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ATR: An Essential Regulator of Genome Integrity

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Preface

Genome maintenance is a constant problem in all cells and a coordinated response to DNA damage is required to maintain cellular viability and prevent disease. The ATR and ATM protein kinases are master regulators of the DNA damage response, signaling to control cell cycle transitions, DNA replication, DNA repair, and apoptosis. Recent studies have provided insights into the mechanisms controlling ATR activation, helped to explain the overlapping but non-redundant activities of ATR and ATM in DNA damage signaling, and clarified the critical functions of ATR in maintaining genome integrity.

Introduction

All cells have elaborate mechanisms to maintain their genomes. DNA can be damaged during replication, by reactive metabolic byproducts as well as environmental mutagens. Responding to and repairing DNA damage is critical for cell viability and disease prevention.

The DNA damage response (DDR) is a signal transduction pathway that coordinates cell cycle transitions, DNA replication, DNA repair and apoptosis. The major regulators of the DDR are the phosphoinositide 3-kinase related protein kinases (PIKKs), including ataxia-telangiectasia mutated (ATM) and ATM and Rad3 related (ATR). ATM and ATR share many biochemical and functional similarities. Both are large kinases with significant sequence homology and a strong preference to phosphorylate serine or threonine residues that are followed by glutamine. Both target an overlapping set of substrates that promote cell cycle arrest and DNA repair. However, ATR is essential for the viability of replicating human and mouse cells, whereas ATM is not[1–3]. ATM functions in response to rare occurrences of double strand breaks. By contrast, ATR is activated during every S-phase to regulate the firing of replication origins, the repair of damaged replication forks and to prevent the premature onset of mitosis[4, 5] (Fig. 1).

Mutations in *ATM* predispose carriers to cancer and are found in approximately 0.5–1% of the population[6, 7]. People with mutations in both alleles of *ATM* suffer from the neurodegenerative and cancer predisposition disorder ataxia-telangiectasia [8]. Mutations in *ATR* are rare and probably only compatible with viability when heterozygous or hypomorphic. While the only clear link between *ATR* gene mutation and disease is in a few patients with the rare Seckel syndrome (characterized by growth retardation and microcephaly)[9], disruptions in the ATR pathway do cause genomic instability, and ATR is activated by most cancer chemotherapies. Furthermore, ATR signaling is a promising target for cancer drug development[10, 11]. This review will focus on ATR signaling in the DNA damage response, and compare and contrast it with the more specialized role of ATM.

Mechanisms of ATR Activation

The broad functions and physiological importance of ATR derive in large part from recognizing the signals that lead to its activation versus that of ATM. Thus, we will address the mechanism of activation in some detail.

Recognizing DNA damage

Although ATR is activated in response to many different types of DNA damage including double strand breaks (DSB), base adducts, crosslinks, as well as replication stress, a single DNA structure may be responsible. The majority of data suggest that this structure contains single-stranded DNA (ssDNA)[12, 13]. Replication protein A (RPA) coats most forms of ssDNA in the cell, including the ssDNA formed during DNA replication and DNA repair[14]. Consistent with this idea, a mutation in the large subunit of RPA in *S. cerevisiae* (*rfa1-t11)* supports replication but is partially compromised in DNA damage responses[15, 16]. Furthermore, depletion of RPA from *Xenopus* egg extracts reduces the association of ATR with chromatin[12]. Finally, RPA-coated ssDNA (hereafter RPA-ssDNA) is important to localize ATR to sites of DNA damage in both human and budding yeast systems[13], although there are some indications of alternative mechanisms[17–19].

ATR recognition of RPA-coated ssDNA depends upon another protein, ATRIP (ATR-interacting protein)[1]. The stability of ATR and ATRIP are linked and their association is not regulated. There are no known differences in the phenotypes that result upon loss of ATR or ATRIP in any organism, suggesting ATRIP should be considered an obligate subunit of the ATR kinase. Biochemical studies indicate that ATRIP binds RPA directly via evolutionarily conserved binding surfaces[20]. The primary interaction involves an acidic alpha-helix in ATRIP that binds in the basic cleft of the N-terminal OB-fold domain of the large RPA subunit[20]. The *rfa1-t11* mutation causes a charge reversal mutation within this basic cleft and is impaired in binding and recruiting Ddc2/ATRIP to a double-strand break site in *S. cerevisiae*[13].

Although RPA-coated ssDNA may be sufficient to localize the ATR-ATRIP complex, it is not sufficient for ATR activation[21–24]. ATR signaling is dependent on co-localization of the ATR-ATRIP complex with the Rad9-Rad1-Hus1 (9-1-1) complex, a heterotrimeric ring-shaped molecule related in structure and sequence to the replicative sliding clamp, PCNA[25]. Like PCNA, the 9-1-1 complex is loaded onto primer-template junctions in an ATP-dependent reaction that involves a damage-specific clamp loader[26–28]. In other words, the 9-1-1 complex recognizes a DNA end that is adjacent to a stretch of RPA-coated ssDNA. The presence of RPA is critical for this reaction as well, and imparts specificity in loading, creating a preference for the 5′-primer end[26, 29]. Indeed, the *rfa1-t11* mutant is defective in loading the *S. cerevisiae* 9-1-1 complex[30], suggesting RPA-coated ssDNA may be recognized by multiple checkpoint sensors.

So in most cases the structure that causes activation of the ATR checkpoint is single-stranded DNA bound to RPA with an adjacent stretch of double stranded DNA presenting a 5′ junction. Consistent with this idea, in *Xenopus* egg extracts ssDNA does not activate ATR but primed ssDNA with a free 5′-primer end is sufficient to promote ATR signaling[24]. This structure is generated during DNA replication when a polymerase stalls as a consequence of helicase and polymerase uncoupling, and in fact, is required for checkpoint activation[23, 31, 32]. It is also possible for the primed ssDNA structure to form during end resection at double-strand breaks, at telomeres, and even during nucleotide excision repair (Fig. 2).

TOPBP1: an ATR activator

How does the 9-1-1 complex stimulate ATR signaling? *In Saccharomyces cerevisiae*, the 9-1-1 complex has been reported to directly activate the ATR kinase[33]. The C-terminal tail of Ddc1 (the yeast orthologue of Rad9) stimulates Mec1/ATR (the yeast orthologue of ATR) under certain *in vitro* conditions. However, it is unclear whether this is the activation mechanism in yeast cells, and there is no evidence that this direct activation occurs in other organisms. Instead, the 9-1-1 complex brings to ATR a critical activator, TOPBP1[34–36]. TOPBP1 is a BRCT domain-containing protein necessary for ATR activation that can dramatically stimulate ATR activity *in vitro*[37]. 9-1-1-mediated recruitment of TOPBP1 relies on a C-terminal tail in RAD9, which is phosphorylated at residue S387 (S373 in xRAD9)[38]. This phosphorylation creates a recognition site for BRCT domains I and II in TOPBP1, thereby bringing TOPBP1 to ATR[34, 35].

TOPBP1 contains an ATR activation domain (AD) located between BRCT domains VI and VII, which interacts with and activates ATR-ATRIP complexes *in vitro*[37]. Indeed, overexpression of this domain by itself activates ATR-ATRIP[37], and fusion of it to PCNA or histone H2B bypasses the need for the RAD17 clamp loader[35]. Although it may be an oversimplification to say that the only role of the 9-1-1 clamp is to bring TOPBP1 to ATR, these observations do suggest that it is the primary role in checkpoint activation. It is interesting to note, however, that the phenotype resulting from loss of ATR is more severe than that resulting from the loss of HUS1 or RAD9[1–3, 39, 40]. This suggests that the 9-1-1 complex is not needed for all functions of ATR.

