

ATM, ATR and DNA-PK: the trinity at the heart of the DNA damage response

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SUMMARY

In vertebrate cells, the DNA damage response is controlled by three related kinases: ATM, ATR and DNA-PK. It has been 20 years since the cloning of ATR, the last of the three to be identified. During this time, our understanding of how these kinases regulate DNA repair and associated events has grown profoundly, although major questions remain unanswered. Here, we provide a historical perspective of their discovery and discuss their established functions in sensing and responding to genotoxic stress. We also highlight what is known regarding their structural similarities and common mechanisms of regulation, as well as emerging non-canonical roles and how our knowledge of ATM, ATR and DNA-PK is being translated to benefit human health.

Introduction

DNA in cells is continually being damaged in many ways. These range from DNA-base mismatches introduced during DNA replication, other base adducts and cross-links generated by chemicals, ultra-violet light (UV)-induced base damage, plus single- and double-strand breaks produced by abortive topoisomerase actions, chemical reactions or ionizing radiation (IR). To pass their genomes on to the next generation intact, all organisms have evolved DNA repair pathways plus associated processes to respond to such damage and, in most instances, repair it faithfully (Ciccio and Elledge, 2010; Jackson and Bartek, 2009). In general, a cell's DNA-damage response (DDR) involves DNA lesion recognition, followed by initiation of a cellular signaling cascade to promote DNA repair, which can be aided by a pause in cell-cycle progression (checkpoint activation).

In concert with such events, cells mediate other responses, including modulation of chromatin structure and transcription, both at sites of DNA damage and more globally. Notably, many DNA lesions become particularly toxic when encountered by the DNA-replication apparatus, necessitating additional DDR mechanisms to deal with such lesions and allow accurate genome duplication. In multi-cellular organisms, certain cells with extensive DNA damage permanently exit the cell cycle (senescence) or undergo programmed cell death (apoptosis), presumably to mitigate the propagation of potentially mutated cells leading to cancer or other age-related pathologies. Notably, DNA lesions also serve as intermediates in certain biological processes, such as DNA demethylation, meiotic recombination and programmed V(D)J recombination, immunoglobulin class-switching and affinity-maturation in the immune system (Alt et al., 2013; Jackson and Bartek, 2009).

Like many intracellular signaling cascades, DNA-damage signaling is driven by protein phosphorylation. All eukaryotic genomes encode at least one of the three members of a family of phosphoinositide 3-kinase (PI3K)-related kinases (PIKKs; (Abraham, 1998)) with principal roles in activating the DDR. In this review, we provide a historical perspective on the identification of the three DDR PIKKs and discuss our current understanding of their functions, mechanisms of activation and regulation, structural similarities and the consequences of their deficiencies. We end by discussing how understanding these kinases is relevant to human health and disease.

A historical perspective

Discovery of DNA-PK

In 1985, it was discovered by accident that addition of double-stranded DNA (dsDNA) to animal-cell extracts increased the phosphorylation of certain proteins (Walker et al., 1985). This finding was serendipitous because the RNA preparations being used to study protein synthesis were contaminated with dsDNA. Fortunately, these seminal experiments provided evidence for the existence of a kinase that could be activated specifically by dsDNA. This DNA-dependent protein kinase (DNA-PK) activity was later partially purified and reported by three independent groups (Carter et al., 1990; Jackson et al., 1990; Lees-Miller et al., 1990). Kinase activity was associated with a particularly large polypeptide of >300 kDa (Carter et al., 1990; Lees-Miller et al., 1990), later named the DNA-PK catalytic subunit (DNA-PKcs; (Blunt et al., 1995)). Subsequent work established that effective DNA-PKcs recruitment to and activation by DNA requires Ku, a heterodimer comprising two subunits of ~70 and 80 kDa (Dvir et al., 1992; Gottlieb and Jackson, 1993). Ku was originally identified as a nuclear factor in human cells reacting with sera from autoimmune disorder patients (Mimori et al., 1981), and so-called after the first two letters of the name of the patient whose serum was used to identify it. Notably, Ku had been shown to preferentially bind dsDNA ends (Mimori and Hardin, 1986), which together with the observation that DNA-PK was activated by linear but not supercoiled plasmid dsDNA in vitro (Jackson et al., 1990), led to the realization that DNA double-strand breaks (DSBs) might also activate DNA-PK in vivo (Gottlieb and Jackson, 1993).

Prior to this, a set of radiosensitive, DSB-repair defective rodent cell lines had been isolated and assigned to various complementation groups, but causative genetic defects had not been identified (Jeggo et al., 1995). Promoted by the potential role for DNA-PK in DSB repair, several groups established that Ku, DNA-PKcs and/or their associated activities were defective in several such cell lines (Blunt et al., 1995; Finnie et al., 1995; Getts and Stamato, 1994; Peterson et al., 1995; Rathmell and Chu, 1994; Smider et al., 1994; Taccioli et al., 1994). Shortly after, DNA-PKcs was found to be absent in a radiosensitive human glioma cell line (Lees-Miller et al., 1995), and mutated in the radiosensitive, severe combined immunodeficient (SCID) mouse identified several years earlier (Blunt et al., 1995; Bosma et al., 1983; Kirchgessner et al., 1995). Taken together, these observations established that DNA-PK was crucial for DSB repair, by a pathway now known as non-homologous end-joining (NHEJ).

Although the genes encoding the Ku70 and Ku80 subunits of DNA-PK had been cloned at this stage (Reeves and Stoeber, 1989; Yaneva et al., 1989), the *PRKDC* gene encoding

DNA-PKcs had not. A breakthrough came in early 1995, when the DNA-PKcs cDNA was cloned and found to encode a colossal ~460-kDa polypeptide (Hartley et al., 1995). Notably, the DNA-PKcs kinase domain was found to be most similar to that of the phospholipid kinase PI3K, rather than a canonical protein kinase. This was surprising, as DNA-PK displayed no detectable activity towards lipids (Hartley et al., 1995). DNA-PKcs also appeared strikingly similar to the product of a recently cloned gene mutated in human patients with a disease called ataxia-telangiectasia (A-T) (Savitsky et al., 1995).

Discovery of ATM

A-T is a rare inherited autosomal-recessive genetic condition (Taylor et al., 2015), characterized by dilated blood vessels (telangiectasia) and progressive neurological decline, resulting in lack of voluntary movement coordination, including gait abnormality (ataxia). A-T patients also display immunodeficiency and predisposition to malignancies, especially lymphoid tumors. After some A-T patients developed severe complications following cancer radiotherapy (Gotoff et al., 1967), it was found that cells from A-T patients are profoundly radiosensitive (Taylor et al., 1975). This radiosensitivity was also apparent at the chromosomal level, suggesting a connection between the gene product mutated in A-T and DNA repair (Taylor et al., 1976). An important subsequent observation was that, while replicative DNA synthesis is markedly inhibited when normal cells are exposed to IR, this does not occur in A-T cells, which instead display radio-resistant DNA synthesis (Houldsworth and Lavin, 1980; Painter and Young, 1980). Later work showed that A-T cells are defective in establishing both the G1/S and G2/M cell-cycle checkpoints in response to IR (Imray and Kidson, 1983; Nagasawa and Little, 1983; Zampetti-Bosseler and Scott, 1981). A major insight into why the G1/S checkpoint is defective in A-T cells came when it was found that the tumor suppressor protein p53 is not induced properly in these cells after IR (Kastan et al., 1992).

When the A-T mutated (*ATM*) gene was identified (Savitsky et al., 1995), it was found that the C-terminus of the predicted ATM protein contained a PI3K-like kinase domain. Although this initially suggested that ATM might function in phospholipid signaling, we now know that it, like DNA-PKcs, is a protein kinase. Compared to proteins known at the time, ATM was found to be most homologous to Tel1, a budding yeast protein involved in controlling telomere length (Lustig and Petes, 1986), and now considered to be the yeast ATM ortholog (Greenwell et al., 1995; Morrow et al., 1995). Significantly, ATM was also found to share homology with two other yeast proteins implicated in DNA repair and cell-cycle

checkpoint control: budding yeast Mec1/Esr1/Sad3, and fission yeast Rad3 (Savitsky et al., 1995).

