, whereas γ H2AX foci kinetics followed similar patterns. It was also demonstrated that formation of 53BP1 foci on heavy ion tracks is influenced by overall chromatin structure in the nucleus [179]. Development of focused ion microbeams in combination with real-time fluorescent microscopy will undoubtedly help solve the intriguing question of ATM activation mechanism(s) in different nuclear regions [180].

Savic et al. [181] demonstrated that only ATM, but not DNA-PK can phosphorylate H2AX at a large distance from the break site and maintain its densities. Interestingly, MDC1 was only essential to promote γ H2AX formation near the sites of DNA DSB, but not at the larger distances. The authors concluded that a soluble pool of active ATM spreads away from the break to ensure continuous phosphorylation of H2AX at distal regions. This theory is in contrast to the previous hypothesis, which suggests that MDC1 is important to promote the spread of ATM-dependent H2AX

phosphorylation alongside chromatin fiber [99]. Importantly, they suggested a “self-reinforcing mechanism” for maintaining γ H2AX densities, which is essential for the retention of active ATM kinase and MDC1 near the sites of DNA DSBs. Iacovoni et al. [182] performed genome-wide analysis of γ H2AX distribution around defined DNA breaks. They demonstrated that large domains of γ H2AX are established bidirectionally from the site of the break in a discontinuous and even asymmetrical fashion. The γ H2AX distribution was influenced by gene transcription since no γ H2AX was found on actively transcribed genes. Importantly, asymmetrical distribution of γ H2AX indicated the existence of chromatin boundaries preventing the spreading of H2AX phosphorylation (see above for hypothesis 1 suggested by the Bonner group). It is not clear what defines the boundary, since no correlation with known chromatin features was found in the analysis of available data. However, the authors speculated that matrix attachment regions (MARs) of chromatin loops might represent an attractive candidate. It would be exceptionally important to determine chromatin distribution of active ATM kinase in the system described by Iacovoni et al. [182].

It is worthwhile to note that the novel tyrosine kinase, Williams syndrome transcription factor (WSTF) was

recently shown to constitutively phosphorylate H2AX on Y142 [183] and this phosphorylation is diminished upon damage-induced S139 phosphorylation by PIKKs. WSTF interacts with PCNA and is recruited to replication foci, together with nucleosome-remodeling factor SNF2H [184]. Interestingly, depletion of WSTF resulted in an increased amount of heterochromatin proteins (HP1 β). WSTF is part of the WINAC chromatin remodeling complex that is required for normal S-phase progression. Since ATM has detectable tyrosine phosphorylation, possibly by c-ABL [157], it would be interesting to check possible direct cross-talk between ATM and WSTF signaling pathways.

The role of DNA damage-induced H2AX phosphorylation has been reviewed extensively (for a more thorough review see [185]). Currently it is believed that H2AX phosphorylation stabilizes the interaction of DSB response proteins, such as 53BP1, BRCA1 and NBS1 at the repair site and acts as an assembly platform to facilitate the accumulation of DNA repair and chromatin remodeling proteins onto damaged chromatin. The γ -H2AX phospho-epitope directly binds to the BRCT repeat of the mediator of DNA damage checkpoint (MDC1/NFBD1) [186]. Early DNA damage-induced phosphorylation of H2AX and MDC1 by ATM serves as a recruiting signal for E3 ubiquitin ligase RNF8 and RNF168 (Fig. 4) leading to new levels of regulation in ATM-mediated DNA damage signaling via protein ubiquitination (for review, see [187]). RNF8 acts together with UBC13, E2 conjugating enzyme to add ubiquitin chains to histone H2AX and other chromatin proteins in the vicinity of the break and serves as a recruitment platform for repair proteins including 53BP1 and BRCA1. Ubiquitinated H2AX serves as an assembly platform to recruit RAP80, and then other DNA repair proteins including Abraxas, BRCA1/BARD1 heterodimer. Interestingly, ubiquitination of H2AX by the UBC13 complex is dependent on its acetylation by TIP60, which regulates release of H2AX from damaged chromatin [188].

γ -H2AX has also been shown to recruit chromatin remodeling complexes INO80, RSC, SWR1(SRCAP) and SWI/SNF to the sites of DNA damage (reviewed in [189, 190]). Recently it was demonstrated that bromodomain of BRG1, a component of SWI/SNF complex binds to γ -H2AX-containing nucleosomes by interacting with acetylated histone H3 rather than γ -H2AX itself, suggesting existence of an additional mechanism that dictates recruitment of chromatin remodeling complexes [191]. Importantly, chromatin remodeling factors are themselves required to allow H2AX phosphorylation since levels of γ -H2AX and formation of γ -H2AX foci are affected by inactivation of SWI/SNF chromatin remodeling complexes [192]. This effect is ATM-independent since SWI/SNF complexes do not influence ATM activation or recruitment to DNA DSBs.

ATM-dependent and independent chromatin changes

Taking into account that ATM itself might influence the ordered changes in chromatin structure after DNA damage, it is currently difficult to discern a hierarchy of events occurring around DNA DSB in chromatin. Interestingly, a model system that introduces defined site-specific DNA breaks in human cells supports the idea that ATM activation by autophosphorylation and recruitment of NBS1 play a pivotal role in orchestrating chromatin changes after DNA damage [135].