The mechanism by which TOPBP1 binding activates ATR is poorly defined. The primary binding site for the TOPBP1 AD on the ATR complex is within ATRIP, and mutations in this ATRIP region block activation[41]. In addition, activation involves amino acids in ATR located between the ATR kinase domain and the FATC domain[41] (Box 1). Mutations in this region, recently named the ATR PRD (PIK-kinase regulatory domain), have no effect on the basal activity of ATR but prevent its activation by TOPBP1, cause checkpoint defects, and mimic a complete deletion of ATR in human somatic cells[41]. Interestingly, this region of ATM and mTOR (another member of the PIKK family) is targeted for post-translational modifications that regulate their activity[42, 43]. The PRD may be part of the conserved architecture of PIK family members that allows for the regulation of these kinases by distinct cellular events (Box 1).

Localization and the two-man rule for ATR activation

The localization of ATR and ATM to sites of DNA damage is a key step in the regulation of these kinases. Sustained colocalization of the ATR-ATRIP and 9-1-1-TOPBP1 complexes at the DNA damage site may increase their local concentration such that a relatively weak interaction between TOPBP1 and ATR-ATRIP is promoted allowing activation (Fig. 1A). In the case of ATM, localization of ATM to the break by the MRN complex and further anchoring of these proteins to this region by additional checkpoint proteins contributes significantly to the damage response[44] (Fig. 1B). Tethering and concentrating the MRN complex in a small region of chromatin is sufficient to activate ATM in the absence of DNA damage[45]. Similarly, in *S. cerevisiae*, artificially colocalizing and concentrating the Ddc1/RAD9 and Ddc2/ATRIP proteins can activate Mec1/ATR in the absence of any DNA damage[46]. Finally, overexpression of the TOPBP1 AD can cause phosphorylation of at least some ATR substrates[20, 37] and induce cellular senescence[47], bypassing the requirement for DNA damage. Thus, the double strand break and primed ssDNA structures may not be strictly required for checkpoint activation and may primarily serve as a scaffold to colocalize and concentrate the proteins needed for ATM or ATR activation respectively. Indeed, RPA-coated ssDNA does not increase the specific activity of ATR as is observed with TOPBP1[20, 48].

Recruitment of the 9-1-1-TOPBP1 and ATR-ATRIP complexes to sites of DNA damage or stalled replication forks are largely independent events[49–53]. This independent recruitment may be an important design element in the signaling pathway because it provides a mechanism to ensure that the checkpoint is only activated when needed by creating a molecular version of a "two-man rule". (A two-man rule is a control system that prevents one person from performing an act such as launching a weapon). By requiring two receptors to sense the problem, inappropriate launching of the checkpoint may be prevented.

So how does this two-man mechanism work in checkpoint activation? We can propose at least two models. First, the requirement for two receptors may enforce a requirement for two signals. Each of the signals may have a chance of being present by itself in normal DNA metabolism, but having both signals simultaneously would be an unusual circumstance that requires checkpoint intervention. However, in some ways this is not a satisfactory explanation. To be most effective the signals should be independent of each other. ssDNA and the 5′ junction do not satisfy this requirement; for example, lagging strand replication produces both a 5′ junction and ssDNA. It is possible to hypothesize some fixes to this problem. Other proteins within the replisome may mask one or more of the signals, or the nature of the 5′ junction during lagging strand replication might not support 9-1-1 loading. (An RNA primer forms the 5′ junction, and it is not clear whether the RNA-DNA duplex is equivalent to a DNA-DNA duplex for 9-1-1 loading[24]). A second problem with the two-signal model is that loading of both ATR-ATRIP and the 9-1-1 complex require part of the same signal–RPA[13, 26–29, 54].

A second model might be that the two receptors actually recognize one signal. If there is one signal with two necessary receptors, then this may force a situation in which the amount of signal becomes critical. For example, the more ssDNA-RPA the more chance both ATR-ATRIP and 9-1-1 will be recruited to the same site, bringing the TOPBP1 activator to ATR. Indeed, larger gaps are more efficient at generating checkpoint activation[24]. Of course, the situation is likely to be more complex than either of these alternative scenarios and could be a combination of both.

Post-translational modifications

Post-translational modifications may regulate the activity of PIKKs as well as localization. Indeed, activation of ATM involves autophosphorylation, an event that may help convert an inactive ATM dimer into active monomers[55]. Several phosphorylation sites have been mapped on ATR and ATRIP (Table 2), and like ATM, ATR-ATRIP also may form a higher order oligomeric complex[56–62]. However, as yet, none of the currently identified modifications has been demonstrated to be a reliable indicator of ATR activation, and there is no data to indicate that the oligomerization status of ATR-ATRIP is regulated. A clear and early mark of ATR activation would be enormously useful for the field, as current approaches to monitoring ATR signaling rely on analysis of downstream substrates. Because the phosphorylation and activation of these substrates requires several additional steps, these are indirect readouts.

Post-translationally modified kinases can often be purified in an activated state. Indeed, ATM isolated from irradiated cells is more activate than the ATM from untreated cells[63, 64]. However, there has been little success in isolating an activated ATR kinase. The Dunphy lab has shown that an activated form of ATR can be found on a DNA template containing single- and double-stranded regions[65]. This activation is salt sensitive, but is not sensitive to treatment with micrococcal nuclease, suggesting it relies upon a protein activator that is lost at higher salt concentrations. It seems likely that the activation observed on these DNA templates results from the ability of the template to bring in TOPBP1. Thus, there is no convincing evidence that a pure, activated form of ATR can be obtained. These observations may indicate that ATR activation is dependent upon continued stimulation by TOPBP1.

Signal amplification

Another theme in kinase signaling is feedback/amplification. Auto-amplification is important in the ATM response and is mediated through ATM-dependent phosphorylation of H2AX, a histone variant that is phosphorylated following DNA damage [44]. MDC1, a BRCT-domain containing protein needed for ATM activation, binds to γH2AX (the phosphorylated form of H2AX) through its tandem BRCT domains and brings more ATM to the DNA damage site (Fig. 1B). In the ATR pathway, the interaction between ATR and TOPBP1 may provide a point for signal auto-amplification. Evidence for this comes from an experiment done in *X. laevis* egg extracts using DNA structures containing double-stranded DNA ends (annealed homopolymers of (dA)70 and (dT)70). These templates activate ATR in an ATM-dependent and RPA-independent manner[66], and this activation requires the phosphorylation of TOPBP1 by ATM on residue S1131, an event that promotes the interaction of TOPBP1 with ATR-ATRIP. While it is unclear what types of DNA damage the (dA)70-(dT)70 polymers mimic *in vivo*, the phosphorylation of TOPBP1 by ATM could facilitate ATR activation at double-strand breaks induced by ionizing radiation (IR) and other agents. This induced interaction between TOPBP1 and ATR-ATRIP may reflect an important component of the ATR signaling response that helps to enhance ATR activation after replication stress as well. Residue S1131 of *X. laevis* TOPBP1 is phosphorylated after replication stress, presumably by ATR, and under physiological conditions or low levels of replication stress, this may provide positive feedback that enhances ATR activation.