Discovery of ATR

Mitosis entry checkpoint 1 (MEC1) is an essential budding yeast gene originally identified in a genetic screen for S and G2 checkpoint defects (Weinert et al., 1994). It was independently retrieved in genetic screens for hypersensitivity to the replication inhibitor hydroxyurea (HU) and the alkylating agent methyl methanesulfonate, and designated *SAD3* or *ESR1*, respectively (Allen et al., 1994; Kato and Ogawa, 1994). The gene was cloned in the latter study, and a C-terminal PI3K-like kinase domain identified. Sequence similarity between Mec1/Esr1/Sad3 (hereafter Mec1) and fission yeast Rad3 was also recognized, this being noteworthy because *rad3* mutants were known to be hypersensitive to UV, IR and HU, and to display checkpoint defects (al-Khodairy and Carr, 1992; Jimenez et al., 1992; Nasim and Smith, 1975; Rowley et al., 1992).

While the *RAD3* gene was sequenced shortly afterwards (Seaton et al., 1992), the sequence was incomplete and the C-terminal kinase domain was not initially identified. Only when the similarity of Rad3 to the newly identified Mec1 and ATM proteins was noted, was the Rad3 locus reanalyzed, its full-length nature determined and its PIKK domain identified (Bentley et al., 1996). This report, and another from a different group, also described the cloning of the human Mec1 and Rad3 counterpart (Bentley et al., 1996; Cimprich et al., 1996). One named it FRAP-related protein 1 (FRP1) because of its similarity to FRAP, another PI3K-related kinase now known as mTOR (Cimprich et al., 1996), while the second named it ATM- and Rad3-related (ATR) (Bentley et al., 1996). Both reports suggested FRP1/ATR (hereafter ATR) to be the ortholog of Mec1 and Rad3, based on sequence similarity and the fact that the ATR cDNA complemented the radiation hypersensitivity of *mec1* budding yeast mutants (Bentley et al., 1996; Cimprich et al., 1996).

With the discovery of ATR, the last of the three vertebrate DDR PIKKs had been identified, and multiple lines of scientific enquiry had converged. Below, we summarize key features of the subsequent two decades of frenzied research, in particular concentrating on the most well-established functions of ATM, ATR and DNA-PK in the DDR.

Common features of ATM, ATR and DNA-PKcs

Domain organization and structural similarities

ATM, ATR and DNA-PKcs are huge polypeptides with similar domain organizations and various common structural features (Figure 1A). In all cases, their kinase domains are C-terminally located, flanked upstream by a FRAP-ATM-TRRAP (FAT) domain and downstream by a PIKK regulatory domain (PRD) and FAT C-terminal (FATC) motif (Bosotti et al., 2000; Mordes et al., 2008). N-terminal to the FAT domain, all PIKKs consist largely of helical solenoid HEAT-repeat domains of variable length that mediate protein-protein interactions (Perry and Kleckner, 2003).

Due to their large size, limited three-dimensional structural information is available on full-length ATM, ATR and DNA-PKcs, with the highest resolution crystal structure for these currently being of DNA-PKcs, at 4.3 Å (Sibanda et al., 2017). This structure revealed that DNA-PKcs consists of three large structural units: an N-terminal unit, a central “circular cradle” structure formed by HEAT repeats, and the C-terminal kinase “head” domain (Figure 1B). Single-particle electron microscopy (EM) studies of Mec1^{ATR} and Tel1^{ATM} suggest that these kinases form similar architectures, with the N-terminal HEAT repeats fashioning a possible cradle region and the FAT-kinase-FATC domains forming a head region (Sawicka et al., 2016).

Substrate specificity

Early biochemical studies on DNA-PK revealed its preference for phosphorylating a serine or threonine residue followed by a glutamine (S/T-Q; (Bannister et al., 1993; Chen et al., 1991; Lees-Miller and Anderson, 1989)). This was later shown to be the case for ATM and ATR as well (Kim et al., 1999). Furthermore, these studies showed that hydrophobic or acidic residues at positions immediately flanking the phosphorylated serine/threonine tend to be positive determinants for targeting, whereas basic residues in their vicinity are generally negative determinants. In line with this, DNA-PK, ATM and ATR share certain substrates and have some overlapping functions, as we discuss in later sections. It should be noted, however, that DNA-PK has also been shown to target Ser/Thr residues within non-S/T-Q contexts in vitro (Jette and Lees-Miller, 2015).

Autophosphorylation

Various S/T-Q motifs are present in ATM, ATR and DNA-PKcs, and all three can autophosphorylate. For DNA-PKcs, multiple autophosphorylation sites have been identified, which mostly cluster in the HEAT-repeat region (Jette and Lees-Miller, 2015). Autophosphorylation of S2056 and T2609 in particular are used to indicate DNA-PKcs activation in human cells (Chan et al., 2002; Chen et al., 2005), although there is some dispute as to whether T2609 may also be phosphorylated by ATM and ATR in certain circumstances (Chen et al., 2007; Jiang et al., 2015; Meek et al., 2007; Yajima et al., 2006). Neither S2056 nor T2609 is required for DNA-PKcs kinase activity but both are important for DNA repair, with current models suggesting that their phosphorylation causes conformational changes that promote DNA-PK disassembly from DSB sites to allow DNA-end ligation (Jette and Lees-Miller, 2015). This is supported by the recent crystal structure of DNA-PKcs in complex with a Ku80 peptide, which suggests that S2056 phosphorylation may regulate Ku80 binding (Sibanda et al., 2017). A third autophosphorylation site, T3950, is located in the kinase domain and may act to switch off DNA-PKcs kinase activity when phosphorylated (Douglas et al., 2007; Sibanda et al., 2017).

For ATM and ATR, roles for autophosphorylation are more controversial. Human ATM phosphorylates itself on S1981, an event proposed to transition the kinase from an inactive dimer into active monomers in response to DNA damage (Bakkenist and Kastan, 2003). While experiments with A-T cells complemented with ATM autophosphorylation mutants, including S1981A, supported this model (Kozlov et al., 2006), subsequent studies found that S1981 does not affect ATM activation *in vitro* and can be uncoupled from ATM monomerization (Dupré et al., 2006; Lee and Paull, 2005). Furthermore, mice expressing mutant ATM that cannot be phosphorylated on this residue (and on additional autophosphorylation sites), are phenotypically normal with no observed defects in ATM-dependent responses (Daniel et al., 2008; Pellegrini et al., 2006). A recent single-particle EM structure of dimeric human ATM also does not support a role for S1981 in ATM activation (Lau et al., 2016). Further work is clearly needed to elucidate the functions of S1981 and other ATM autophosphorylation events.

Like ATM and DNA-PKcs, ATR autophosphorylates *in vitro* (Cliby et al., 1998). One ATR autophosphorylation site is T1989 (Liu et al., 2011; Nam et al., 2011), although there is disagreement about whether T1989 plays a major role in ATR function. T1989 is located in the highly-conserved ATR FAT domain, but the site itself and surrounding residues are not

well-conserved, even in mammals. Nevertheless, this site can be a useful marker for ATR activation. Commercial antibodies are also available against another phosphorylation site, S428, in the ATR N-terminal region, but this site is not induced upon ATR activation nor required for ATR-dependent phosphorylation events (Liu et al., 2011; Nam et al., 2011), so caution should be employed before using this mark as a readout of ATR activity.

Recruitment to DNA damage sites

ATM, ATR and DNA-PKcs must be tightly regulated to prevent aberrant activation that could lead to toxic DNA repair, cell cycle arrest, senescence or apoptosis. Indeed, each kinase requires a specific protein co-factor for stable recruitment to DNA damage sites (Figure 2): this is NBS1 for ATM (Falck et al., 2005), ATRIP for ATR (Zou and Elledge, 2003), and Ku80 for DNA-PKcs (Gell and Jackson, 1999; Singleton et al., 1999). Although each kinase requires a distinct factor, a common principle applies to their recruitment because NBS1, ATRIP and Ku80 all share a related C-terminal motif required for PIKK binding, probably via interactions with the PIKK HEAT repeats (Ball et al., 2005; Falck et al., 2005; Spagnolo et al., 2006).

DNA-PKcs: regulator of DNA repair by NHEJ

Mechanism of DNA-PKcs activation and its function in cells

DNA-PKcs is activated when it is recruited to DSBs by Ku, as described above. The crystal structure of the Ku heterodimer revealed it to be basket-shaped, with a central cavity that accommodates a dsDNA end (Walker et al., 2001). DNA-PK thus acts as a sensor for DSBs, and its major role is to promote NHEJ (Jette and Lees-Miller, 2015). NHEJ repairs most DSBs in mammalian cells, except when they occur at DNA replication forks when homologous recombination (HR) is preferentially used (Beucher et al., 2009; Karanam et al., 2012). As its name implies, NHEJ involves ligation of two broken DNA ends without needing a repair template. Although classical NHEJ is often described as error-prone and mutagenic (for example in the context of gene-editing by CRISPR-Cas9 (Hsu et al., 2014)), in reality it is remarkably efficient and mostly accurate (Bétermier et al., 2014). Only when classical NHEJ fails must cells use an alternative end-joining pathway such as that mediated by DNA polymerase θ (POLQ), which can introduce extensive mutations (van Schendel et al., 2015).