However, another piece of evidence suggests that local changes in chromatin structure precede the localization and accumulation of early DNA damage sensors at the sites of the breaks [193]. Using a photoactivatable GFP-H2B fusion protein to monitor chromatin dynamics at the sites of DNA DSB, very rapid ATP-dependent chromatin de-compaction was observed in the damaged area. This local chromatin expansion was not influenced by ATM or H2AX suggesting the existence of early chromatin events facilitating recruitment and activation of DNA damage sensing proteins. The “Access-Repair-Restore (ARR) hypothesis” proposed that disruption of chromatin structure is required before DNA repair can proceed [194]. Recent data demonstrated the existence of both ATM-dependent and ATM-independent events controlling widespread chromatin changes accompanying DNA damage. Indeed, major structural proteins of chromatin-HP1 β [195] and HMGN1 [196] as well as histone post-translational modifications have been suggested as early determinants of DNA damage response. GFP-HP1 β quickly moves to sites of laser-induced DNA DSBs from surrounding chromatin areas [195]. This movement increases the amount of available H3 methylated on K9, which in turn recruits histone acetyltransferase TIP60 required for ATM activation as discussed above [96]. Release of HP1 from chromatin is dependent on its phosphorylation by casein kinase2 (CK2). While there is some discrepancy in the interpretation of HP1 dynamics near damage sites in hetero- and euchromatic regions [197], it is likely that the model mentioned above is correct [6]. Notably, a series of casein kinase 2 phosphorylation events important for initiation of DNA damage signaling have been described [101]. However, specific complexes of CK2 involved in mediating IR-induced ATM activation have not been isolated. In contrast, CK2 has been found to be in a complex with chromatin transcriptional elongation factor FACT (hSpt16 and SSRP1 heterodimer), involved in UV-induced p53 phosphorylation (S392) [198]. The CK2/TATA binding protein (TBP) complex, involved in transcriptional repression of RNA polymerase III after DNA damage is also known to exist [199]. Curiously, CK2 was identified

as a negative regulator of neuronal CDK5 kinase [200], which is involved in ATM activity regulation in neuronal cells.

Another important event in the initiation of the DNA damage signaling cascade involves the requirement of HMGN1 for ATM recruitment to chromatin [196]. HMGN1 regulates the ATM interaction with chromatin in both damaged and normal states. HMGN1 modulates an increase in levels of acetylated histone H3K14 after DNA damage and therefore orchestrates a global ATM response in the nucleus. In the absence of HMGN1, ATM shows increased accumulation on chromatin even if DNA damage is not present.

Recruitment and activation of ATM kinase to DNA DSBs causes further changes in chromatin structure. ATM phosphorylation of KAP1 (KRAB-associated protein, TIF1 β , KRIP-1 or TRIM28) leads to DNA damage induced chromatin relaxation [201]. It probably facilitates access of many DNA damage response proteins to chromatin and sustains phosphorylation of H2AX by activated ATM. KAP1 is a molecular scaffold that facilitates the assembly of silencing complexes of Krüppel-associated box zinc finger proteins (KRAB-ZFPs). KAP1 functions as transcriptional corepressor for ZBRK1, a KRAB-ZFP member. As KAP1 itself cannot bind DNA directly, the RING finger-B box-coiled-coil domain of KAP1 associates with the KRAB domain of ZBRK1, repressing the transcription of the DNA damage-responsive genes GADD45 α and p21. DNA damage-inducible KAP1 S824 phosphorylation by ATM also represses KAP1 SUMOylation, leading to the de-repression of genes involved in cell cycle control (p21 and GADD45 α) and apoptosis (BAX, PUMA, NOXA) [202]. Recently, Li et al. (2010) found that PP1 phosphatase interacts with KAP1 to modulate its response to DNA damage [203]. PP1 α interacts with KAP1 under normal conditions to ensure dephosphorylation of S428, whereas PP1 β was recruited to KAP1 under DNA damage conditions. The concerted actions of PP1 α and PP1 β tightly regulate dephosphorylation of KAP1 at S824 and ensure its SUMOylation to counteract the effect of ATM kinase [203]. SUMOylation of KAP1 is important for the recruitment of NuRD/Mi2 histone deacetylase complex Mi2- α and histone methyltransferase SETDB1 to chromatin [204]. KAP1 also recruits heterochromatin protein 1 (HP1) to histones through a PXVXL motif [205]. Silencing through KRAB-ZNF-KAP1-HP1 recruitment to chromatin creates inheritable epigenetic mark, raising interesting questions about role of ATM signaling in “chromatin memory”. Importantly, a key role of ATM signaling in the repair of DNA DSB in heterochromatin has recently been suggested [206].

Yet another global effect of ATM on chromatin can be exerted through transcriptional regulation. Studies

employing microarray technology pointed to the existence of ATM-dependent transcriptional events after DNA damage [207]. However, only recently mechanisms mediating the effects of ATM on transcription have begun to emerge [208]. After DNA damage, ATM is able to initiate silencing over large areas of chromatin. ATM prevents chromatin decondensation required for transcription by acting to ubiquitylate histone H2A through E3 ubiquitin ligases RNF8 and RNF168. This new ATM-mediated event was called DISC (double-strand break-induced silencing in *cis*). An influence of ATM on RNA polymerase I transcription has also been demonstrated [209]. In addition to the above functions, a unique role of ATM kinase in the regulation of telomere chromatin has been extensively reviewed and readers are referred to an excellent treatise on this topic [210].

