ATR: an essential regulator of genome integrity

Karlene A. Cimprich* and David Cortez‡

Abstract | Genome maintenance is a constant concern for cells, and a coordinated response to DNA damage is required to maintain cellular viability and prevent disease. The ataxia-telangiectasia mutated (ATM) and ATM and RAD3-related (ATR) protein kinases act as master regulators of the DNA-damage response by signalling to control cell-cycle transitions, DNA replication, DNA repair and apoptosis. Recent studies have provided new insights into the mechanisms that control ATR activation, have helped to explain the overlapping but non-redundant activities of ATR and ATM in DNA-damage signalling, and have clarified the crucial functions of ATR in maintaining genome integrity.

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

Seckel syndrome
A rare autosomal-recessive disorder that is characterized by microcephaly, mental retardation and growth retardation. One form is caused by mutations in the ataxia-telangiectasia mutated and RAD3-related (*ATR*) gene.

*Department of Chemical and Systems Biology, Stanford University School of Medicine, Clark Center, 318 Campus Drive, W350B, Stanford, California 94305-5441, USA. ‡Department of Biochemistry, Vanderbilt University School of Medicine, 613 Light Hall, 23rd @ Pierce Avenue, Nashville, Tennesee 37232, USA.
e-mails:
cimprich@stanford.edu;
david.cortez@vanderbilt.edu

All cells have elaborate mechanisms to maintain their genomes. DNA can be damaged by reactive metabolic by-products and by environmental mutagens. Responding to and repairing DNA damage is crucial for cell viability and disease prevention.

The DNA-damage response is a signal-transduction pathway that coordinates cell-cycle transitions, DNA replication, DNA repair and apoptosis. The major regulators of the DNA-damage response are the phosphoinositide 3-kinase (PI3K)-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 for phosphorylating Ser or Thr residues that are followed by Gln. 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 (DSBs). By contrast, ATR is activated during every S phase to regulate the firing of replication origins and 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 0.5–1.0% 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 result in viability when they are heterozygous or hypomorphic. Although the only clear link between *ATR* gene mutation and disease exists in a few patients with the rare Seckel syndrome (characterized by growth retardation and microcephaly)[9], disruptions in the ATR pathway cause genomic instability, and ATR is activated by most cancer chemotherapies. Furthermore, ATR signalling is a promising target for cancer drug development[10,11]. This review will focus on ATR signalling 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 the signals that lead to its activation. We therefore address the mechanism of ATR activation in some detail.

Recognizing DNA damage. Although ATR is activated in response to many different types of DNA damage, including DSBs, base adducts, crosslinks and replication stress, a single DNA structure might be responsible. Most 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 that is formed during DNA replication and DNA repair[14]. A mutation in the large subunit of RPA in *Saccharomyces cerevisiae* (*rfa1-t11*) supports replication but is partially compromised in DNA-damage responses[15,16]. Furthermore, depletion of RPA from *Xenopus laevis* egg extracts reduces the association of ATR with chromatin[12]. Finally, RPA-coated ssDNA (hereafter referred to as RPA–ssDNA) is important for the localization of ATR to sites of DNA damage in both human and budding yeast systems[13], although there are some indications of alternative mechanisms[17–19].
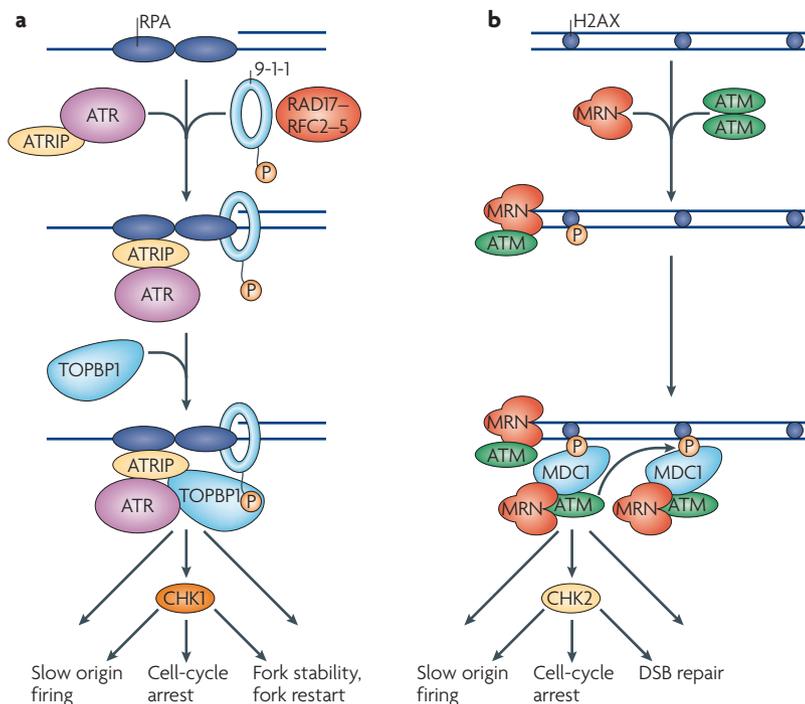
Figure 1 | **Simple models for ATR and ATM activation. a** | Two complexes, RAD9–RAD1–HUS1 (also known as 9-1-1) and one comprising ataxia-telangiectasia mutated and RAD3-related (ATR) kinase and ATR-interacting protein (ATRIP), are independently recruited to the junction of the 5′ primer with single-stranded DNA (ssDNA). Replication protein A (RPA) binds ATRIP and directs the RAD17–replication factor C (RFC) clamp loader complex to load the 9-1-1 checkpoint clamp at the 5′ primer junction. Loading of the 9-1-1 complex brings the ATR activator topoisomerase-binding protein-1 (TOPBP1) to the damage site through an interaction that involves two breast cancer-1 C-terminal (BRCT) domains of TOPBP1 and the phosphorylated C-terminal tail of RAD9 (see main text). TOPBP1 binds and activates ATR in an ATRIP-dependent manner, leading to phosphorylation of the downstream checkpoint kinase-1 (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 the recruitment of the meiotic recombination protein-11 (MRE11)–RAD50–Nijmegen breakage syndrome protein-1 (NBS1) (MRN) complex and the separation of the dimeric, inactive form of ataxia-telangiectasia mutated (ATM) to a monomeric, phosphorylated form. This monomeric form of ATM binds the MRN complex at the double-strand break (DSB) and is further activated by the DNA and MRN complex. Activated ATM then phosphorylates the C-terminal tail of the histone variant H2AX. Phosphorylated H2AX (γH2AX) binds to mediator of DNA damage checkpoint protein-1 (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, including CHK2. Phosphorylation of downstream targets leads to cell-cycle arrest, inhibition of origin firing in S phase and DSB repair.

