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ATR signaling at a glance

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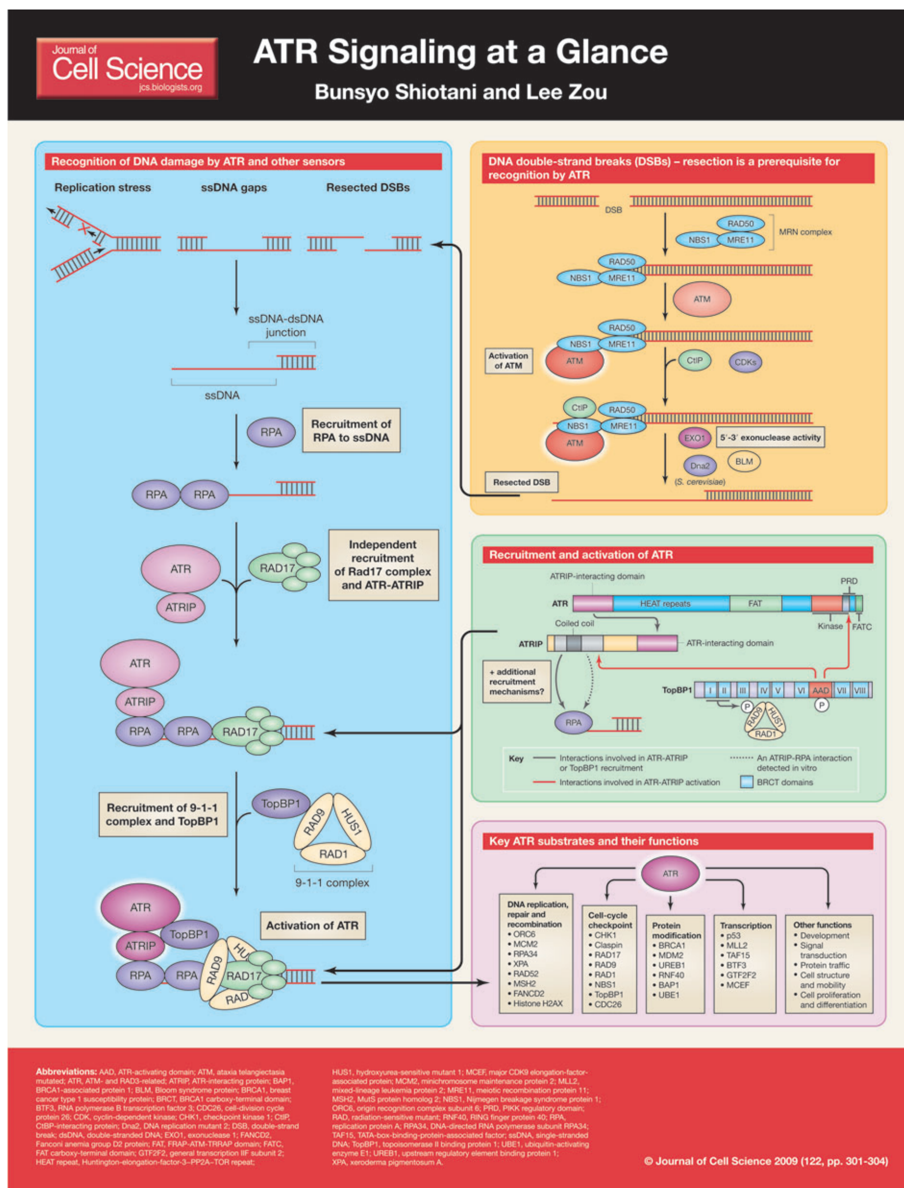
The maintenance of genomic integrity is crucial for the survival of all organisms. In humans, compromised genomic integrity contributes to genetic disorders, aging and cancers. The task of safeguarding the genome is accomplished by the concerted action of a number of cellular processes, including DNA replication, DNA repair, senescence and apoptosis. Many, if not all, of these processes are regulated and coordinated by the DNA-damage checkpoint, which is a complex signaling network that is triggered by DNA damage or genomic instability. Two phosphoinositide 3-kinase-like protein kinases (PIKKs) – ataxia telangiectasia mutated (ATM), and ATM- and Rad3-related (ATR) – are master regulators of two major checkpoint pathways (Harper and Elledge, 2007). ATM is primarily activated by DNA double-strand breaks (DSBs), whereas ATR responds to a much broader spectrum of DNA damage, including DSBs and many types of DNA damage that interfere with DNA replication (Cimprich and Cortez, 2008; Zou, 2007). In contrast to ATM, ATR has a crucial role in stabilizing the genome during DNA replication and is essential for cell survival (Brown and Baltimore, 2003). Here, we summarize the recent findings on ATR signaling in four areas: sensing of DNA damage; activation of the ATR kinase; interplay between ATR and ATM; and phosphorylation of ATR substrates.