Mechanistically, NHEJ is initiated by Ku and DNA-PKcs binding to DSBs, with the ensuing DNA-PK holoenzyme promoting DNA end-tethering (Graham et al., 2016). Additional NHEJ core factors are subsequently recruited for the ends to be closely aligned and

ligated, including XRCC4 (Li et al., 1995), XLF (Ahnesorg et al., 2006; Buck et al., 2006), and DNA ligase IV (LIG4; (Critchlow et al., 1997; Grawunder et al., 1997)). The NHEJ machinery including DNA-PKcs is stabilized on damaged chromatin by PAXX (Ochi et al., 2015), a second XRCC4-like core NHEJ factor (Figure 3).

Some DSBs repaired by NHEJ require additional accessory proteins such as DNA polymerases and nucleases for their repair. One such factor is the endonuclease Artemis (Moshous et al., 2001), which interacts with DNA-PKcs to promote repair of a subset of DSBs (Ma et al., 2002; Riballo et al., 2004; Rooney et al., 2003). Indeed, in addition to its end-tethering role, a major function of DNA-PKcs is to recruit Artemis to promote DNA-end processing (Jiang et al., 2015; Malu et al., 2012).

DNA-PKcs kinase activity is important for NHEJ, and various putative DNA-PK substrates have been reported, including other NHEJ factors (Jette and Lees-Miller, 2015). However, it is not yet clear whether NHEJ requires DNA-PKcs to phosphorylate any proteins other than itself. While DNA-PK inhibition does not block recruitment of downstream factors such as LIG4 (Jiang et al., 2015), it does prevent formation of short-range synaptic complexes (Graham et al., 2016). This indicates that DNA-PKcs is a physical block to NHEJ and that autophosphorylation relieves this to allow DNA-end alignment and completion of repair.

Physiological roles of DNA-PKcs in development

During lymphocyte development, DSBs are generated in immunoglobulin and T-cell receptor loci to generate immune-receptor diversity by V(D)J and class-switch recombination (Alt et al., 2013). As such DSBs require NHEJ for efficient repair, defects in most core NHEJ factors cause severe immunodeficiency in mammals. PAXX and XLF are exceptional in this regard, as both must be simultaneously lost to produce substantial immune defects in mice due to their partial functional redundancy in B cells (Balmus et al., 2016; Kumar et al., 2016; Lescale et al., 2016; Liu et al., 2017). Like PAXX, ATM is also crucial for V(D)J recombination in the absence of XLF, as discussed below.

In addition to abrogating B and T cell development, specific mutations in DNA-PKcs that inhibit its kinase activity also cause profound neurological defects (Jiang et al., 2015; Woodbine et al., 2013), phenotypes that are also associated with deficiencies in certain other NHEJ components (Balmus et al., 2016; Gao et al., 1998; Saito et al., 2016). While this suggests that NHEJ factors play important roles in repairing DSBs that arise during

differentiation of neural progenitor cells, the nature and etiology of such DSBs remain to be determined.

ATM: master regulator of cellular responses to DSBs

ATM activates a DSB-signalling cascade

ATM is the apical kinase responsible for global orchestration of cellular responses to DSBs, which include DNA repair, checkpoint activation, apoptosis, senescence, and alterations in chromatin structure, transcription and pre-mRNA splicing (Shiloh and Ziv, 2013). To achieve this, ATM phosphorylates hundreds of substrates in response to DNA damage (see for example (Matsuoka et al., 2007)), although it is unclear what proportion of these is functionally important. Most ATM substrates are probably also phosphorylated by ATR in response to replication stress, and a few such as histone H2AX by DNA-PKcs as well. Notably, ATM phosphorylates and activates other protein kinases that phosphorylate yet more substrates, meaning that ATM-dependent signaling events are not just restricted to factors directly phosphorylated by ATM (Shiloh and Ziv, 2013). A well-characterized example of this is the CHK2 kinase, which is activated by ATM and phosphorylated on multiple sites including T68 (Ahn et al., 2000; Brown et al., 1999; Matsuoka et al., 1998; 2000; Melchionna et al., 2000). While CHK2-T68 phosphorylation is routinely used to indicate ATM activation, it is worth noting that this modification may not be solely ATM-dependent in all circumstances (Matsuoka et al., 2000). Checkpoint signaling is largely intact in CHK2-deficient cells, but they are resistant to IR, highlighting roles for ATM and CHK2 in triggering apoptosis (Hirao et al., 2002; Takai et al., 2002).

Activation of p53 by ATM

The tumor suppressor p53 is stabilized in response to DNA damage to activate a transcriptional program that can lead to cell cycle arrest in G1, senescence or apoptosis (Shiloh and Ziv, 2013). ATM can phosphorylate p53 on multiple sites, with modification of S15 having been particularly well-studied (Banin et al., 1998; Canman et al., 1998; Siliciano et al., 1997). S15 phosphorylation was proposed to inhibit interaction of p53 with the ubiquitin ligase MDM2, resulting in rapid p53 stabilization and activation in response to DSBs (Shieh et al., 1997); however, subsequent studies have questioned this model and indicated that ATM-dependent phosphorylation of MDM2 may be more important for p53 stabilization (Cheng and Chen, 2010). It is important to note, however, that ATM controls p53 stability via multiple

mechanisms by phosphorylating not just p53 itself but also other proteins that directly or indirectly influence p53 stability (Shiloh and Ziv, 2013). One such protein is the p53 co-factor hnRNP K, which is phosphorylated by ATM in response to DSBs and protected from MDM2-mediated degradation (Moumen et al., 2013; 2005). As with various ATM-dependent processes, ATR can activate similar pathways in response to other types of genotoxic stress (Lakin et al., 1999; Moumen et al., 2005; Tibbetts et al., 1999).

Mechanism of ATM activation

ATM is recruited to chromatin in response to DSBs (Andegeko et al., 2001; Bekker-Jensen et al., 2006), in a process that requires ATM binding to the C-terminus of NBS1 (Falck et al., 2005), a component of the MRE11-RAD50-NBS1 (MRN) complex (Carney et al., 1998). This complex can stimulate ATM kinase activity directly in vitro (Lee and Paull, 2004; 2005), and is required for optimal ATM signaling in cells (Carson et al., 2003; Uziel et al., 2003), indicating that MRN both recruits ATM to DNA lesions and stimulates ATM kinase activity once there. However, the exact mechanism whereby MRN activates ATM is still not understood. MRN/DSB-independent means of ATM activation also exist, for example in response to oxidative stress or chromatin changes (Guo et al., 2010; Kaidi and Jackson, 2013).

Early biochemical studies with immunoprecipitated endogenous ATM from cell extracts demonstrated that pre-treating cells with DSB-inducing agents results in a more active ATM kinase (Banin et al., 1998; Canman et al., 1998). This may be explained, at least in part, by ATM autophosphorylation, as well as the existence of regulatory mechanisms promoting ATM activation following DSB-associated chromatin changes. Upon DNA damage, exposed nucleosomes bearing the H3K9me3 histone mark are recognized by the TIP60/KAT5 acetyltransferase (Sun et al., 2009), which is thus recruited to damaged chromatin and shielded from dephosphorylation (Kaidi and Jackson, 2013). Phosphorylated TIP60 is activated and stimulates ATM by acetylating its FATC motif on K3016 (Sun et al., 2005; 2007). This process may be promoted by FOXO3A and inhibited by NOTCH1, which compete for binding to the ATM C-terminus (Adamowicz et al., 2016). Notably, the enzyme mediating TIP60 phosphorylation is the c-Abl tyrosine kinase (Kaidi and Jackson, 2013), which is itself phosphorylated and activated by ATM in response to DNA damage (Baskaran et al., 1997; Shafman et al., 1997). These data thus suggest the existence of a positive feedback loop that reinforces ATM activation.