Common themes and outstanding questions

Common regulatory themes for all of the PIK kinases continue to emerge, suggesting that we should look to translate discoveries about the regulation of one of the kinases into our research on the others (Box 2). Although a nice model for ATR activation has emerged from the current body of work, the picture is undoubtedly more complex. For example, ATR may also respond to stalled transcriptional complexes[67, 68]. Similarly, our understanding of TOPBP1 regulation and function is incomplete. TOPBP1 BRCT domains I and II bind phosphorylated RAD9[34, 35], but BRCT domain V is needed for the recruitment of TOPBP1 to nuclear foci[69]. In addition, there is evidence that BRCT domains VII and VIII are also important for checkpoint activation[70] so the regulation of TOPBP1 may be complex. Finally, although TOPBP1 is needed for the phosphorylation of RAD1 by ATR in *X. laevis* egg extracts, the C-terminal tail of Rad9 is not, suggesting that the 9-1-1-TOPB1 interaction is not needed for all ATR-dependent phosphorylation events[71]

In addition, several experimental observations do not yet fit the model that multiple lesions produce a single ATR-activating DNA structure. Several results indicate that there may be other means of activating ATR-ATRIP, which are secondary to or work with RPA-ssDNA. Surprisingly, deletion of the primary RPA-binding surface in human, *X. laevis* and *S. cerevisiae* ATRIP proteins yields only mild defects in ATR signaling[20, 54, 59]. The *rfa1-t11* mutation (which alters the corresponding ATRIP-binding surface on RPA[20]) has no apparent effect on Ddc2/ATRIP recruitment to stalled replication forks and is largely proficient in supporting the replication checkoint[30]. Additional RPA-ssDNA interaction motifs on ATRIP[72], direct DNA interactions, or even additional proteins may help explain these results. Indeed, in *S. pombe*, Cdc18 (the homologue of human CDC6, a protein required for initiation of DNA replication) anchors ATR-ATRIP to chromatin in the presence of replication stress, an event that is necessary for the long-term maintenance of checkpoint activation[18]. In human cells, a new checkpoint protein, Cep164, was shown to regulate ATR-ATRIP foci formation and signaling[73], and mismatch repair proteins may recruit ATR some DNA adducts using an RPA-independent mechanism[19]. Even the lesion itself may contribute to activation. ATR can bind to UV-damaged DNA, and this DNA can stimulate ATR activity in a partially reconstituted system[17, 48, 74]. Finally, there are still a number of other proteins that have been

implicated in ATR regulation through as yet undefined mechanisms including PP5, p18, and SNIP1[75–77]. Some of these regulatory activities could help produce or maintain the checkpoint-activating nucleic acid structure[78]. Others may regulate ATR in response to specific DNA lesions. Fitting all of these observations into a unified ATR activation model will require much more research.

ATR Signaling

Once an active ATR complex is assembled at a DNA lesion or stalled replication fork, signaling to coordinate cell cycle, repair, and replication begins. The list of ATR substrates is rapidly expanding due to large-scale proteomic profiling methodologies[79–82]. The best studied is the serine-threonine kinase CHK1.

ATR activation of CHK1

CHK1 activation requires phosphorylation by ATR on S317 and S345, which appears to be a reliable indicator of CHK1 activation[83–85]. Unlike many checkpoint proteins, which act at the sites of DNA damage, CHK1 functions to signal DNA damage to the rest of the nucleus. Presumably, it is transiently localized to sites of DNA damage, where it is activated. Claspin, an “adaptor” or “mediator” protein found at the replication fork, is critical for this activation, functioning to bring ATR and CHK1 together[86]. Claspin interacts with CHK1 in a damage-dependent manner, and this interaction requires the phosphorylation of Claspin on at least two sites (S864 and S895 in xClaspin)[87]. Though this phosphorylation is ATR-dependent, the responsible kinase may not be ATR itself since neither serine resides in a consensus ATR phosphorylation site. Unlike TOPBP1, which is needed for the phosphorylation of a number of ATR effectors, it is unclear whether any other ATR substrates require Claspin for their phosphorylation[88]. Claspin binds to phosphorylated Rad17 (a component of the 9-1-1 clamp loader) and this interaction is important for sustaining CHK1 phosphorylation[89]. Since Rad17 phosphorylation is ATR-dependent[90], this may provide another mechanism for signal amplification. In addition to Claspin, a second replication fork associated complex, Tim/Tipin, may also mediate activation of CHK1 by ATR[91, 92].

Once phosphorylated, CHK1 is released from chromatin to phosphorylate its substrates[93]. Key CHK1 targets for controlling cell cycle transitions are the CDC25 phosphatases[94]. Human cells have three CDC25 proteins that regulate cell cycle transitions by removing the inhibitory phosphorylations on cyclin-dependent kinases. CHK1 phosphorylation of the CDC25 proteins inhibits their activity and prevents CDK activation[95–97]. This is a major checkpoint mechanism that prevents entry into mitosis.

ATR signaling through CHK1 is also critical for regulating replication. ATR signaling slows DNA replication at least in part by inhibiting origin firing. ATR-dependent inhibition of origin firing is important even in the absence of added exogenous replication stress agents[98, 99] and is critical to reduce the rate of DNA synthesis under DNA damaging conditions[100–106]. The precise mechanism by which ATR signaling regulates replication origins is not clear but may be at least partly through the regulation of CDK2-cyclin E kinases by the CHK1-CDC25 pathway. There is also some evidence that in mammalian cells and *S. pombe* systems ATR signaling slows replication fork elongation[91, 107] but the precise mechanism is unknown.

ATR substrates at the replication fork

While ATR phosphorylation of CHK1 helps to spread the damage signal, many of the critical functions of ATR are on chromatin and more specifically at the site of the stalled replication fork (Fig. 3). These activities promote replication fork stability and recovery of stalled forks to ensure completion of replication.

The ATR-dependent substrates that maintain fork integrity are poorly understood. Evidence from a variety of systems suggests that replisome components such as Polε and PCNA dissociate from stalled forks in the absence of ATR signaling[103, 108]. Additional substrates include the replication factor C complex, RPA1 and RPA2, the MCM2-7 complex, MCM10, and several DNA polymerases[79, 90, 109–114]. The consequences of most of these phosphorylation events are unknown. However, by examining replication in *Xenopus* egg extracts under stressful conditions, the Costanzo group recently discovered a functional role for MCM2 phosphorylation[115]. MCM2 is a component of the MCM2-7 replicative helicase that unwinds the DNA duplex. ATR phosphorylates MCM2 on S108 and xMCM2 on the equivalent site, S92[111, 112]. MCM2 phosphorylation creates a docking site for Polo-like kinase (Plk) 1. PLK1 recruitment then promotes recovery of DNA replication in extracts treated with replication inhibitors[115]. Plk1 activity near a stalled replication fork may promote neighboring origins to fire through regulating the chromatin association of CDC45, association of which is a pre-requisite for unwinding and recruitment of DNA polymerase. By promoting origin firing, PLK1 overrides the ATR/CHK1-dependent inhibition of origin firing that is the major cause of decreased replication rates in damaged cells.

Why would ATR signaling both inhibit and promote origin firing? One possibility is suggested by the observation that dormant origins do fire under conditions of replication stress as a way of ensuring complete replication[116, 117]. It is possible the ATR-MCM2-PLK1 mechanism acts locally to promote replication near the stalled fork while an ATR-CHK1 signaling pathway operates throughout the nucleus to slow overall rates of DNA synthesis. Indeed, it is interesting to note that PLK1 suppresses CHK1 activation somewhat[115]. It may be that the local phosphorylation of MCM2 at stalled forks allows PLK1 to suppress CHK1 activation near these forks, further promoting the completion of replication in these problem areas. Although further testing of this idea is required, such a model would allow one to reconcile the apparently contradictory effects of ATR on origin firing and make use of both the global and local components of ATR signaling.