It is clear that ATM kinase plays a key role in all aspects of chromatin maintenance. Chromatin remodeling by ATM after DNA damage, in particular through phosphorylation of H2AX, starts a multitude of signaling pathways leading to cell cycle checkpoints and DNA repair activation and ultimately culminating in the restoration of a normal chromatin state. However, the story has become more complex, recent studies suggest that many unknown chromatin epigenetic events are possibly involved in DNA DSB recognition and repair (reviewed in [189, 190]). It is tempting to speculate that extension of the “histone code” hypothesis to non-histone chromatin proteins will bring into light a new repertoire of proteins involved in ATM-dependent signaling. Notably, the “histone code” principle in the regulation of non-histone protein, histone methyltransferase G9a has already been demonstrated [211]. Therefore, interplay of methylation, phosphorylation and acetylation of non-histone proteins will provide finely tuned switches in ATM-dependent signaling pathways. In this regard, it would be interesting to see how these concepts can be applied to “hub proteins”, found to modulate multiple signaling pathways in *C. elegans* [212]. All of these proteins act as chromatin regulators including, Mys-1 (TIP60), trr-1 (TRRAP), dpy-22 (transcriptional mediator protein MED12/TRAP230), hmg-1.2 (HMG1/2), egl-27 (MTA1, a subunit of the nucleosome remodeling and histone deacetylase (NURD) complex) and din-1 (transcriptional co-repressor for nuclear receptors SHARP/SPEN) and their activity seems to be conserved in evolution. The involvement of TIP60, TRRAP, HMG1 and MTA (see above) in DNA damage response is well documented and some evidence exist for possible role of SHARP [213]. ATM interactions with “hub proteins” can provide an attractive explanation to the multiplicity of cell signaling defects observed in A-T cells. Further studies will undoubtedly bring new surprising discoveries on the role of ATM in maintaining nuclear structure and function.

Oxidative stress and ATM cytoplasmic signaling

Oxidative stress (OS) is caused by an imbalance between the production of reactive oxygen species (ROS) and a biological system's ability to readily detoxify the reactive intermediates or easily repair the resulting damage. While it was recognized quite early that ATM might play roles distinct from just the recognition of DNA damage [42, 214], the biochemical mechanisms underlying the response of ATM to oxidative stress were described only recently [41, 215, 216]. The DNA damage-independent signaling by ATM is ultimately linked to ongoing debate about the nuclear vs cytoplasmic localization of ATM, particularly in neuronal cells. It is well established that a significant amount of ATM is present in the cytoplasm of neuronal cells [217]. Consistent with this, there is mounting evidence for an important role of cytoplasmic ATM in oxidative stress signaling for the survival and functioning of neurons [215, 218, 219].

Early reports of a putative cytoplasmic function of ATM in neuronal cells were questioned by studies demonstrating an essential role of nuclear DNA damage signaling in these cells [220]. It is hardly surprising that this debate recently produced new insights into ATM function in the cytoplasm. Investigation of ATM localization during the differentiation process in SH-SY5Y cells showed cytoplasmic translocation of ATM [221]. Moreover, ATM was shown to participate in insulin signaling by preventing apoptosis in serum-starved SH-SY5Y cells. More recently, Li et al. demonstrated association of ATM with synaptosomes and showed that cytoplasmic ATM modulates synaptic function [216]. These authors demonstrated that ATM forms a complex with the synaptic vesicle proteins Synapsin-I and VAMP2. ATM was also found to be associated with ATR kinase in the cytoplasm. ATM was able to phosphorylate Synapsin-I, while ATR was responsible for VAMP2 phosphorylation, these phosphorylations modulated ATM interaction with synaptic vesicle proteins. Importantly, phosphorylation of ATM on S1981 after DNA damage was detected in the nucleus of neuronal cells, but not in the cytoplasm. This suggests that cytoplasmic ATM is either not activated by DNA damage, or mechanisms not involving S1981 autophosphorylation exist for ATM kinase activation in cytoplasm of neuronal cells. ATM is not listed so far in synaptosome proteomics data sets [222]. It is worthwhile to note that Cdk5 kinase, which activates ATM in neuronal cells, is found in synaptosomes [223].

More recently, oxidative stress has been shown to induce activation of ATM in cytoplasm [215]. The authors speculated that ATM kinase might serve as a direct sensor of ROS in cytoplasm through unknown biochemical mechanisms, possibly involving oxidation of reactive cysteine residues in ATM to alter its confirmation (Fig. 5).

They demonstrated that ROS initiate signalling from ATM to LKB1, AMPK and TSC2 to suppress activity of the mTOR kinase complex (mTORC1) to maintain homeostasis of organelles by activating autophagy. In this scenario, it was previously demonstrated that radiation causes inactivation of mTOR signaling, nuclear translocation of mTOR and increases autophagy in some cell types [224]. Non-transformed cells also respond to radiation by inhibiting mTOR-dependent protein synthesis [225].