Roles of ATM in DSB repair

While most (~80%) IR-induced DSBs outside of S phase are repaired by NHEJ independently of ATM (Beucher et al., 2009), a minority is repaired by a pathway requiring ATM, DNA-PK, the MRN complex and Artemis (Riballo et al., 2004). This subset may include DSBs in heterochromatic regions and those with blocked ends that require processing (Alvarez-Quilón et al., 2014; Goodarzi et al., 2008). Mechanistically, MRN-dependent ATM activation may trigger phosphorylation of DNA-PKcs to promote Artemis recruitment and activation (Goodarzi et al., 2006; Jiang et al., 2015), a model supported by the demonstration that Ku and MRN can simultaneously localize to the same DSB site in cells (Britton et al., 2013). Together with PAXX, ATM plays at least one important additional role in NHEJ that is not yet clear, but which is redundant with XLF and is therefore not normally apparent (Balmus et al., 2016; Zha et al., 2011). Given that ATM substrates on chromatin involved in DNA-end protection and synapsis, such as H2AX and 53BP1, also share redundant functions with XLF in NHEJ, it is possible that the role of ATM in NHEJ relates to DSB-end bridging (Kumar et al., 2014b).

ATM also promotes HR, the other major DSB repair pathway. HR requires DSB-end processing to produce tracts of single-stranded DNA (ssDNA) that, once bound by the recombinase RAD51, invade another DNA molecule (normally a sister chromatid) that serves as a repair template (Kowalczykowski, 2015). This process, termed DNA-end resection, is a key determinant of DSB repair pathway choice (Chapman et al., 2012), and is stimulated by ATM (Adams et al., 2006; Cuadrado et al., 2006; Jazayeri et al., 2006; Myers and Cortez, 2006). In part, this stimulation occurs via ATM directly phosphorylating the MRN-interacting protein CtIP, a key factor required for resection initiation (Sartori et al., 2007; Shibata et al., 2011; Wang et al., 2013). ATM-mediated CtIP phosphorylation may also impact on later stages of HR, as it promotes the removal of Ku from single-ended DSB ends at collapsed replication forks to allow the strand invasion step of HR to proceed (Britton et al., 2013; Chanut et al., 2016). Despite these connections, it is important to note that ATM is not essential for HR and that HR-driven processes can take place in its absence.

ATM initiates and sustains chromatin-based DDR signaling

DSBs trigger rapid phosphorylation of S139 in the C-terminal tail of histone variant H2AX (Rogakou et al., 1998). This modification, termed γ H2AX, is primarily mediated by ATM in response to DSBs and forms the foundation of a chromatin-based signaling cascade involving phosphorylation, ubiquitylation and other post-translational modifications (Scully and Xie,

2013) (Figure 4). The major reader of the γ H2AX mark is MDC1, which possesses C-terminal tandem BRCT domains that specifically bind γ H2AX at DSB sites (Stucki et al., 2005). There, MDC1 is stabilized on chromatin by ATM, which phosphorylates MDC1 on T4 to promote MDC1 dimerization via its FHA domain (Jungmichel et al., 2012; Liu et al., 2012). MDC1 is constitutively phosphorylated on multiple other residues by CK2, which are recognized by the FHA-BRCT region of NBS1 to promote MDC1-MRN retention on γ H2AX-containing chromatin (Chapman and Jackson, 2008; Melander et al., 2008; Spycher et al., 2008). An attractive model is that MRN recruitment by MDC1 leads to further recruitment of ATM via its interaction with NBS1, leading to additional γ H2AX formation and MDC1-MRN-ATM recruitment, thus spreading the assembly along chromatin and amplifying DDR signaling.

At DSB sites, ATM phosphorylates MDC1 on T-Q-X-F motifs that are recognized by the FHA domain of the ubiquitin ligase RNF8, thus promoting RNF8 retention on damaged chromatin (Huen et al., 2007; Kolas et al., 2007; Mailand et al., 2007). With the ubiquitin-conjugating enzyme UBC13, RNF8 stimulates ubiquitylation of the linker histone H1 (Thorslund et al., 2015). Ubiquitylated H1 is recognized by ubiquitin-binding domains in another ubiquitin ligase, RNF168, which ubiquitylates K15 of H2A-type histones to promote recruitment of the scaffold protein 53BP1 to channel DSB repair towards NHEJ (Fradet-Turcotte et al., 2013; Mattioli et al., 2012; Thorslund et al., 2015). In turn, 53BP1 recruits further proteins by mechanisms that involve ATM. One such factor, PTIP, associates directly with the 53BP1 extreme N-terminus in a manner requiring PTIP BRCT domains and ATM-dependent phosphorylation of 53BP1 on S25 (Jowsey et al., 2004; Muñoz et al., 2007). A second, RIF1, interacts with 53BP1 independently of PTIP but in a manner requiring phosphorylation of the 53BP1 N-terminus by ATM; ensuing 53BP1 complexes containing PTIP, RIF1, REV7/MAD2L2 and other factors promote DSB repair by NHEJ through mechanisms that are not yet clear (Hustedt and Durocher, 2016).

Some 53BP1 functions are antagonized by BRCA1, the product of a highly penetrant breast and ovarian tumor suppressor gene (Miki et al., 1994). ATM, the loss of which also predisposes to breast cancer (Swift et al., 1987), functionally phosphorylates BRCA1 on multiple residues in response to DNA damage (Cortez et al., 1999). BRCA1 exists in several protein complexes with discrete functions in maintaining genome stability as well as other processes, but its major function seems to be to promote HR at the expense of 53BP1-mediated NHEJ (Hustedt and Durocher, 2016). The complex mechanisms whereby 53BP1 and BRCA1 assemblies counteract each other and otherwise function to maintain genome integrity—and

how such processes are regulated by ATM and subject to cell-cycle control (e.g. (Orthwein et al., 2015))—are still emerging and beyond the scope of this review.

Notably, γ H2AX-marked chromatin is transcriptionally inactive (Chou et al., 2010; Polo et al., 2012), possibly to help prevent collisions between DNA repair and transcription machineries. Such transcriptional silencing may also counteract formation of DNA-RNA hybrids such as R loops (Britton et al., 2014). Depending on its cellular context, transcriptional silencing can depend on ATM, ATR and/or DNA-PKcs (Britton et al., 2014; Pankotai et al., 2012; Shanbhag et al., 2010), with ATM being particularly important for nucleolar DNA silencing (Kruhlak et al., 2007). Mechanistically, ATM-dependent transcriptional silencing of RNA polymerase II depends on phosphorylation of the BAF180 subunit of the PBAF SWI/SNF chromatin remodeling complex (Kakaroukas et al., 2014).

Another class of ATM- and H2AX-dependent, transcriptionally repressed chromatin foci are 53BP1-OPT domains, which form specifically in G1 phase cells around DNA lesions carried over from the previous cell cycle (Harrigan et al., 2011; Lukas et al., 2011). OPT domains disappear as cells enter S phase and may contain under-replicated DNA, which is shielded from end-processing by 53BP1 until the next round of DNA replication starts.

Other roles for γ H2AX foci may be to concentrate factors in “liquid-demixed” zones, where DSB clustering may enhance DNA-repair efficiency (Altmeyer et al., 2015; Caron et al., 2015). γ H2AX chromatin domains might also define boundaries to avert over-resection of DSBs and counteract limitations in repair-factor supply (Ochs et al., 2016). Importantly, while ATM predominates in generating γ H2AX under many circumstances, in some contexts, ATR and/or DNA-PKcs can substitute partly or entirely. For example, during male mammalian meiosis, ATR-mediated γ H2AX generation globally represses almost all transcription on the non-recombining X-Y chromosome pair (Royo et al., 2013).

ATR: an essential kinase involved in DNA replication stress responses

ATR activation is a multi-step process

ATR is the apical DNA replication stress response kinase, phosphorylating many substrates in response to agents such as UV (see for example (Matsuoka et al., 2007)). Unlike ATM and DNA-PKcs, ATR is essential in proliferating cells (Brown and Baltimore, 2000; de Klein et al., 2000), which hampered efforts to study its functions until the recent development of selective small-molecule ATR kinase inhibitors (Fokas et al., 2014). Nonetheless, previous work using *Xenopus* egg extracts, RNA interference or cells expressing hypomorphic ATR

variants has been highly informative. One such cell type is derived from patients with Seckel syndrome, a hereditary form of microcephalic dwarfism caused by an *ATR* pre-mRNA splicing mutation that results in very low *ATR* protein levels (O'Driscoll et al., 2003).

In contrast to *ATM* and *DNA-PKcs*, which respond primarily to *DSBs*, *ATR* is activated by a much wider range of genotoxic stresses. This is because *ATR* is recruited, via its partner protein *ATRIP*, to extended tracts of *ssDNA* coated with the *ssDNA*-binding protein complex, replication protein A (*RPA*) (Zou and Elledge, 2003) (Figure 5). Such *RPA*-coated *ssDNA* is generated by nucleolytic processing of various forms of damaged DNA (Raderschall et al., 1999), or by helicase-polymerase uncoupling at stalled replication forks (Byun et al., 2005).