ATR and DNA repair

A final class of ATR substrates comprises those that regulate DNA repair. Specifically, ATR signaling may regulate recombination at stalled and collapsed replication forks. ATR phosphorylates several proteins that regulate recombination including BRCA1, WRN and BLM[118–121]. Recombination can be used to re-initiate replication but can also cause genome instability if not regulated properly. In *S. pombe*, deletion of the RecA-like, recombinase protein, RAD51 prevents aberrant strand-exchange events that occur at stalled replication forks of checkpoint-deficient cells[122]. It is still unclear how ATR-dependent phosphorylation of recombination proteins alters their activities; however, the effect may be to limit the types and frequencies of crossover events. ATR also targets the Fanconi-anemia protein FANCD2 to regulate inter-strand crosslink repair. ATR-dependent FANCD2 phosphorylation promotes its monoubiquitination and localization to damage foci[123]. Finally, ATR phosphorylates the nucleotide excision repair protein XPA to regulate its intracellular localization[124]. Thus, ATR signaling promotes repair of a variety of DNA lesions.

PIK kinase redundancy and crosstalk

Most ATR substrates can also be phosphorylated by ATM, and the major functions of ATR and ATM in cell cycle control are overlapping and redundant. So why is one kinase not sufficient? A simple but incomplete answer to this question is that ATM and ATR respond to different types of DNA damage, DSBs for ATM and replication stress for ATR. More specifically, the kinases sense different DNA structures–DNA ends [ATM] vs. ssDNA [ATR]. However, to think of ATM and ATR as interchangeable kinases that simply see different inputs ignores much of the complexity in the DNA damage response that is enabled by having two

kinases. In particular, the ability of one DNA damage type to be converted into another, the kinetics of the ATM vs. ATR checkpoint responses, and crosstalk between the pathways suggests both unique and interdependent roles for these kinases.

ATR and the DSB response

Although ATR is primarily a replication stress response kinase, it is also activated by DSBs. Thus, agents such as ionizing radiation (IR) activate both ATM and ATR. However, ATM is activated rapidly irrespective of the cell cycle, whereas ATR is activated more slowly and predominantly in S and G2 phase cells[125].

The slower and cell cycle-dependent ATR activation at a double-strand break site is a consequence of the need for CDK-dependent DSB end resection that reveals a larger single-stranded region. Importantly, several recent publications have demonstrated a requirement for ATM in end resection and recruitment of ATR[125–128]. A second level of crosstalk may occur at the level of TOPBP1. TOPBP1 is phosphorylated by ATM, and phosphorylated TOPBP1 is a more efficient activator of ATR[66].

These data point to the importance of ATM in ATR activation. This is easily seen in experiments using cells from patients with ataxia-telangiectasia. In these cells, shared ATM and ATR substrates are not phosphorylated efficiently in response to IR because ATM is absent causing a delay in end resection. Eventually, ATM-independent end resection does occur allowing ATR to recognize the damage [125–127]. The consequence of this interdependency is that ATM-deficient cells have severe cell cycle checkpoint defects in response to IR. The importance for active ATR signaling in the IR-induced checkpoint response can be visualized in systems where ATR is inactivated conditionally[1, 129]. In these circumstances, checkpoint responses to IR are compromised. ATM is often described as the kinase that initiates the checkpoint response and ATR is the kinase that maintains it. This is an oversimplification because in wild-type cells, end resection occurs rapidly and ATR is activated within 10–15 minutes.[125, 126]. Thus, even the initiation of the G2/M checkpoint is impaired in the absence of ATR[129]. Therefore, it is more appropriate to think of ATM and ATR as partners in the DSB response.

ATM and replication

If ATR partners with ATM to promote checkpoint signaling at a DSB site, then the obvious question to ask is whether ATM functions at a stalled replication fork? Certainly, ATM does signal at collapsed replication forks where DSBs are often formed. This is easiest to observe in cells in which ATR signaling has been inactivated resulting in the accumulation of collapsed forks, γH2AX phosphorylation, and ATM activation[129]. However, this ATM function is still a DSB response, leaving unanswered the question whether ATM functions at forks that stall without generating a DSB?

There are data that indicate that ATM and the ATM-activating MRE11, RAD50 NBS1 (MRN) complex does have important activities at replication forks. The most convincing data is from *Xenopus* egg extracts. xATM and xMRE11 are important to prevent double-strand breaks at stalled forks during replication[108, 130]. Inhibition of ATM increases the rate of DNA replication and ATM is transiently activated as DNA replication proceeds in the egg extract[98]. The data from human or mouse systems is less convincing although MRN components do localize to replication forks especially in response to agents that cause fork stalling[131]. It is formally possible that ATM and MRE11 prevent the accumulation of DSBs during replication by promoting rapid repair after they occur. In this scenario, ATM and MRE11 should still be thought of as DSB sensing proteins and their involvement in replication limited to when DSBs form at a collapsed fork. It is also possible that MRE11 has functions

at replication forks independent of ATM. In contrast to ATM, MRE11, like ATR, is essential for life and all known mutations are hypomorphic[132–134]. The MRN complex has been found in some ATR-ATRIP complexes and may function upstream of ATR signaling events[135, 136].

If ATM does function to regulate replication in the absence of DSBs, does its activity in these cases depend on ATR? The answer is unclear although there is one report that ATM may be directly phosphorylated by ATR[137]. Interestingly, ATR has also been reported to phosphorylate another PIK kinase, DNA-PKcs, in response to UV-induced replication stress[138]. Clearly, the role of ATR upstream of ATM and other PIK kinases needs further investigation.

Crosstalk downstream of ATR

The overlap in substrate specificity for ATM and ATR is also an important point of crosstalk. Most substrates that have been examined such as BRCA1 and p53 are phosphorylated by both kinases[118, 139–141]. However, there is evidence of some unique specificities. In particular, CHK1 and CHK2 may be exclusively ATR and ATM substrates respectively. Indications of crosstalk at the level of the CHK kinases (such as ATM-dependent CHK1 phosphorylation after IR[142]) are likely to be largely due to interconversion of the DNA lesions. End resection converts DSBs into ssDNA structures that activate ATR while nucleases can cleave ssDNA to yield DSBs that activate ATM (Fig. 4).

ATR's Essential Function

Although ATM and ATR may collaborate in many ways, the organismal and cellular phenotypes that result from the loss of ATM and ATR are dramatically distinct: the loss of ATR causes rapid lethality at the earliest embryonic stages[2, 3], whereas people with no functional ATM can live for decades. What then is the essential function of ATR? As already detailed, ATR responds primarily to primed ssDNA implying that the formation of this structure in unperturbed cell cycles is at the heart of the matter. One can thus envision at least two answers to this question.

One possibility is that ATR is activated at low levels in every cell cycle as a result of normal Okazaki fragment-dependent lagging strand synthesis, during which the activating structure may be repeatedly produced. This activation may be required to enforce the order of S and M phase and to control origin timing[5]. In yeast, however, separation of function mutants indicate that Mec1/ATR's essential function has little to do with cell cycle arrest or origin firing, but is related to its fork stabilization activity[143].