In contrast, a pathway relaying signals from mTOR to ATM kinase has also been described recently [226]. Inhibition of mTOR activity with rapamycin or inhibition of protein synthesis causes an increase in phosphorylation of ATM-dependent substrates. In addition, ATM was recently reported to inhibit mTORC1 activity in response to hypoxia [227] and to stimulate expression of HIF-1 α (hypoxia-inducible transcription factor) and REDD1 (regulated in development and DNA damage responses1, target gene for HIF-1). ATM phosphorylation of HIF-1 α on S696 controlled its stability however, the molecular details of how this is achieved remains elusive. It is also not clear how these findings relate to mechanisms of cytoplasmic ATM activation described by Alexander et al. and Guo et al. [41, 215]. Interestingly, several years ago radiation was shown to cause accumulation of HIF-1 and activation of HIF-1 [228] by two pathways dependent on reoxygenation: accumulation of ROS and disassembly of stress granules.

Yet another important signaling pathway to trigger activation of cytoplasmic ATM was recently proposed in response to excessive excitation of neurons that can induce metabolic oxidative stress [219]. Membrane bound tyrosine kinases including platelet-derived growth factor receptor β (PDGFRB) were required to trigger activation of ATM under such conditions, which is independent of the response to genotoxic stress (Fig. 5). The authors demonstrated that expression of PDGFRB is induced by transcription factor ZFH3 (ATBF1), which in turn is controlled by ATM signaling through CREB after retinoic acid-induced differentiation of neuronal cells. Importantly, excessive excitation of neurons by kainic acid (KA), a glutamate receptor agonist, leads to ATM activation in the cytoplasm via induced oxidative stress. Interestingly, observations made on ATM activation in the cytoplasm of deep cerebellar nuclei (DCN) indicated DCN are intimately connected with the normal functioning of Purkinje cells. KA caused formation of vacuolar structures in the neurons of DCN and intense staining for microtubule associated protein light chain 3 (LC3 protein), indicative of an autophagic response [219]. The authors speculated that autophagy acts as a protective mechanism for the functions of neurons by clearing dysfunctional organelles. It would be extremely interesting to further study the mechanism of

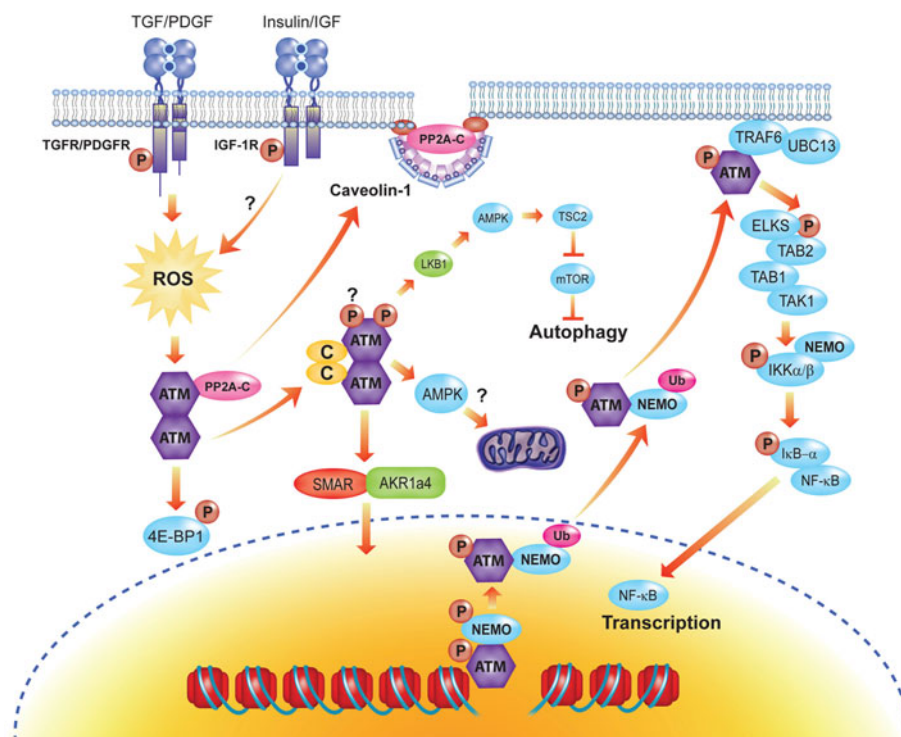


Fig. 5 Cytoplasmic and oxidative stress signaling pathways mediated by ATM. Selected pathways of ATM-dependent signaling in cytoplasm are shown. Various extra- and intra-cellular stimuli resulting in ROS production lead to ATM kinase activation independently of DNA damage signaling pathway (see text). ROS activate ATM kinase in the cytoplasm through oxidation of cysteine residues resulting in formation of catalytically active dimer. Sequestration of inhibitory phosphatases (PP2A-C) to caveolin-enriched lipid rafts in the membrane might also play a role in the ATM activation process. ATM signals through LKB1 and AMPK kinases to suppress activity of the mTORC1 kinase complex and activates autophagy. ATM is also involved in AMPK-dependent mitochondrial biogenesis

activation of ATM-LKB1-AMPK-mTORC1 autophagic pathway in neuronal cells.