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Sensing of DNA damage

The initial step in ATR activation is the recognition of DNA structures that are induced by DNA damage, such as single-stranded DNA (ssDNA) and junctions between ssDNA and double-stranded DNA (dsDNA). ssDNA is a DNA structure that is commonly induced by DNA damage and by interference with DNA replication. An increased amount of ssDNA is generated at DNA replication forks when the coordination between DNA polymerase activity and DNA helicase activity is compromised (Byun et al., 2005; Walter and Newport, 2000). In addition, ssDNA gaps are induced by several types of DNA repair, such as nucleotide excision repair, mismatch repair and long-patch base excision repair (Friedberg, 2003; Lopes et al., 2006). ssDNA is also present at DSBs that have been trimmed by exo- or endonucleases through a process called resection (Lee et al., 1998). When ssDNA is generated at sites of DNA damage or at stalled DNA replication forks, it is always accompanied by junctions between ssDNA and dsDNA. Circular ssDNA that is annealed with primers, which possesses both ssDNA and ssDNA-dsDNA junctions, is sufficient to activate the ATR-mediated DNA-damage checkpoint response in *Xenopus laevis* egg extracts (MacDougall et al., 2007).

In all eukaryotes, ssDNA that is induced by DNA damage is first detected by replication protein A (RPA), which is an ssDNA-binding protein complex. ATR-interacting protein (ATRIP), which is the regulatory partner of ATR, binds directly to RPA-coated ssDNA (RPA-ssDNA) and thereby enables the ATR-ATRIP complex to localize to sites of DNA damage (Ball et al., 2005; Namiki and Zou, 2006; Zou and Elledge, 2003). The N-terminal RPA-interacting domains of human ATRIP and its *Saccharomyces cerevisiae* homolog Ddc2

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are important for the recruitment of ATRIP and Ddc2 to sites of DNA damage (Ball et al., 2007; Ball et al., 2005; Zou and Elledge, 2003). Additional interactions between ATR-ATRIP and RPA, as well as the interactions between ATR-ATRIP and other proteins, might also contribute to the association of ATR-ATRIP with damaged DNA (Ball et al., 2005; Namiki and Zou, 2006; Yoshioka et al., 2006). The localization of ATR-ATRIP to sites of DNA damage, however, is by itself not sufficient to activate the DNA-damage-checkpoint response fully; instead, it requires the functions of additional ATR regulators, including RAD17, RAD9, RAD1 and HUS1. RAD17 is a protein that is structurally related to RFC1 (the largest subunit of the five-subunit replication complex RFC), and it forms an RFC-like complex with the four small subunits of RFC (RFC2-RFC5). By contrast, RAD9, RAD1 and HUS1 form a ring-shaped trimeric complex (the 9-1-1 complex) that resembles proliferating cell nuclear antigen (PCNA), a processivity factor for DNA polymerase. During DNA replication, RFC recognizes primer-template junctions and recruits PCNA onto DNA; similarly, during the DNA-damage response, the RAD17 complex recruits 9-1-1 complexes onto damaged DNA. Both RFC and the RAD17 complex require RPA for their function (Ellison and Stillman, 2003; Majka et al., 2006a; Zou et al., 2003). Unlike RFC, which specifically recognizes 3′ dsDNA-ssDNA junctions, the RAD17 complex preferentially recruits 9-1-1 complexes to 5′ dsDNA-ssDNA junctions, which are associated with resected DSBs and stressed replication forks (Ellison and Stillman, 2003; Majka et al., 2006a; Zou et al., 2003). The recognition of DNA damage by ATR-ATRIP and the RAD17 complex is largely independent (Kondo et al., 2001; Melo et al., 2001; Zou et al., 2002); in *S. cerevisiae*, colocalization of Ddc2 (which is homologous to ATRIP) and Ddc1 (which is homologous to RAD9) at artificial binding sites activates the DNA-damage checkpoint in the absence of DNA damage (Bonilla et al., 2008), suggesting that a crucial function of DNA damage in checkpoint activation is to bring ATR-ATRIP and the 9-1-1 complex together.