The second possibility is that there are abundant sources of stalled forks in unperturbed cells due to endogenous damage and sequences that are difficult to replicate. ATR would thus be needed to stabilize these structures and promote their restart when collapse occurs. In effect then, the essential function of ATR may be to respond to DNA damage or replication stress, just as it does when cells are stressed by a DNA damaging agent or replication inhibitor. On this point, it should be noted that the essential function of Mec1/ATR can be rescued in unstressed cells by mutations that increase deoxynucleotide levels[144, 145]. Since Mec1/ATR regulates deoxynucleotide production by regulating ribonucleotide reductase, the loss of Mec1/ATR may create the very conditions (stalled forks) that require Mec1/ATR to be resolved.

Although these are not mutually exclusive models, it seems likely that the second possibility is true. After all, ATR did not evolve to respond to the doses of radiation and chemicals used in the laboratory to study signaling. DNA is a fairly stable molecule, but the large amount of it in the nucleus (3 billion base pairs for human cells) provides ample opportunities for chemical

modifications such as nicks, base modifications and crosslinks[146]. Repair enzymes constantly correct these lesions but undoubtedly some are encountered by replication forks prior to repair. In addition, some DNA, DNA-RNA, and DNA-protein structures may stall replication even in the absence of any change in nucleic acid chemistry[147–153]. In some of these cases, the helicase may not uncouple from the polymerase so ATR would not be activated, but in many circumstances, uncoupling does occur and the checkpoint is required for fork stabilization. Even on the relatively small circular *E. coli* genome it is estimated that nearly all replication forks encounter lesions and must be repaired during every round of replication even in normal growth conditions[154]. For mammals, sister chromatid exchange rates suggest that approximately 10 DSBs per cell division form at replication forks[155]. The frequency of fork stalling is undoubtedly much higher.

Defects in ATR-dependent activities at stalled forks may be a major cause of genetic instability. Stalled forks are usually not problematic in eukaryotic cells with multiple replication origins since a fork converging from the other direction can complete replication. In the absence of ATR, however, a stalled fork will collapse and double-strand breaks accumulate[156]. Chromosomal fragile sites may be examples of this phenomenon. Elevated rates of fragile site breakage are observed when cells are treated with low doses of replication stress agents. Therefore, fragile sites are thought to represent chromosomal regions that are particularly difficult to replicate or perhaps are difficult to repair if replication forks collapse in these areas[157]. ATR-deficient cells have high levels of fragile site breakage[158]. Similar fragile sites are thought to exist in *S. cerevisiae* cells and are associated with slow replicating zones that have high rates of fork stalling especially in Mec1/ATR-deficient cells[159]. Thus, the consequences of defective ATR-dependent replication regulation are DSBs and, eventually, cell death.

Conclusions and Future Directions

The first described cell cycle checkpoint (the S-phase checkpoint) was discovered partly due to an analysis of cells from patients with ataxia-telangiectasia that had defects in ATM function[160, 161]. Cloning of the *ATM* gene opened an entire field of checkpoint signaling[8]. By contrast, the study of ATR lagged behind because ATR is essential for cell viability and the intimate connection between ATR and DNA replication make experimentation difficult. However, concerted efforts in multiple biological systems has yielded a good description of ATR loss of function phenotypes; the first outlines of the ATR activation mechanism; and rapidly increasing numbers of ATR substrates and signaling pathways.

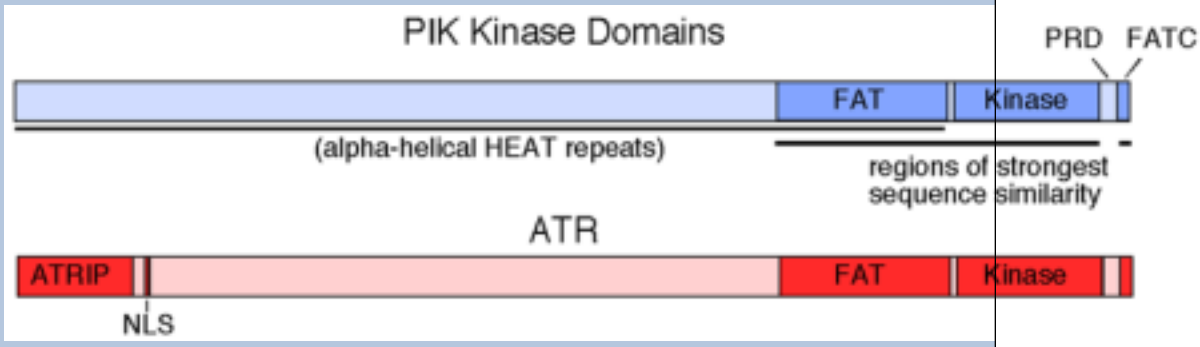
Of course, there are still many unanswered questions. Many experimental observations suggest levels of complexity beyond the ssDNA-ATR activation model including additional protein regulators and even alternative mechanisms depending on the type of DNA lesion. Especially critical will be a better understanding of how post-translational modifications regulate ATR complexes and how TOPBP1 is regulated. A true mechanistic understanding will require structural information. The large size of the kinase may continue to foil attempts at crystallizing the entire complex, but domain mapping and mutagenesis studies have matured and may enable smaller sub-domain structures to be solved and interpreted.

Although genetic studies have yielded increasing evidence for critical ATR activities at replication forks and biochemical experiments have identified large numbers of substrates, we remain largely ignorant of the mechanisms by which this signaling translates into increased fork stability and how it regulates origin firing and fork elongation.

Finally, the relevance of ATR signaling to human disease will be an increasing area of research focus. Although homozygous loss of function mutations in ATR are likely to be exceedingly

rare, mutations in ATR regulators and downstream substrates might be expected in cancer and other diseases linked to genome instability. The effects of heterozygous ATR mutations also need to be examined carefully since mouse studies suggest that ATR is a gene dosage-dependent tumor suppressor in specific genetic backgrounds[162]. The observation that the DNA damage response is activated in premalignant lesions at least in part due to replication stress[163, 164] makes understanding how ATR promotes genome maintenance a critical research goal. ATR signaling may act as a barrier to cancer development by promoting DNA repair, cell cycle checkpoints, apoptosis and cellular senescence in hyperplastic lesions with DNA integrity challenges. Furthermore, the ATR pathway may be a useful target for new drug development[10, 11], and it is important to remember that the effects of many current cancer treatments such as radiation and many chemotherapeutic agents are modulated by the DNA damage response. Thus, we should expect an increasing effort to translate our rapidly expanding knowledge of ATR signaling into the clinic.

Box 1. Functional domains of PIK Kinases



The PIK kinase family contains five active kinases, ATR, ATM, DNA-PKcs, mTOR, and SMG-1. Unfortunately, the lack of high-resolution structural information has prevented a mechanistic understanding of PIK kinase regulation. Low resolution, electron microscopy structures of DNA-PKcs and ATM show that these proteins fold into large globular structures suggesting that distant regions in the primary sequence may come together in space[165–169]. However, these structures do not yet provide enough information to recognize where individual domains reside or how the kinases are regulated. Nonetheless, the domain architecture of the PIK kinases is similar (see figure), suggesting that different regulatory inputs may alter the kinases in similar ways to cause activation. The kinase domain is flanked by two regions of high sequence similarity named the FAT and FATC domains in all the kinases. The FAT domain is part of a long N-terminal region predicted to fold into an extended alpha-helical HEAT repeat structure[170]. The small FATC domain of at least some of the kinases is interchangeable[171].