More recently, Guo et al. [41, 229] explored the mechanism of ATM activation by oxidative stress. They demonstrated that ATM is activated by ROS in a completely novel way, entirely different from the mechanism of its activation by DNA damaging agents. ATM can be directly activated by hydrogen peroxide and forms an active dimer cross-linked with several disulfide bonds (Fig. 5). A critical cysteine residue, C2991, is located in the FATC domain of ATM kinase and is essential for activation of ATM by hydrogen peroxide. Importantly, mutant ATM (C2991L) retained its ability to be activated by MRN complex in the presence of DNA, but was deficient in ROS-mediated ATM activation when expressed in A-T cells [41]. Similarly, they found that the naturally occurring ATM mutant, R3047X, which is missing the last ten amino acids of the FATC domain, could not be activated in the presence of oxidative stress, but could be efficiently

activated by DNA DSBs. In a follow up study, the authors also demonstrated that oxidative stress blocks ATM activation by DNA DSBs through abolishing the DNA-binding activity of the MRN complex [229]. Hydrogen peroxide did not influence ATM-MRN interaction, but dramatically decreased MRN complex binding to DNA. The authors proposed a model where oxidative stress plays a role in the DNA damage-induced mechanism of ATM activation. ROS generated by IR play a role in immediate activation of ATM kinase, which is ROS concentration-dependent. Cellular ROS detoxification systems then reduce the extent of oxidative stress-induced activation of ATM and activate the “switch” to MRN- and DNA-damage dependent pathway of ATM activation.

ATM has also recently been implicated in the mitochondrial biogenesis pathway mediated through AMPK activation [230]. This pathway, however, differs from the ATM-LKB-AMPK cascade described above, and probably represents the effects of prolonged treatments with

activated by DNA DSBs. In a follow up study, the authors also demonstrated that oxidative stress blocks ATM activation by DNA DSBs through abolishing the DNA-binding activity of the MRN complex [229]. Hydrogen peroxide did not influence ATM-MRN interaction, but dramatically decreased MRN complex binding to DNA. The authors proposed a model where oxidative stress plays a role in the DNA damage-induced mechanism of ATM activation. ROS generated by IR play a role in immediate activation of ATM kinase, which is ROS concentration-dependent. Cellular ROS detoxification systems then reduce the extent of oxidative stress-induced activation of ATM and activate the “switch” to MRN- and DNA-damage dependent pathway of ATM activation.

etoposide or hydrogen peroxide (16 h). Cell lines of A-T patient show signs of mitochondrial dysfunction, including defects in mitochondrial structure, decreased membrane potential, and respiratory activity [231]. It is of interest that several ATM substrates demonstrate mitochondrial translocation (CREB, p53) and affect mitochondrial functions (HMGA1). HMGA1 translocates to mitochondria in the S-G2 phases of cell cycle [232] and levels of HMGA1 are correlated with mitochondrial mass, levels of ROS and repair of mtDNA damage. The role of ATM-dependent phosphorylation of HMGA1 in the mitochondrial biogenesis pathway remains to be explored.

Another intriguing pathway that involves nuclear to cytoplasmic shuttling of ATM is DNA-damage induced activation of NF- κ B pathway [63]. The authors reported that ATM shuttles to the cytoplasm and that ATM and various post-translational modifications of NEMO/IKK γ (NF- κ B essential modulator/I κ B kinase) were required for DNA damage-induced NF- κ B activation (Fig. 5). They also demonstrated that ATM associates with and phosphorylates IKK causing its SUMOylation and ubiquitin-dependent nuclear export. ATM exported to the cytoplasm forms a complex with IKK (I κ B kinase) and activates its kinase activity. IKK phosphorylation of I κ B (NF- κ B inhibitor) activates NF- κ B and causes its translocation into nucleus resulting in transcriptional activation of genes involved in regulation of DNA damage-dependent apoptosis. An extension of this study into an investigation of NF- κ B activation by other types of damage found that ATM activated by chloroquine or hypotonic shock was also capable of phosphorylating NEMO and activating the IKK–NF- κ B pathway [233]. An important role of poly-ADP ribose polymerase 1 (PARP1) in DNA damage-induced activation of NF- κ B signaling pathway has also been demonstrated recently [61]. The authors show that PAR synthesis by PARP after DNA damage promotes dynamic assembly of NEMO (IKK γ), ATM and PIAS γ -SUMO E3 ligase into the nucleoplasmic signalosome. PAR-binding motifs of ATM [59] are important for assembly of the signalosome and for NEMO SUMOylation by PIAS γ . A recent report provided convincing evidence that several proteins known to be involved in IKK activation by cytokine receptors, such as TRAF6, Ubc13, TAK1 and TAB2, are important for IKK activation by DNA damage induced by ionizing radiation [234]. The authors demonstrated that ATM is exported to the cytoplasm and plasma membrane following DNA damage (Fig. 5). In the cytoplasm, ATM engages TRAF6 to activate the kinase complex of TAK1-TAB1-TAB2/(TAB3), which then activates IKK–NF- κ B signaling. Not only is the binding of ATM to TRAF6 important to initiate signaling, but also ATM recruitment to the membranes causes activation of TAK kinase complex by promoting cytoplasmic TAB2

accumulation, probably through direct phosphorylation of TAB2 at S168. Their evidence also suggests that NEMO monoubiquitination by cIAP1, which is regulated by a nuclear complex of PARP, ATM and PIAS γ , is required for IKK activation by DNA damage, but not by TNF α . The authors propose a model in which DNA damage-induced IKK activation requires both TAK1 activation and NEMO ubiquitination.