ATR recruitment to *RPA*-*ssDNA* is not sufficient for optimal activation, but also requires the presence of activator proteins and *ssDNA*/*dsDNA* junctions. The best-characterized *ATR* activator is *TopBP1*, which contains an *ATR*-activation domain that stimulates *ATR* kinase activity (Kumagai et al., 2006) via contacts with both *ATRIP* and the C-terminal *PRD* in *ATR* (Mordes et al., 2008). As with *ATR* itself, *TopBP1* loss or an inactivating mutation in the *TopBP1* *ATR*-activation domain is lethal in mammalian cells (Jeon et al., 2011; Zhou et al., 2013). One important interaction of *TopBP1* is its binding to the C-terminal tail of the *RAD9* subunit of the *RAD9*-*RAD1*-*HUS1* (9-1-1) complex (Delacroix et al., 2007; Lee et al., 2007), a ring-shaped heterotrimer that is loaded onto *RPA*-*ssDNA*/*dsDNA* junctions by the *RAD17*-*RFC* complex (Ellison and Stillman, 2003; Zou et al., 2002; 2003). However, while the *TopBP1*-*RAD9* interaction is important for *ATR* activation, it is not essential for *TopBP1* recruitment to stalled replication forks (Lee and Dunphy, 2010; Yan and Michael, 2009), suggesting that this must be mediated by other, potentially overlapping, mechanisms. In line with this, other factors including *MRN* and *BLM* have been implicated in *TopBP1* recruitment (Blackford et al., 2015; Duursma et al., 2013); but it is likely that *TopBP1* localization to damaged DNA is complex, requiring numerous protein-protein interactions influenced by cell-cycle stage and genotoxic insult.

Recently a second *ATR*-activator protein, *ETAA1*, was identified and found to contain an *ATR*-activation domain similar to that of *TopBP1* (Bass et al., 2016; Feng et al., 2016; Haahr et al., 2016). Unlike *TopBP1*, *ETAA1* is recruited to *RPA*-*ssDNA* via direct binding to *RPA*. It is thus tempting to speculate that *TopBP1* and *ETAA1* may be recruited to different types of aberrant DNA structures to stimulate *ATR* towards discrete sets of substrates.

Roles for ATR in checkpoint signaling

A key ATR function is to phosphorylate and activate the protein kinase CHK1 (Guo et al., 2000; Hekmat-Nejad et al., 2000; Liu et al., 2000; Zhao and Piwnica-Worms, 2001). When activated, CHK1 promotes proteasomal degradation of CDC25A, a phosphatase that removes inhibitory modifications from cyclin-dependent kinases (CDKs) (Bartek et al., 2004). CDC25A inactivation by CHK1 in response to genotoxic stress thereby slows or arrests cell-cycle progression by reducing CDK activity. This presumably allows more time for DNA repair so that cells do not enter mitosis prematurely, or if the damage is too extensive, for activation of senescence or apoptotic pathways. The importance of the ATR-CHK1-CDC25A axis for cell survival is highlighted by the lethality associated with ATR or CHK1 inhibition being circumvented by CDC25A inactivation (Carette et al., 2011; Ruiz et al., 2016).

In addition to preventing premature mitotic entry, it was previously thought that ATR played a role in regulating replisome stability (Cortez, 2015). However, recent data using methods that allow observation of global replisome stability genome-wide, suggest that in both human and yeast cells, the replisome is not disassembled when cells lacking ATR/Mec1 experience replicative stress (De Piccoli et al., 2012; Dungrawala et al., 2015). Instead, ATR may prevent replication fork collapse primarily through the following mechanisms. First, by inhibiting CDKs, ATR signaling restrains replication origin firing (Costanzo et al., 2003). This is important to avoid exhaustion of replication and repair factor pools, particularly RPA (Toledo et al., 2013). Second, ATR limits replication fork collapse by directly targeting helicases such as SMARCAL1, which can produce fork configurations that are recognized and cleaved by SLX4/MUS81 nuclease complexes (Couch et al., 2013; Forment et al., 2011; Ragland et al., 2013). Finally, ATR regulates deoxyribonucleotide availability in mammalian cells by promoting upregulation of the ribonucleotide reductase subunit RRM2 at the transcriptional and post-translational levels in response to DNA damage (Buisson et al., 2015; D'Angiolella et al., 2012; Pfister et al., 2015). This is highlighted by studies showing that an extra copy of *RRM2* protects against some of the genome instability associated with ATR loss in mice (López-Contreras et al., 2015).

ATR activates the Fanconi anemia pathway

While ATR phosphorylates many of the same substrates as ATM (Matsuoka et al., 2007), it may also have unique targets—particularly those functioning in pathways connected to replication fork repair. One of these is the Fanconi anemia (FA) pathway, which promotes

repair of DNA interstrand crosslinks (ICLs) (Ceccaldi et al., 2016). FA is a rare hereditary genetic disorder characterized by bone marrow failure and cancer predisposition, with some patients also displaying microcephaly and dwarfism features reminiscent of ATR-Seckel syndrome. These common characteristics may reflect a requirement for ATR in ICL repair by promoting FANCD2 mono-ubiquitylation, a key step in the FA pathway (Andreassen et al., 2004). This is probably achieved via phosphorylation of FANCI by ATR (Ishiai et al., 2008), an event that may also play a more general role in the replication stress response by regulating dormant origin firing (Chen et al., 2015).

Cross-talk between ATM, ATR and DNA-PKcs

The G1/S, intra-S and G2/M checkpoints induced by DNA damage signaling are similar in that the end-point of all three is to inhibit CDK activity. DSBs are not resected to generate significant amounts of RPA-ssDNA in G1 (Jazayeri et al., 2006), so the G1/S checkpoint is believed to be controlled primarily by ATM rather than ATR. By contrast, both ATM and ATR contribute to establishment and maintenance of the intra-S and G2/M checkpoints, in part because ATM-dependent DNA-end resection provides the RPA-ssDNA signal for ATR recruitment and activation (Adams et al., 2006; Cuadrado et al., 2006; Jazayeri et al., 2006; Myers and Cortez, 2006). This ATM-dependent ATR activation is one example of functionally important cross-talk between PIKKs. As discussed earlier, ATM- and ATR-dependent phosphorylation of DNA-PKcs may be important for DNA repair, and recent evidence suggests that DNA-PK also phosphorylates ATM to restrain its activity (Zhou et al., 2017). Furthermore, ATR has been reported to phosphorylate ATM in response to UV exposure (Stiff et al., 2006).

Additional biological roles for ATM, ATR and DNA-PKcs

Activation in response to telomere dysfunction

Under normal circumstances, chromosome ends do not trigger DNA damage signaling, as telomeric DNA is bound by the shelterin complex, which inhibits ATM, ATR and DNA-PK via multiple mechanisms (De Lange, 2009). For instance, shelterin component TRF2 promotes formation of a T-loop structure that sequesters the telomeric DNA end, thereby blocking MRN and Ku binding and inhibiting ATM and DNA-PK activities, respectively. Furthermore, the POT1 component of shelterin binds telomeric ssDNA, thus excluding RPA and preventing ATR recruitment. In cells lacking telomerase, telomeres become progressively shorter after

each DNA replication cycle, eventually leading to telomere dysfunction and ATM/ATR-mediated cell cycle arrest, followed by senescence (d'Adda di Fagagna et al., 2003).

Connections between ATM and transcriptional stress

Regulators of RNA metabolism are frequently identified in screens for DDR components (e.g. (Paulsen et al., 2009)). Some appear to have dual roles in transcription and DNA repair (Polo et al., 2012), while others may be targeted by PIKKs to modulate RNA metabolism. Indeed, emerging evidence suggests the spliceosome is both a target and an effector for ATM in response to R-loop formation (Tresini et al., 2015). Interestingly, the helicase senataxin, which promotes R loop resolution (Skourti-Stathaki et al., 2011), is mutated in ataxia-ocular apraxia 2 (Moreira et al., 2004). This form of ataxia is reminiscent of A-T but patients are not radiosensitive and do not get lymphoid cancers. These findings raise the possibility that at least some neurological phenotypes associated with ATM deficiency may be linked to defective resolution of DNA-RNA hybrids.