The PIKK regions that lack sequence similarity among paralogs but retain sequence similarities in orthologs might provide opportunities for unique regulatory mechanisms. Indeed, the poorly conserved N-terminal regions of the kinases mediate interactions with the protein cofactors such as ATRIP, Nbs1, and Raptor[54, 172–174]. Furthermore, the PIK regulatory domain (PRD) between the kinase and FATC domains is poorly conserved between family members but highly conserved within orthologs in different organisms. This region is not essential for basal kinase activity but is a regulatory domain in at least ATM, mTOR, and ATR[41–43].

Box 2. Common Regulatory Themes of the PIK Family of Atypical Kinases

Kinase	Partner	Nucleic Acid	Activator(s)	PTM Regulation
ATR	ATRIP	RPA-ssDNA	TOPBP1	Phosphorylation
ATM	Nbs1	DSB	Mre11/Rad50 and DNA	Phosphorylation and Acetylation
DNA-PKcs	Ku70/80	DSB	Ku70/80 and DNA	Phosphorylation
SMG1	UPF1	mRNA	UPF2 and exon junction complex	?
mTOR	Raptor/Rictor	-	mLST8, Rheb-GTP	Phosphorylation

Since all PIK kinases share a similar domain architecture and sequence similarity, it might be expected that the mechanisms controlling their regulation would be similar. Indeed, there is increasing evidence that most if not all of these kinase are regulated by localization, a protein or protein/nucleic acid activator, and post-translational modifications (see Table).

DNA-PKcs, ATR, and ATM localize to DNA through their interactions with Ku70/80, ATRIP, and Nbs1 respectively[13, 54, 175–179]. SMG-1 localizes to mRNAs with premature termination codons to mediate non-sense mediated mRNA decay at least in part through an interaction with its major substrate UPF1[180]. mTOR also binds one of two protein cofactors (Raptor and Rictor) forming two signaling complexes mTORC1 and mTORC2 resepectively[174, 181, 182]. mTOR may signal from intracellular membranes, but it is unclear whether Raptor and Rictor regulate this localization. Each of the PIK kinases are also regulated by a protein or protein and nucleic acid activator (Mre11/Rad50 + DNA for ATM[183], Ku + DNA for DNA-PKcs[175, 176], TOPBP1 for ATR[37], UPF2 and the exon-junction complex for SMG-1[180], and mLST8 with Rheb-GTP for mTORC1[184, 185]). Post-translational modifications including phosphorylation and acetylation have been described on all of the kinases although in many cases the key sites and the mechanisms of regulation remain to be identified. Finally, there is at least one common regulator of all 5 PIK kinases as well as the related transformation/transcription domain-associated protein (TRRAP). Tel2 binds all six proteins and regulates their stability[186]. Common regulatory mechanisms for the PIK kinases suggests that advances in understanding the regulation of one of the kinases may be informative for the other family members.

Online Summary

- ATR is a member of the PIK family of protein kinases that includes ATM, and regulates DNA damage responses to maintain genome integrity.
- A common DNA structure–single stranded DNA (ssDNA) with a 5′ double stranded primer junction is responsible in most instances for ATR activation.
- ATR binds to a protein co-factor, ATRIP, that regulates ATR localization and activation.
- The TOPBP1 protein directly activates ATR-ATRIP complexes. Its recruitment to DNA lesions is promoted by the 9-1-1 checkpoint clamp.
- ATR signals to regulate DNA replication, cell cycle transitions, and DNA repair through the phosphorylation of hundreds of substrates including CHK1 and the MCM helicase complex.

- ATM and ATR have overlapping but non-redundant functions in the DNA damage response. Crosstalk between these pathways often occurs as a consequence of inter-conversion of the activating DNA lesions.
- ATR is essential for the survival of most replicating cells perhaps due to the ubiquitous presence of DNA lesions and replication stress.

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Glossary terms

Seckel Syndrome
: A rare autosomal recessive disorder characterized by microcephaly with mental retardation and growth retardation. One form is caused by mutations in *ATR*

Replication stress
: A problem during DNA replication caused by DNA lesions, inadequate deoxynucleotide supplies, or other difficulty that interferes with replication fork movement

Replication protein A
: A hetero-trimeric single-stranded DNA binding protein complex with multiple activities in nucleic acid metabolism

Clamp loader
: A protein complex that binds and then assembles a protein clamp onto the DNA at a 3′-OH primer end for DNA replication or 5′-P primer end for checkpoint signaling

End resection
: The nuclease dependent removal of base pairs at a double-strand break to leave an extended ssDNA end with a recessed 5′ end

Nucleotide excision repair
: A process in which a small region of the DNA strand that surrounds a bulky DNA lesion is removed from the DNA helix as an oligonucleotide

BRCT domain
: An evolutionarily conserved phospho-serine/threonine-interaction motif that was first identified in the carboxy-terminal part of BRCA1 and, subsequently, in several other checkpoint mediators

Replisome
: A multi-protein complex at the junction of the DNA replication fork that contains all the enzymes required for DNA replication

Cellular senescence
: A nearly irreversible stage of permanent G1 cell-cycle arrest, which is linked to morphological changes (flattening of the cells), metabolic changes and changes in gene expression

Polo-like kinase
: An evolutionarily conserved serine/threonine kinase with functions in mitosis and checkpoint signaling

Collapsed replication fork
: A blocked replication fork that has lost components of the replisome

MCM Complex
: A complex of 6 mini-chromosome maintenance proteins (MCM2-7) that functions together with accessory proteins as the replicative helicase to unwind double-stranded DNA

MRN Complex
: A DSB-sensing complex containing Mre11, Rad50, and Nbs1 that is important to recruit and activate ATM

Hypomorphic
: A mutation that reduces, but does not completely eliminate, the function of a gene

HEAT repeat
: A tandemly repeated, 37–47 amino acid long module that forms an extended alpha-helical structure. Named for four proteinshuntingtin, elongation factor 3 (EF3), protein phosphatase 2A (PP2A) and TOR1

Nonsense-mediated mRNA decay
: A pathway that ensures that mRNAs bearing premature stop codons are eliminated as templates for translation

Exon-junction complex
: A complex of proteins that is deposited as a consequence of pre-mRNA splicing 20–24 nucleotides upstream of splicing-generated exon–exon junctions of newly synthesized mRNA