Yet another signaling event leading to the activation of ATM by oxidative stress was recently described. Oxidative stress is generated by smoking and causes senescence in lung fibroblasts. Volonte et al. [235] found that caveolin-1 can translocate phosphatase PP2A-C to caveolar membrane after oxidative stress. Protein Phosphatase 2A catalytic subunit is an inhibitor of ATM, and its sequestration from the nucleus leads to ATM activation [28]. It seems ATM is also capable of influencing the activity of scaffold/matrix-associated region-1-binding protein (SMAR1) and NADPH-dependent aldehyde reductase (-AKR1a4) complex in cytoplasm [236]. ATM activation by oxidative stress leads to dissociation of the SMAR1–AKR1a4 complex and translocation of SMAR1 into nucleus (Fig. 5), where it binds to p53 and regulates cell cycle arrest.

The effects of ATM-mediated responses to oxidative stress play a major role in physiology of stem and neuronal cells. Early on, ATM was demonstrated to be important for differentiation of neuronal lineages from mouse adult neural progenitor cells [237] and was shown to be distributed in the nuclear (80%) and cytoplasmic fractions (20%) of neural stem cells. Cells from the adult hippocampus of *Atm* $-/-$ mice failed to differentiate into neurons or oligodendrocytes in vitro [237]. However, studies of immortalized human neural stem cell line (see below) after knockdown of ATM expression showed almost normal differentiation of progenitors into all three neuronal lineages [238].

Ito et al. (2004) [239] discovered that ATM can protect hematopoietic stem cells (HSC) from oxidative stress damage and allow them to maintain their self-renewal potential. Interestingly, HSC are usually found in the hypoxic areas of the bone marrow, and therefore are sensitive to perturbations in oxidative metabolism. HSCs from *Atm* $-/-$ mice demonstrated higher levels of damaging ROS than cells from wild type animals. Administration of antioxidant, *N*-acetyl-L-cysteine (NAC) reverses the damage and promotes the self-renewal capacity of *Atm* $-/-$ HSCs. This pathway operates through p16INK4a-Rb signaling. It is not clear why A-T patients do not show defects in HSC maintenance, it is possible that human stem cells have additional mechanisms to protect them against oxidative stress damage. The same group later found that increased intracellular ROS activates the p38 MAPK signaling pathway in mouse HSC [240] and demonstrated the

essential role of FOXO3a transcription factor in promoting HSC self-renewal [241]. FOXO3a is known to modulate normal ATM expression [242] and contributes to regulation of ROS levels in murine HSC. Interestingly, FOXO3a was found to be a direct modulator of ATM kinase activity [243]. FOXO3a binds to the FAT domain of ATM kinase and modulates levels of S1981 autophosphorylation and ATM-dependent signaling. A similar mechanism of ROS response through p38 MAPK signaling operates in neural stem cells [244]. Using *Atm* $-/-$ mice, the authors demonstrated increased levels of ROS and elevated activity of p38 MAPK in neural stem cells and defects in their proliferation capacity. Treatment of *Atm* $-/-$ neurospheres with the antioxidant *N*-acetyl cysteine (NAC) or p38 kinase inhibitor resulted in normal proliferation. Loss of ATM in neurospheres reduced levels of active Erk1/2 and Akt kinases. The same authors demonstrated defects in neurons and astrocytes in *Atm* $-/-$ mice mediated by oxidative stress [245] and probably representing a loss and decrease in proliferation of neural stem cells. ATM was found to play a major role in the biology of melanocyte stem cells [246]. Excessive DNA damage causes stem cell depletion via their differentiation, which results in premature hair graying. Signs of premature aging are often observed in A-T, and indeed the authors demonstrated that the loss of ATM in mice predisposes melanocyte stem cells to ectopic differentiation even after low doses of irradiation (3 Gy). Persistence of IRIF in stem cells of *Atm* $-/-$ mice points to defects in DNA repair mechanisms, which probably lead to the differentiation of melanocyte stem cells. The authors suggested that ATM signals to a specific “stemness checkpoint”, which controls quality and fate of stem cells [246].

Carlessi et al. [238] explored the potential of immortalized human neural stem cell line (ihNSC) as in vitro model system to investigate the role of ATM gene in neurogenesis. The differentiation of ihNSC into neuronal lineages was characterized by the upregulation of ATM/DNA-PK and down regulation of ATR and Chk1. However, shRNA-mediated depletion of ATM in ihNSC did not influence their proliferation, self-renewal capacity or chromosomal stability. Depletion of ATM did not affect ihNSC differentiation into neurons and astrocytes, and only marginally influenced differentiation into GaclC-positive oligodendrocytes and sensitivity of glial cells to oxidative stress.

Recent studies in human embryonic stem cells (hES) and induced pluripotent stem cells (iPS) demonstrated specific features of DNA damage and oxidative stress response in these cells [247–249]. Importantly, both hES and iPS cells show the existence of cellular signaling pathways that prevent the accumulation of ROS and that maintain a low number of mitochondria [249].

Interestingly, the authors noted some minor differences in the expression of mitochondrial biogenesis genes between hES cells and iPS clones. Taking into account possible involvement of ATM in the mitochondrial biogenesis pathway, it would be extremely interesting to investigate mechanisms of ROS-induced ATM activation in these cell types. Since levels of ROS are ultimately linked to cell fate decisions in stem cells (self-renewal, differentiation or apoptosis), the recent finding of an ATM kinase role as a direct redox sensor in the cells will have wide-ranging implications in the field of cell biology, including future therapeutic applications of iPS cells.