ATR as a sensor of mechanical forces

Chromatin dynamics generate mechanical forces that can be transmitted to the nuclear envelope via lamin-associated chromatin domains. In yeast, Mec1^{ATR} facilitates DNA replication by releasing such domains from the nuclear envelope as replication forks approach them, thereby avoiding generation of aberrant DNA topology that could lead to fork reversal (Bermejo et al., 2011). ATR is also activated at the nuclear envelope in human cells in response to mechanical stress in a manner apparently independent of RPA (Kumar et al., 2014a). Intriguingly, HEAT repeats, such as those in PIKKs, can act as elastic connectors (Grinthal et al., 2010), suggesting that ATR and possibly other PIKKs may sense mechanical forces directly.

Connections between PIKKs and viral infection

Viruses cause various human diseases and are significant contributors to cancer (Weitzman and Weitzman, 2014). Viral genomes and their replication intermediates can resemble damaged DNA to the host cell, which could trigger DNA-damage responses that might limit the productivity of viral infection. Presumably as a consequence, many viruses encode proteins that specifically inhibit ATM, ATR and/or DNA-PK activity, either completely or partially. A common strategy is to hijack cellular ubiquitylation pathways to target PIKKs (Lees-Miller et

al., 1996), or their activator proteins such as TopBP1 and the MRN complex (Blackford et al., 2010; Stracker et al., 2002), for proteasomal degradation.

Small molecule inhibitors of ATM, ATR and DNA-PK as potential therapeutic agents

As the DDR can be activated at early stages in tumorigenesis due to oncogene-induced replication stress or processes such as telomere shortening, this has suggested that cell-cycle arrest or cell death enforced by PIKK signaling may serve as a barrier to tumorigenesis (Bartkova et al., 2005; 2006; d'Adda di Fagagna et al., 2003; Gorgoulis et al., 2005). In line with this, ATM is frequently mutated in human cancers (Macheret and Halazonetis, 2015). Why then, should inhibiting PIKKs be a promising avenue for cancer treatment? The answer lies in the fact that precisely because rapid, unregulated cell division and genome instability are hallmarks of cancer (Hanahan and Weinberg, 2011), tumor cells are inherently vulnerable to additional replication stress, exogenous DNA damage and/or DDR inhibition. This probably in large part explains therapeutic successes with radiotherapy and various DNA-damaging chemotherapeutics, and also contributes to the anti-cancer properties of emerging DDR-enzyme inhibitors (O'Connor, 2015). Small molecule inhibitors are now available against all three PIKKs, and have entered phase I or II clinical trials both as single agents and in combination with radiotherapy or traditional chemotherapeutics (Brown et al., 2017; O'Connor, 2015). ATR is a particularly attractive target for anti-cancer therapy, as tumor cells are likely to rely heavily on ATR for survival due to their high burden of replication stress. This provides both the rationale for the use of ATR inhibitors in multiple types of cancers, and provides an explanation for the fact that ATR is not a strong tumor suppressor despite its role in maintaining genome stability (Lecona and Fernandez-Capetillo, 2014).

Furthermore, genome instability in many tumors is caused by defects in certain DNA repair pathways, such as HR. This offers the potential for targeted therapies to exploit the concept of synthetic lethality, whereby loss of one cellular pathway results in high reliance on another pathway that is not essential under normal settings. While this is perhaps best exemplified by the use of PARP inhibitors to target HR-deficient tumors (Bryant et al., 2005; Farmer et al., 2005), it is notable that growing evidence highlights how loss of ATM and other DDR factors also leads to PARP-inhibitor sensitivity in cancers (Lord and Ashworth, 2017). Interestingly, and perhaps not surprisingly given their somewhat overlapping functions, synthetic lethal relationships exist between ATM and DNA-PKcs (Gurley and Kemp, 2001; Sekiguchi et al., 2001), and between ATM and ATR (Balmus et al., 2012; Reaper et al., 2011);

although apparently not between ATR and DNA-PKcs (Middleton et al., 2015). Such relationships might be exploited therapeutically by specific DDR-PIKK inhibitor drugs in certain cancers deficient in ATM, ATR or DNA-PK.

Finally, we note that our growing appreciation of DDR-PIKK biology is suggesting therapeutic opportunities in other disease areas. ATM inhibition has, for instance, been suggested as a potential anti-retroviral strategy (Lau et al., 2005). Furthermore, there is a growing realization that hyperactive DDR signaling is associated with various neurodegenerative diseases, as highlighted by recent work showing that ATM inhibition ameliorates pathologies in models of Huntington's disease (Lu et al., 2014).

Conclusions and future perspectives

Since their discovery over 20 years ago, our understanding of ATM, ATR and DNA-PK has improved dramatically. Nevertheless, major questions still remain. One key issue is the current lack of high-resolution DDR-PIKK structures. Another is separating out functionally important phosphorylation events from the many residues that are modified simply because they by chance conform to DDR-PIKK consensus motifs. New and emerging technologies, such as super-resolution microscopy, cryo-electron microscopy, CRISPR-Cas9 and affordable high-throughput sequencing will no doubt help address these and other issues. Combining such advances with selective PIKK inhibitors plus our growing understanding of PIKK functions in normal cells and in disease will surely pave the way for exciting new dimensions in DDR-PIKK research, and hopefully better treatments for cancer as well as other diseases associated with genome instability and/or aberrant DDR-PIKK function.

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FIGURE LEGENDS

Figure 1. (A) PIKK domain organization. Colored boxes represent indicated protein domains; numbers represent amino acid residues. Major PIKK phosphorylation sites are indicated with an encircled letter P. HEAT = huntingtin, elongation factor 3, A subunit of protein phosphatase 2A, TOR1; FAT = FRAP-ATM-TRRAP; PRD = PIKK regulatory domain; FATC = FAT C-terminal. **(B) Crystal structure of DNA-PKcs.** Subdomains are colored as follows: N-terminus, blue; circular cradle, green; FAT domain, pink; kinase domain, yellow; FATC domain, light pink. Reproduced with permission from (Sibanda et al., 2017).

Figure 2. Model for PIKK recruitment and activation in response to DNA damage. DNA-PKcs is recruited and activated by Ku-bound DSB ends. ATM is activated and recruited to DSBs by the MRE11-RAD50-NBS1 (MRN) complex. ATR is recruited to RPA-coated ssDNA by its stable binding partner ATRIP.

Figure 3. Model for the role of DNA-PK in NHEJ. DNA-PKcs is recruited and activated by Ku-bound DSB ends to promote NHEJ. Please note that this model does not reflect the relative numbers of NHEJ factors that may be present at DSBs.

Figure 4. ATM activation promotes a signaling cascade on damaged chromatin. ATM is recruited and activated by the MRN complex at DSBs. A phosphorylation-acetylation cascade sustains ATM activation by c-Abl and chromatin-bound TIP60/KAT5. ATM phosphorylates histone H2AX and MDC1 to activate a phosphorylation-ubiquitylation signaling cascade mediated by RNF8 and RNF168 that results in 53BP1 recruitment. ATM phosphorylates 53BP1 to promote recruitment of its effectors, which are counteracted by BRCA1 and CtIP, also ATM substrates. P = phosphorylation; Me = methylation; Ub = ubiquitylation. See main text for further details. ATM and the MRN complex are shown in yellow. Factors in peach promote NHEJ, and those in green promote HR. Those that promote both or neither are in blue. Rectangular boxes indicate kinases, spheres all other proteins.

Figure 5. ATR recruitment and activation of S phase checkpoint signaling. ATR is recruited to RPA-ssDNA and activated by TopBP1 or ETAA1. ETAA1 is recruited directly to RPA-coated ssDNA. TopBP1 is recruited via mechanisms that are not yet clear, but its role in

ATR activation requires interaction with the RAD9-HUS1-RAD1 (9-1-1) clamp complex, which is loaded onto ds/ssDNA junctions by the RAD17/RFC2-5 clamp loader. ATR signaling activates the CHK1 kinase and restrains fork processing enzymes. CHK1 activation causes CDC25A degradation, leading to inhibition of CDK activity, slowing of cell cycle progression, inhibition of late origin firing and increased nucleotide availability, in part by upregulation of RRM2. ATR and its recruitment factors are shown in blue. Factors with a positive role in ATR stimulation or which are activated by ATR are in green, while those that are inhibited by ATR are shown in peach. See text for further details.