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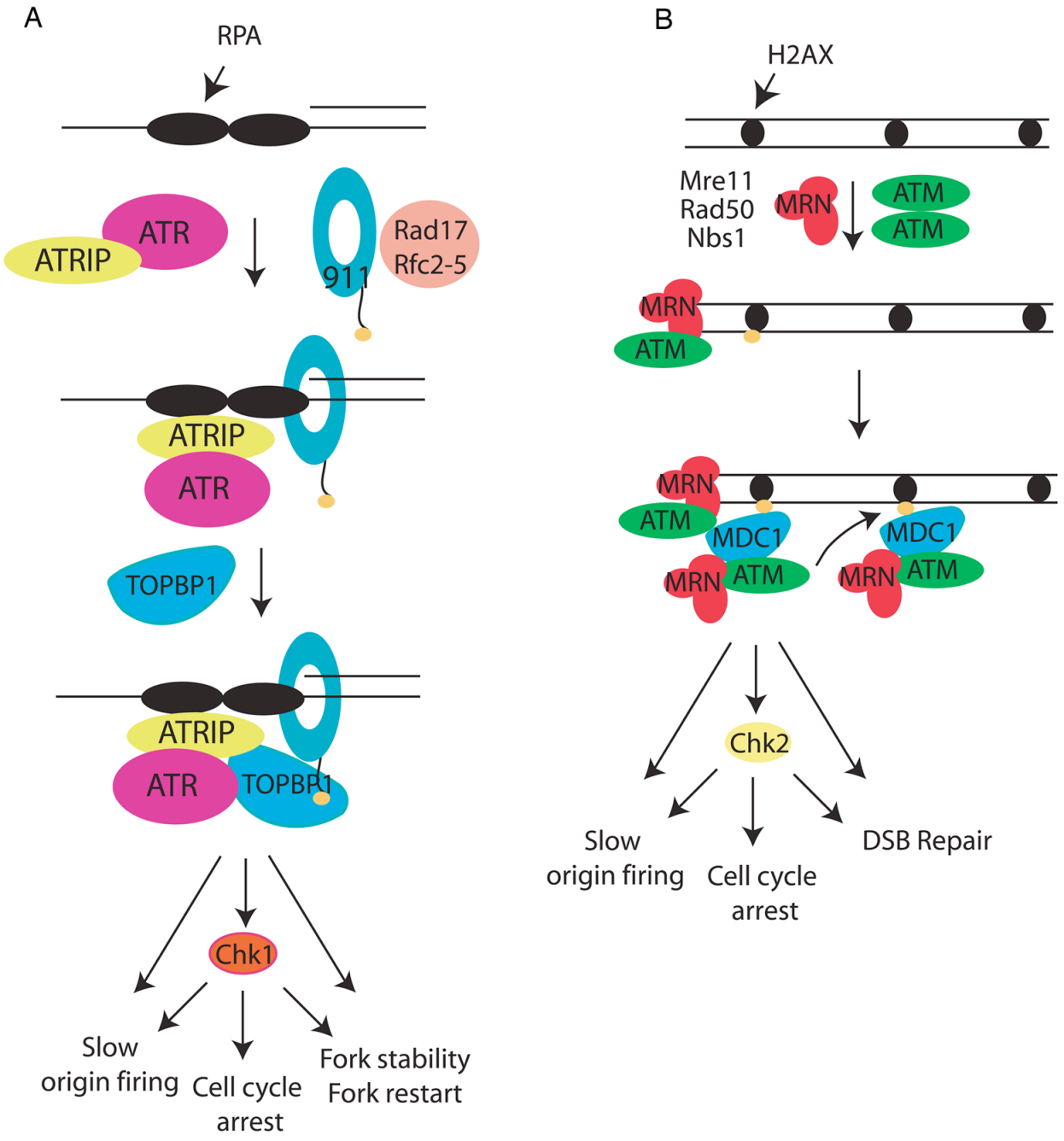

Figure 1. Simple models for ATR and ATM activation

(A) The ATR-ATRIP complex and the 9-1-1 complex are recruited to the ssDNA-5′ primer junction independently. RPA binds ATRIP and directs the Rad17-RFC complex to load the 9-1-1 checkpoint clamp at the 5′ primer junction. Loading of 9-1-1 brings the ATR activator TopBP1 to the damage site through an interaction involving two BRCT domains of TopBP1 and the phosphorylated C-terminal tail of Rad9 (see text). TopBP1 binds and activates ATR in an ATRIP-dependent manner, leading to phosphorylation of the downstream kinase Chk1 and other ATR effectors. In response to DNA damage or replication stress, ATR and its effectors ultimately slow origin firing and induce cell cycle arrest as well as stabilize and restart stalled replication forks. (B) Formation of a double-stranded DNA end leads to recruitment of

the MRN complex and dissociation of the dimeric, inactive form of ATM to a monomeric, phosphorylated form. This monomeric form of ATM binds the MRN complex at the DSB, and is further activated by the DNA and MRN complex. Activated ATM then phosphorylates the carboxy terminal tail of the histone variant, H2AX. Phosphorylated H2AX (γ-H2AX) binds to MDC1 through the BRCT domains of MDC1, leading to recruitment of additional ATM/MRN complexes and further H2AX phosphorylation. The activated ATM also phosphorylates downstream targets, among which are Chk2. Phosphorylation of these proteins leads to cell cycle arrest, inhibition of oirgin firing in S phase and double-strand break repair.

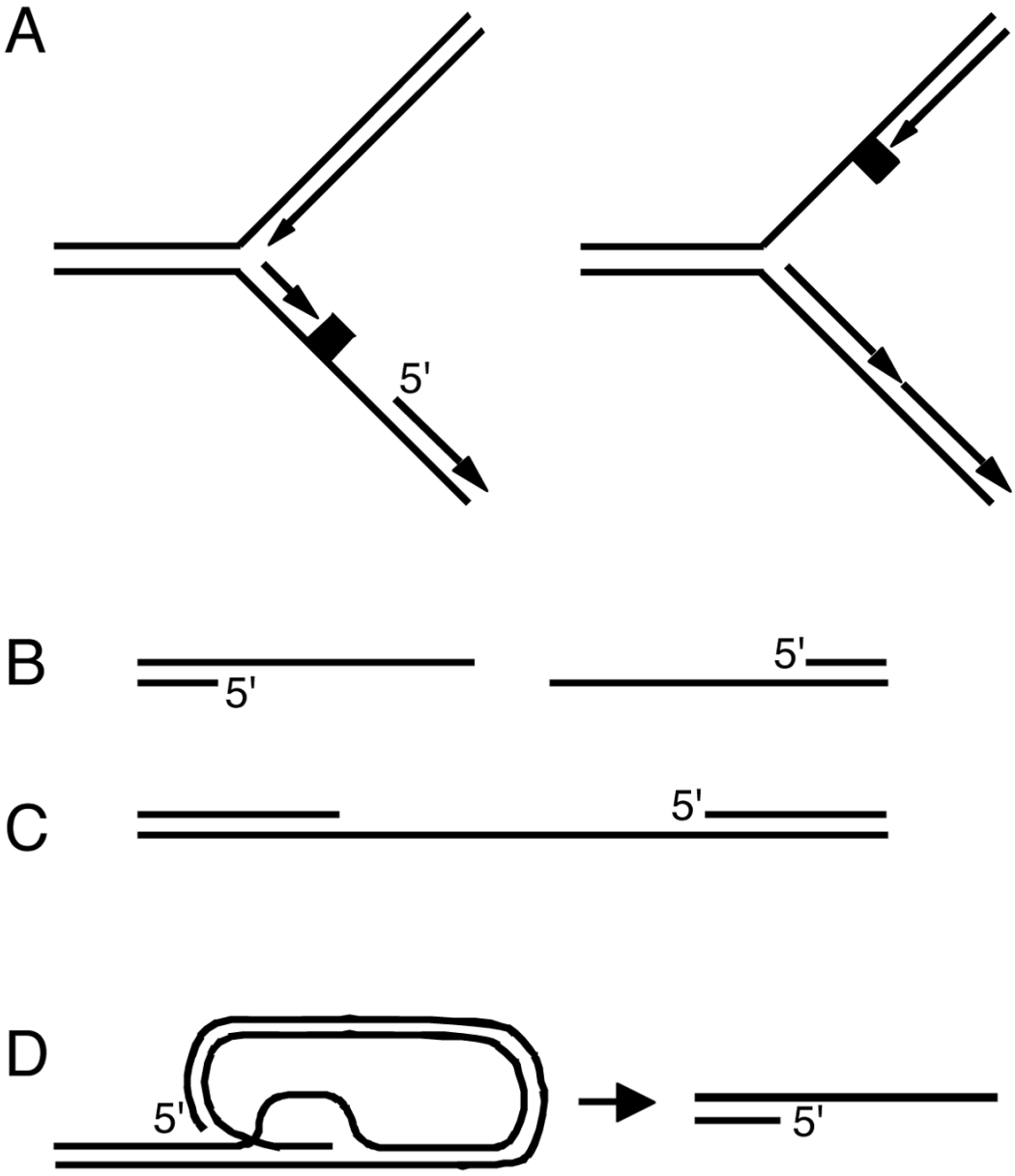

Figure 2. A common DNA structure activates ATR

ssDNA gaps with a 5′ primer end are formed during nucleic acid metabolism. Most noteably, helicase/polymerase uncoupling occurs at replication forks upon encountering lesions that stall the polymerase but not the more permissive helicase. A lesion on the lagging strand would immediately leave a gap with a 5′ primer end. Lesions on the leading strand would require repriming to generate the gapped structure. End resection of DSBs and intermediates in nucleotide excision DNA repair also form the ATR activating structure. Finally, telomere erosion such that the normal telomere capping mechanism is removed produces a recessed 5′ end adjacent to telomeric ssDNA.