ATM, insulin, and extracellular signaling

A number of A-T patients demonstrate symptoms of insulin resistance diabetes and glucose intolerance reminiscent of metabolic syndrome. Early work showed a defect in the binding affinity of insulin receptors to insulin in A-T cells [250]. A-T cells express low levels of insulin-like growth factor-I receptor (IGF-IR), which can be restored by expression of ATM cDNA [251]. On the other hand, decreasing IGF-IR expression in mouse melanoma cells by ATM antisense RNA lowered the ATM protein level, abolished the induction of ATM kinase activity by DNA damage and caused an increase in their radiosensitivity [252].

Several years ago, Yang and Kastan [253] reported a cytosolic role of ATM in insulin signaling. They showed that insulin can activate ATM kinase activity and the absence of ATM negatively affected insulin signaling in certain cell types. ATM was shown to phosphorylate 4E-BP1 (eIF-4E-binding protein 1) on S111 (Fig. 5). Therefore, a direct role of ATM in insulin signaling pathway controlling translation initiation was demonstrated. However, it is clear that the ATM pathway does not have exclusive control of translation initiation through 4E-BP1 after insulin treatment, and probably cooperates with Akt-mTOR signaling through unknown cross-talk mechanism [253]. It is tempting to speculate that the activation of cytoplasmic ATM by ROS [41] might play a role in the insulin signaling through ATM. The effects of ROS on insulin receptor signaling have been demonstrated (reviewed in [254], see also [255]). It is also possible to propose as yet unidentified membrane signaling events involving ATM kinase as demonstrated for ATM mediated DNA damage-induced activation of NF- κ B pathway [234]. It is interesting to note that insulin treatment causes rapid reorganization of the plasma membrane and targeting of receptors into newly formed caveolin-enriched lipid rafts. While particular aspects of the role of caveolae and caveolins in insulin signaling are still an issue of much debate [256], nonetheless oxidative stress has been shown to cause

caveolin-1-mediated translocation of phosphatase PP2A-C to caveolar membrane [235]. This process might influence ATM activation through sequestration of inhibitory phosphatase. In addition, the presence of DNA-PK was detected in lipid rafts in response to radiation [257]. Therefore, ATM recruitment to membranes demonstrated recently [234], might be an example of more general signaling pathways involving ATM recruitment to particular lipid rafts.

The regulation of insulin signaling by ATM is quite complex. ATM is also involved in phosphorylation of S473 on Akt after insulin stimulation and use of siRNA against ATM or ATM inhibitor exhibited reduced insulin-stimulated Akt phosphorylation [258]. Interestingly, it was shown that DNA damage (IR) also induces ATM-dependent S473 Akt phosphorylation. However, ARK5 kinase (NUAK1, AMPK-related protein kinase 5) was proposed as an ATM upstream kinase in the Akt signaling pathway [154]. ARK5 is activated by Akt *in vitro* and can directly phosphorylate ATM after glucose deprivation, suggesting feedback mechanism from Akt to ATM [154]. Importantly, ATM signaling was shown to be important in metabolic syndrome and modulation of ATM kinase activity by chloroquine was demonstrated to improve clinical parameters such as glucose tolerance and atherosclerosis [259]. It is interesting to note that insulin receptor signaling plays a role in neuronal signal transduction including potential roles in neurodegenerative and neuronal development disorders (reviewed in [260]).

Early on in A-T research it became clear that extracellular signaling is involved in defining certain aspects of the A-T phenotype. As mentioned above, insulin signaling was shown to be defective in A-T cells [250]. In addition, various other ligands were found to be involved in mediating ATM-dependent cellular signaling. Lymphocytes of A-T patients showed defects in mitogen mediated signaling, e.g., poor proliferative response to phytohaemagglutinin (PHA) [261] and reduced proliferative response to T-cell mitogens and low IL-2 production [262]. Fibroblasts from A-T patients also have increased requirements for EGF/FGF-like growth factors [263] and have a defect in K⁺ channel signaling [264]. In addition, defective signaling through B-cell antigen receptor [265], as well as defects in T-cell receptor signal transduction [266] have also been demonstrated, suggesting that various extracellular stimuli mediate signaling in an ATM-dependent manner. In this scenario, ATM is also activated after treatment of cells with TGF. TGF signals via type II and type I serine/threonine kinase receptors (TGFRII and TGFRI). Activated TGF receptor complex subsequently phosphorylates receptor activated (R) Smad proteins, Smad2 and Smad3, which together with the common-mediator (Co) Smad4 are transported to the nucleus where they act as transcriptional

regulators for specific genes. Smad7 shuttles between the nucleus and the cytoplasm. It is localized in the nucleus in the absence of TGF but is trafficked to the cell membrane upon the receptor ligand interaction. Smad7 co-exists with γ H2AX in DNA damage induced foci and was obligatory for proper cell cycle checkpoints to prevent genetic instability [267]. Smad7 inhibits R-Smad activation by binding to the active TGF β complex. Moreover, Smad7 can couple with the ubiquitin ligase Smurf1, which mediates ubiquitination and degradation of the receptors. It has been proposed that radiation induced activation of ATM and its downstream components is regulated by TGF and TGFRI1 concomitantly [268]. However, these findings are contentious since Wiegman et al. [269] were able to demonstrate the stimulatory effect of TGF β -1 on the immediate activation of ATM signaling pathway following radiation, but they failed to find a role for the activation of TGF β -Smad signaling in this response.