FIGURE 1

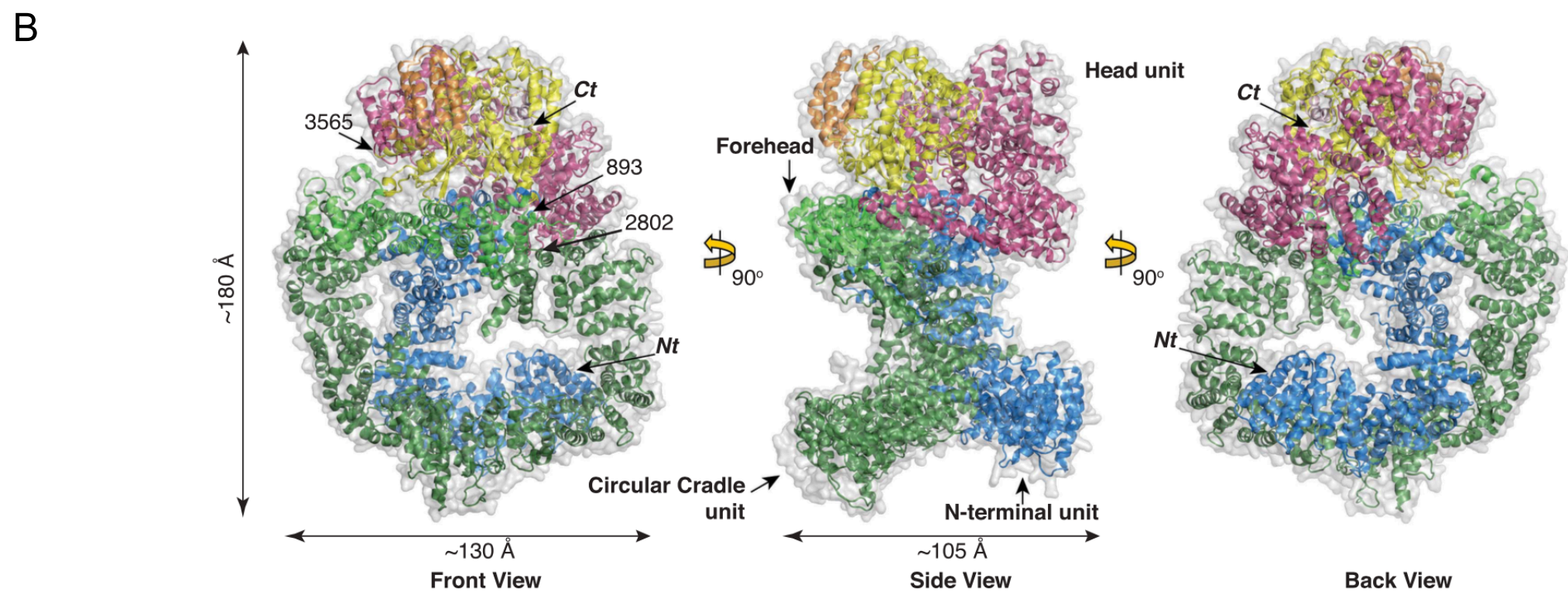
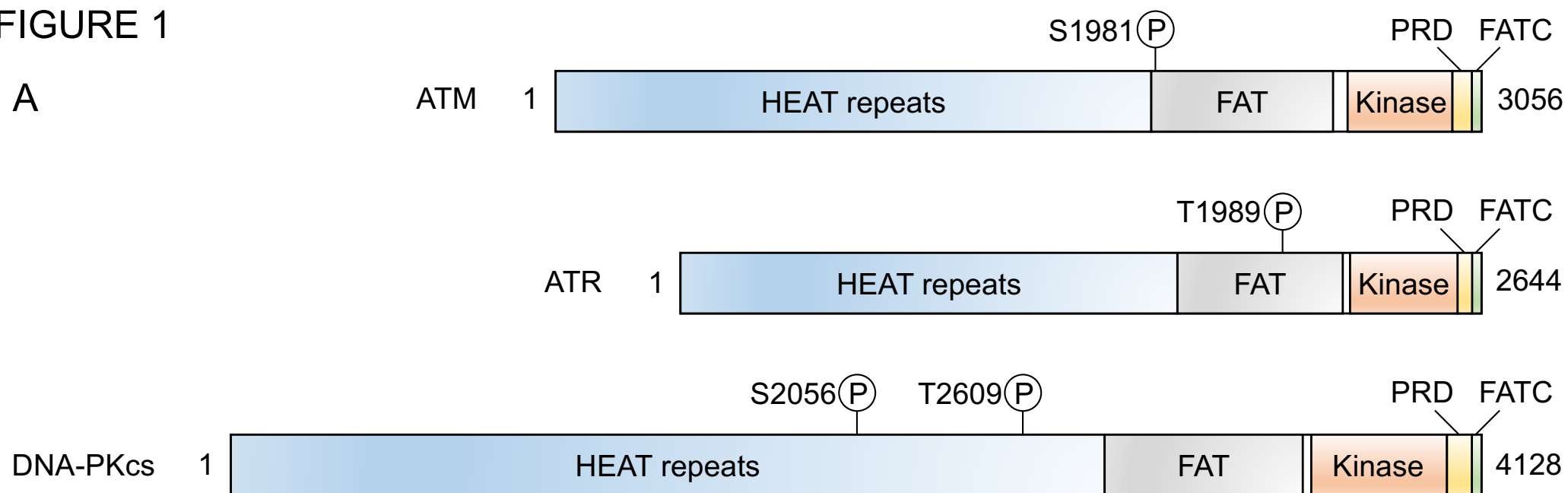


FIGURE 2

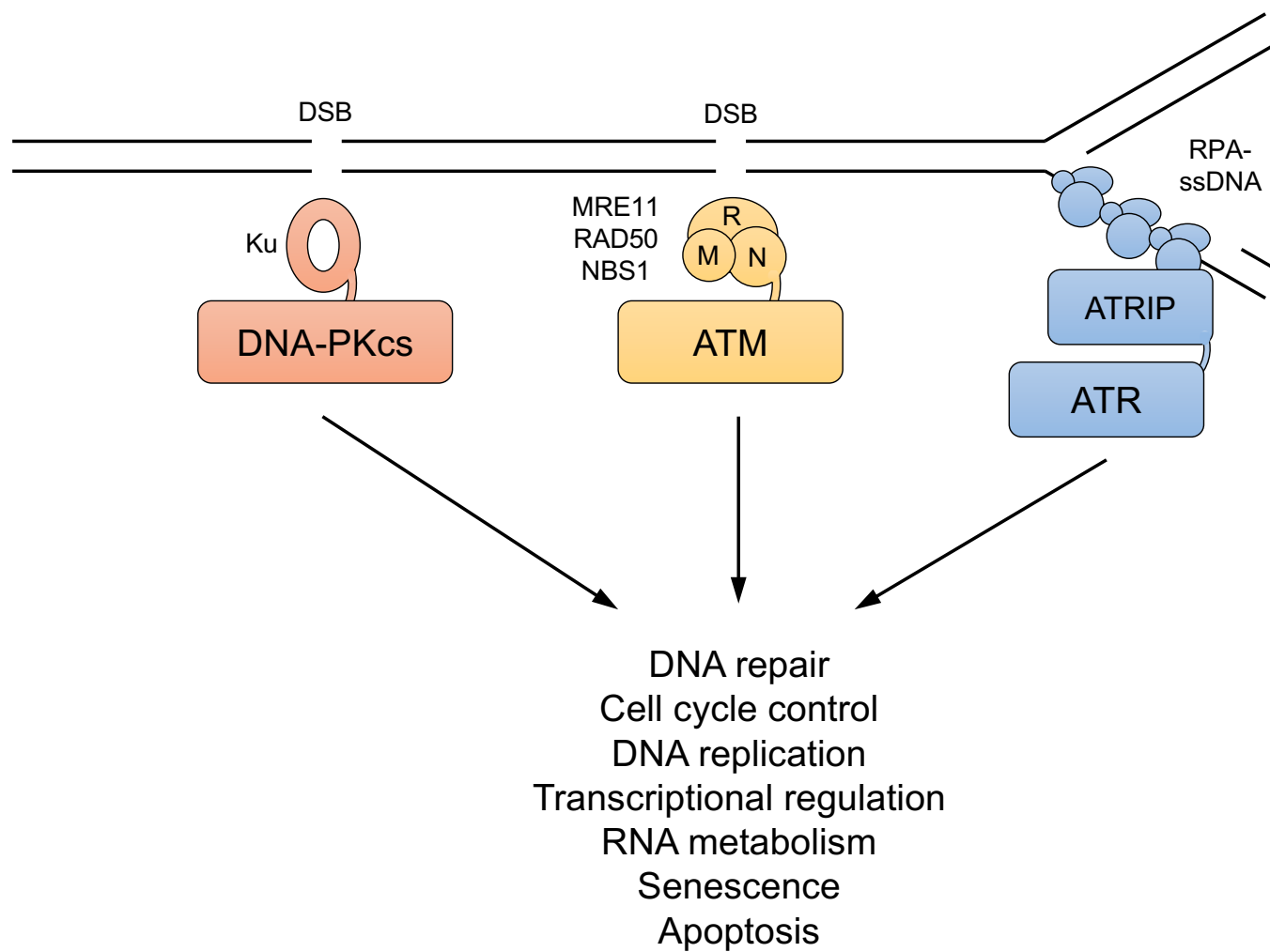


FIGURE 3

Step 1: DSB induction

Step 2: Ku loading

Step 3: DNA-PKcs recruitment
& long-range DNA end synapsis

Step 4: Recruitment of downstream NHEJ
core factors, DNA-PKcs phosphorylation
& short-range DNA-end synapsis