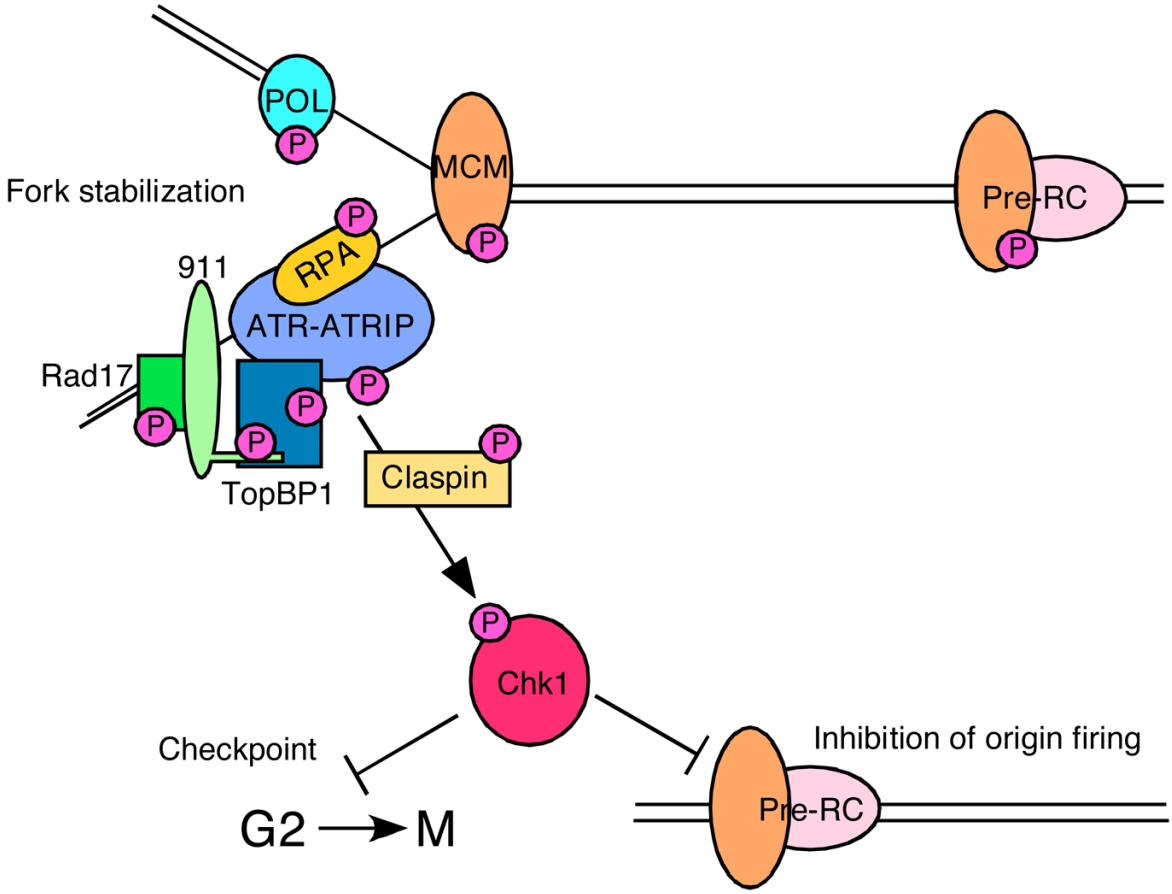

Figure 3. ATR phosphorylates numerous substrates to regulate replication and cell cycle transitions

A major ATR substrate is the checkpoint kinase CHK1. CHK1 phosphorylation releases it from chromatin and increases its kinase activity. CHK1 has numerous substrates some of which regulate cell cycle transitions and replication origin firing. Many ATR substrates are at the replication fork. In most cases, the consequences of phosphorylation remain unknown but likely contribute to fork stabilization. As discussed in the text, ATR-dependent MCM2 phosphorylation regulates binding to PLK1 which may promote the completion of DNA replication near the stalled fork.

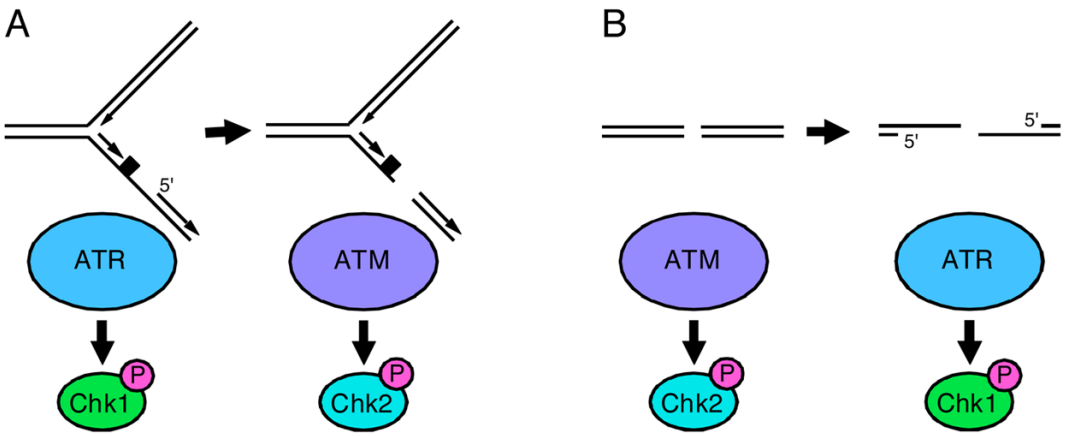

Figure 4. Inter-conversion of ATR- and ATM-activating DNA lesions

(A) Stalled replication forks activate ATR. Nucleases can cleave stalled forks causing DSBs to form which activate ATM. The rate at which DSBs form at stalled forks is greatly increased in cells with defective ATR signaling. (B) DSBs activate ATM but will also activate ATR as a consequence of DNA end resection. This process is ATM- and cell cycle-dependent such that most ATR activation by double-strand breaks occurs in S and G2 phase cells. CHK1 and CHK2 are primarily ATR and ATM substrates respectively.

Table 1

Phosphorylation sites on ATR-ATRIP

Site	Kinase	Damage Inducible?	Function	Reference
ATR				
S428	?	?	?	
ATRIP				
S68/S72	ATR	Yes	?	Itakura, 2004[187]
S224	CDK2	No	G2/M checkpoint regulation	Myers, 2007[188]
S239	?	No	G2/M checkpoint regulation	Myers, 2007[188] and Venere, 2007[189]