Notably, ATM can be activated in epithelial cells after treatment with TGF β as indicated by an increase in autophosphorylation of ATM in TGF β -treated cells [268]. TGF-treated prostate cancer (LNCaP) cell line also showed a significant enhancement of ATM autophosphorylation. However, abrogation of negative regulators of TGF signaling pathway including NEDD4 and SMURF1 geared up the process of ATM activation and attenuated genomic instability in these cells [270]. TGF β was shown to induce γ H2AX foci in primary human astrocytes (NHA) and T98G glioma cells [271]. This effect was abrogated by DMSO or filipin treatment suggesting the involvement of ROS in TGF β -mediated signaling. It remains to be determined if activation of cytoplasmic ATM by ROS is also involved in transmission of cytokine signals [41] (Fig. 5). Extracellular signals mediate complex responses of tissues to stress and damage and impart increasing diversity to multi-step activation of ATM.

Since the moment of discovery of the ATM gene, it became clear that many functions and signaling pathways of ATM kinase should be explained in the light of ATM role in determining complex phenotypes observed in A-T patients. In this review, we attempted to untangle the complex web of ATM activation pathways with the purpose to pinpoint the role of ATM in multistep disease initiation and progression in different tissues.

Future directions and knowledge gaps

It is exciting to note from the above discussions that tremendous strides are being made in improving the understanding of ATM dynamics in response to diverse signals. There is compelling evidence that supports an essential role of ATM kinase in maintenance of genome

stability in response to DNA damage. The mechanisms of ATM activation are tightly controlled, involving a series of complex steps, including post-translational modifications, dimer-monomer transitions, interactions with targeting subunits and re-localization to diverse cellular functional compartments. We are just beginning to understand these mechanisms.

It is clear that we are still missing the full picture of ATM post-translational modifications (PTMs) in various tissues and organs in response to various stimuli. Undoubtedly, the progress in proteomic approaches to simultaneously identify all possible PTMs on a given protein will shed light on ATM activation mechanisms. The picture has started to emerge of possible hierarchy and crosstalk between identified ATM PTMs (phosphorylation, acetylation, cysteine modifications). It remains to be seen how these and yet unidentified PTMs could be assembled into logical and testable model(s) of ATM kinase responses to different stress signals. It is tempting to speculate that we might see the emergence of unifying hypothesis of the role of PTMs in ATM regulation similar to the “histone code” hypothesis proposed for histone modifications in chromatin. Such models will require clear experimental evidence for functional significance of all identified ATM modifications. An important step in this direction will be resolution of current discrepancies in several observations between murine and human A-T experimental models (e.g., role of phosphorylation sites, fate of stem cells, neuronal degeneration). Creation of new experimental systems (new model organisms, A-T patients-derived stem cells) will open a new frontier in A-T research.

Taking into account the intimate link between ATM kinase, the MRN complex and DNA damage in chromatin, we should expect a proliferation of cross-field experimental approaches to investigate the role of ATM in the chromatin environment. Combination of biochemical, mass-spectrometry and live-imaging microscopy techniques will continue to be used in deciphering the early steps of ATM kinase activation by DNA DSBs. While the role of ATM as DNA damage recognition sensor will remain unchallenged, the expansion of studies of ATM functions in various aspects of chromatin maintenance should be a priority. How many proposed activators/inhibitors of ATM activity can fit in a combined model of its regulation in the chromatin environment remains to be seen. It is clear that full understanding of this process at a biochemical level will be dependent on our success in obtaining the X-ray structure of ATM kinase and its complex with interacting partners, including MRN. Taking into account promising results achieved with the resolution of structures of DNA-PKcs and MRN complex, we should see progress in the ATM field sooner rather than later.

While the search for ATM substrates produced an impressive and ever-growing list of proteins involved in every aspects of cellular metabolism, it is also clear that addressing the functional significance of every interacting partner in every cell type relevant to pathology in A-T will be a challenging task. It will be interesting to see how data accumulated in massive phosphoproteomic screens for ATM substrates can be applied to dissect aspects of A-T phenotypes that are relevant to clinical practice. With the overwhelming focus of ATM research on DNA damage signaling in the past 10 years, the appreciation of damage-independent roles of ATM as cytoplasmic protein involved in ROS sensing is just starting to emerge. Unification of models of ATM nuclear and cytoplasmic activation and signaling will depend on extension of our current experimental approaches to new cell types, in particular to cells of the nervous system since neurodegeneration is one of the most debilitating aspects of A-T phenotype. Notably, the study of the role of ATM in neuronal cell types and development of nervous system (particularly Purkinje cells) is emerging as a major focus of A-T research, which is strongly supported by A-T patients and their parents.

No therapy exists at present to prevent or cure the progression of A-T [2]. Existing therapeutics cannot stop progressive neurodegeneration and cancer predisposition in A-T patients. However, treatment with antioxidants and phytonutrients [272], read-through drugs to help produce active ATM kinase in patients with truncating mutations [273] and genetic modification of stem cells from A-T patients all have been suggested as potential treatment strategies.

Here, we have tried to demonstrate a complex signaling web that underlies the heterogeneous response of ATM protein kinase to stress. Taking into consideration the current understandings of push and pull between multiple roles of ATM, there is an urgent necessity to tailor therapies with promising clinical outcomes.

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