Step 5: DNA-end ligation
& NHEJ complex disassembly

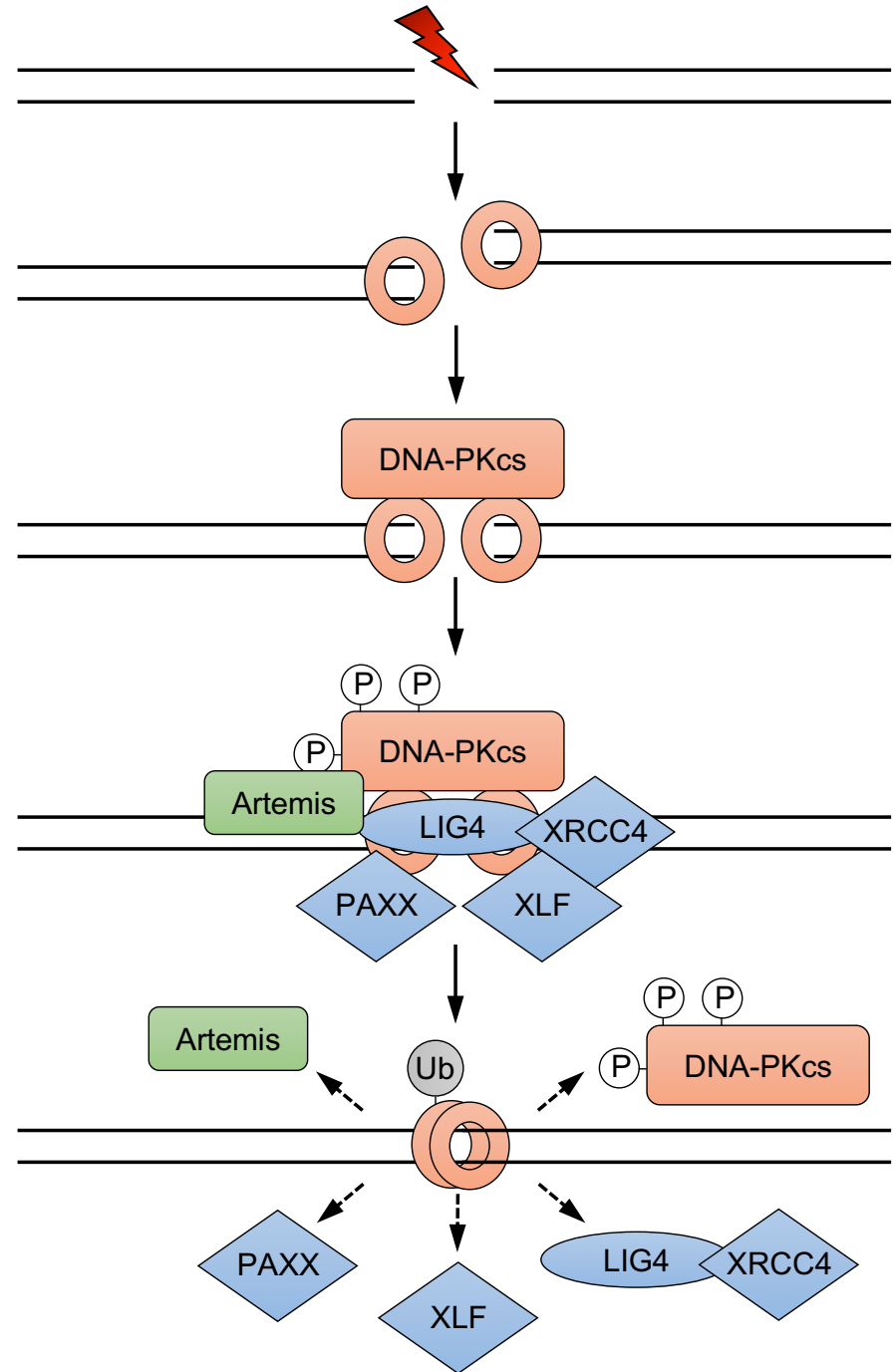


FIGURE 4

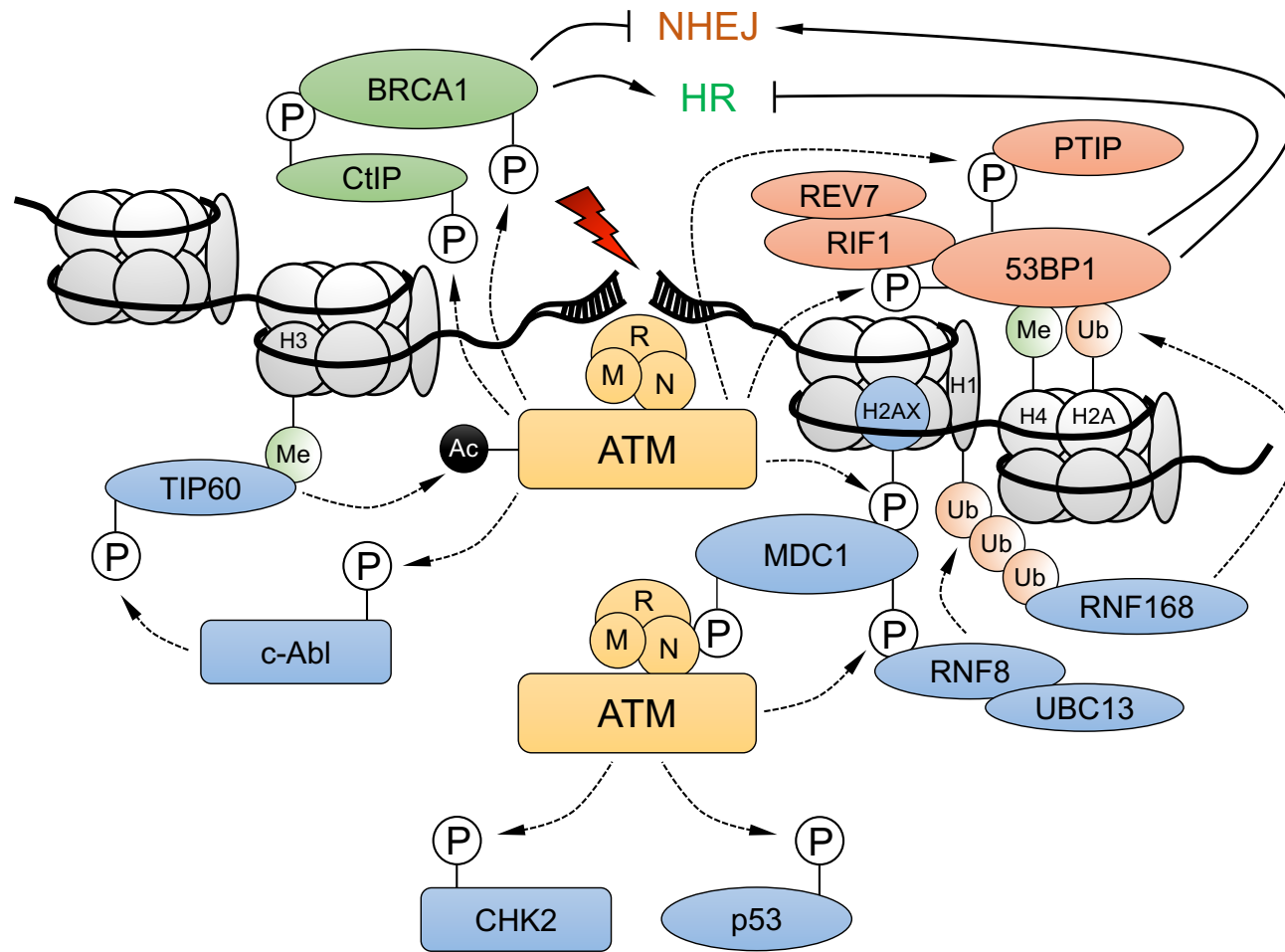


FIGURE 5