Activation of the ATR kinase by TopBP1

Following the recruitment of ATR-ATRIP and the 9-1-1 complex, the kinase activity of ATR is stimulated at sites of DNA damage. When it is loaded onto DNA, the *S. cerevisiae* Ddc1-Rad17-Mec3 complex (which contains homologs of the three components of the 9-1-1 complex) directly stimulates the kinase activity of the Mec1-Ddc2 complex (which contains homologs of ATR and ATRIP) (Majka et al., 2006b). Whether the 9-1-1 complex can stimulate ATR-ATRIP activity in other organisms is still unclear. In both human and *Xenopus*, DNA topoisomerase II binding protein 1 (TopBP1) stimulates the kinase activity of ATR-ATRIP even in the absence of DNA (Kumagai et al., 2006). Dpb11, the *S. cerevisiae* homolog of TopBP1, also stimulates the kinase activity of Mec1-Ddc2 (Mordes et al., 2008b; Navadgi-Patil and Burgers, 2008). Thus, TopBP1 is a direct and evolutionarily conserved stimulator of ATR-ATRIP.

How does TopBP1 interact with ATR-ATRIP? In both *Xenopus* and humans, the ATR-activating domain (AAD) of TopBP1 was mapped to a segment of the protein that lies between two BRCA1 C-terminal domains (BRCTs) – BRCT VI and BRCT VII (Kumagai et al., 2006). Inactivating mutations in the AAD diminished the DNA-damage-checkpoint response in *Xenopus* extracts, showing that the stimulation of ATR-ATRIP activity by TopBP1 is an important event during checkpoint activation (Kumagai et al., 2006). In humans, the AAD of TopBP1 interacts with the ATR-ATRIP complex via an internal region of ATRIP and the PIKK regulatory domain (PRD) near the C-terminus of ATR (Mordes et al., 2008a). The interaction between the AAD of TopBP1 and the PRD of ATR, as well as the interaction between the AAD of TopBP1 and ATRIP, are important for the stimulation of ATR by the AAD (Mordes et al., 2008a).

How is the stimulation of ATR-ATRIP by TopBP1 regulated by DNA damage? In humans and *S. cerevisiae*, TopBP1 and Dpb11 were found to interact with RAD9 and Ddc1, respectively (Makiniemi et al., 2001; Wang and Elledge, 2002). Subsequent studies have shown that both human and *Xenopus* TopBP1 associate with the C-terminus of RAD9 through the BRCT I and BRCT II domains of TopBP1 and phosphoserine 387 of RAD9 (Delacroix et al., 2007; Lee et al., 2007). This interaction between RAD9 and TopBP1, which is required for checkpoint activation in *Xenopus* extracts and chicken cells, might provide a means to recruit TopBP1 to sites of DNA damage. Nonetheless, the phosphorylation of certain ATR substrates, such as RAD1, is dependent on TopBP1 but not on the C-terminus of RAD9 in *Xenopus* extracts (Lupardus and Cimprich, 2006), which suggests that TopBP1 can be regulated by DNA damage through alternative mechanisms that are independent of the C-terminus of RAD9. Fusion of the AAD of TopBP1 with PCNA or histone H2B directly localizes the domain to chromatin and bypasses the requirement for RAD17 for activation of the DNA-damage checkpoint, which indicates that a crucial function of RAD17 and 9-1-1 in ATR activation is to bring TopBP1 to sites of DNA damage (Delacroix et al., 2007).

In addition to stimulating the kinase activity of ATR, TopBP1 is a substrate of ATR and ATM. Serine 1131 of *Xenopus* TopBP1 is phosphorylated by ATR in response to replication inhibition, or by ATM in the presence of the synthetic DNA structures that are generated in the presence of poly deoxyadenine (poly-dA) and poly deoxythymidine (poly-dT) (Hashimoto et al., 2006; Yoo et al., 2007). The phosphorylation of serine 1131 enhances the interaction between TopBP1 and ATR-ATRIP, which suggests that TopBP1 phosphorylation promotes a feed-forward signaling loop that amplifies ATR-mediated signals (Yoo et al., 2007).

Interplay between ATR and ATM

Although ATR can respond directly to interference with DNA replication, its activation at DSBs is dependent on ATM (Jazayeri et al., 2006; Myers and Cortez, 2006). When they are generated in cells, DSBs are recognized by a protein complex that comprises meiotic recombination protein 11 (MRE11), RAD50 and Nijmegen breakage syndrome protein 1 (NBS1). The MRN11-RAD50-NBS1 (MRN) complex recruits and stimulates ATM, and is crucial for the function of ATM at DSBs (Berkovich et al., 2007; Falck et al., 2005; Kitagawa et al., 2004; Lee and Paull, 2005; You et al., 2005). ATM is rapidly activated by DSBs throughout the cell cycle, whereas ATR is activated more slowly, and predominantly during the S and G2 phases (see below) (Jazayeri et al., 2006). Thus, the activation of ATM by DSBs is necessary, but not sufficient, to activate ATR.

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The function of ATM in ATR activation is, at least in part, attributable to its role in DSB resection. ATM is required for the resection of DSBs by exo- and endonucleases, which generates long stretches of ssDNA adjacent to the breaks (Jazayeri et al., 2006). The exo- and endonucleases involved in DSB resection include the MRN complex (an exo- and endonuclease), CtIP (an endonuclease and an activator of the exonuclease activity of MRN) and EXO1 (an exonuclease) (Buis et al., 2008; Sartori et al., 2007; Schaetzlein et al., 2007). In addition to these nucleases, the DNA helicase BLM is implicated in DSB resection (Gravel et al., 2008). Recent studies have suggested that the resection of DSBs occurs in a two-step manner (Mimitou and Symington, 2008; Zhu et al., 2008). During the initial stage, the MRN complex and CtIP function together to generate short stretches of ssDNA at DNA ends. Subsequently, the DSBs that have been processed by MRN and CtIP are further resected by two redundant mechanisms involving EXO1 and BLM, respectively. In *S. cerevisiae*, Dna2, which possesses both DNA helicase and endonuclease activities, might function with the BLM homolog Sgs1 during the second stage of resection (Mimitou and Symington, 2008; Zhu et al., 2008). Most, if not all, of the factors involved in the two-step DSB resection are required for efficient activation of ATR, suggesting that long stretches of ssDNA at DSBs are needed for ATR activation. Although ATM is clearly required for DSB resection, the precise function of ATM is still unknown.

In addition to ATM, the cyclin-dependent kinases (CDKs) are important regulators of DSB resection (Ira et al., 2004; Jazayeri et al., 2006; Zierhut and Diffley, 2008). In *S. cerevisiae*, the phosphorylation of Sae2 (the functional homolog of CtIP) by CDKs is needed for efficient DSB resection (Huertas et al., 2008). The activation of CDKs during the S and G2 phases of the cell cycle might facilitate ATR activation during these periods. Deletion of Ku70 and Ku80, which form a protein complex that inhibits the binding and function of the MRN complex at DSBs, enables DSB resection even in G1 (Clerici et al., 2008). Thus, CDKs might promote DSB resection by alleviating the inhibitory effects of Ku70 and Ku80 on this process.

Phosphorylation of ATR substrates

The phosphorylation of ATR substrates has a clear role in the transduction of DNA-damage signals and in the effects of ATR on downstream processes such as cell-cycle arrest and DNA repair. Many of the proteins that are involved in ATR signaling – including ATRIP, RPA, RAD17, the 9-1-1 complex and TopBP1 – are phosphorylated by ATR during checkpoint activation. The best-characterized ATR substrate is the checkpoint kinase 1 (Chk1), an effector kinase of ATR in the ATR-mediated DNA-damage-checkpoint pathway (Bartek and Lukas, 2003). Chk1 is not only important for the checkpoint response during S phase, but is also crucial for the stability of DNA replication forks. In response to DNA damage, Chk1 is phosphorylated by ATR at multiple sites, which stimulates the kinase activity of Chk1 and releases it from chromatin. Activated Chk1 phosphorylates numerous substrates including Cdc25A and Cdc25C, which are involved in cell-cycle arrest at the G1-S and G2-M transitions, respectively.

The phosphorylation of Chk1 by ATR is mediated by claspin, which is a possible component of DNA replication forks (Osborn and Elledge, 2003). Even in the absence of DNA damage, claspin and Chk1 stabilize each other (Chini et al., 2006; Mamely et al., 2006; Yang et al., 2008). In response to DNA damage, claspin is phosphorylated by ATR and by an unidentified kinase in an ATR-dependent manner (Kumagai and Dunphy, 2003; Yoo et al., 2006). The ATR-mediated phosphorylation of claspin promotes the phosphorylation of Chk1 by ATR and might directly stimulate the kinase activity of Chk1 (Kumagai et al., 2004). The level of claspin is tightly regulated during the cell cycle – it is degraded in the G2 and M phases by the Skp1–Cullin1–F-box (SCF) ubiquitin ligase through a Polo-like kinase 1 (Plk1)-regulated mechanism (Mailand et al., 2006; Peschiaroli et al., 2006) and is degraded in G1 by the anaphase-promoting complex (APC) (Bassermann et al., 2008). In response to DNA damage, the degradation of claspin in G2 is disrupted (Bassermann et al., 2008). The ability of claspin to accumulate during the S and G2 phases ensures efficient Chk1 activation in this cell-cycle window if DNA is damaged. The claspin-mediated phosphorylation of Chk1 provides an example of how the phosphorylation of ATR substrates can be regulated at multiple levels.

Several recent large-scale proteomic studies have identified hundreds of proteins that are phosphorylated at [S/T]Q sites by ATM or ATR (Matsuoka et al., 2007; Mu et al., 2007; Smolka et al., 2007; Stokes et al., 2007). Among these substrates, some were shown to be preferentially phosphorylated by ATR. Given that ATM is required for the activation of ATR by DSBs, some of the substrates might be phosphorylated by ATR in an ATM-dependent manner. Many ATM or ATR substrates are involved in DNA-damage signaling, DNA replication and DNA repair. In addition, many ATM or ATR substrates function in protein modification (such as ubiquitylation), transcriptional regulation, developmental processes, cell structure, mobility, proliferation and differentiation. These studies reveal that ATM and ATR regulate a much broader spectrum of cellular processes than was previously appreciated.

Conclusions and perspectives

In the past decade, tremendous progress has been made in understanding how ATR is activated and how it protects genomic stability. Although the framework of the ATR pathway has clearly emerged, the function and regulation of this pathway are yet to be fully appreciated. It is clear that ATR activation is regulated at multiple levels, such as the localization of ATR, the stimulation of ATR kinase activity and the recognition of ATR substrates. How these different mechanisms function in concert is still poorly understood.

Despite the identification of many ATR regulators, little is known about the biochemical details of ATR activation. We also know little about the organization of the ATR pathway in the contexts of DNA replication and various types of DNA repair. The key substrates of ATR that mediate the relevant cellular processes, such as the stabilization of stalled replication forks, remain to be identified. The recent proteomic studies discussed above have extended the network of cellular processes that might be orchestrated by ATR, and the functions of ATR in regulating and coordinating these processes await further investigation. Finally, because ATR is an essential protein, its functions in animals have only been explored to a limited degree (Ruzankina et al., 2007). We anticipate that combined biochemical, cell-biological and genomic studies on ATR will attest to its role as a central safeguard of the genome.

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