Replication stress
A problem during DNA replication that is caused by DNA lesions, inadequate deoxynucleotide supplies or other difficulties that interfere with replication-fork movement.

Replication protein A
(RPA). A heterotrimeric single-stranded DNA-binding protein complex with multiple activities in nucleic acid metabolism.

ATR recognition of RPA–ssDNA depends on another protein, ATR-interacting protein (ATRIP)[1]. The stabilities of ATR and ATRIP are linked and their association is not regulated. There are no known differences in the phenotypes that result from the loss of ATR or ATRIP in any organism, suggesting that ATRIP should be considered an obligate subunit of ATR. Biochemical studies indicate that ATRIP binds RPA directly through evolutionarily conserved binding surfaces[20]. The primary interaction involves an acidic α-helix in ATRIP that binds to the basic cleft of the N-terminal oligonucleotide/oligosaccharide binding (OB)-fold domain of the large RPA subunit[20].

The *rfa1-t11* mutation causes a charge reversal in this basic cleft and impairs the binding and recruitment of DNA damage checkpoint protein-2 (Ddc2; the yeast orthologue of ATRIP) to a DSB site in *S. cerevisiae*[13].

Although RPA–ssDNA might be sufficient to localize the ATR–ATRIP complex, it is not sufficient for ATR activation[21–24]. ATR signalling is dependent on colocalization of the ATR–ATRIP complex with the RAD9–RAD1–HUS1 complex (also known as 9-1-1), a heterotrimeric ring-shaped molecule that is related in structure and sequence to proliferating cell nuclear antigen (PCNA)[25], a replicative sliding clamp. As with 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–ssDNA. The presence of RPA is also crucial for this reaction and imparts specificity in loading, thereby 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], which suggests that RPA–ssDNA might be recognized by multiple checkpoint sensors.

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

TOPBP1: an ATR activator. How does the 9-1-1 complex stimulate ATR signalling? In *S. cerevisiae*, the 9-1-1 complex has been reported to directly activate ATR[33]. The C-terminal tail of Ddc1 (the yeast orthologue of RAD9) stimulates mitosis entry checkpoint protein-1 (Mec1; the yeast orthologue of ATR) under certain *in vitro* conditions. However, it is unclear whether this represents the activation mechanism *in vivo*, and there is no evidence that this direct activation occurs in other organisms. Instead, the 9-1-1 complex brings a crucial activator, topoisomerase-binding protein-1 (TOPBP1), to ATR[34–36]. TOPBP1 is a breast cancer-1 (BRCA1) C-terminal (BRCT)-domain-containing protein that is necessary for ATR activation *in vitro*[37]. 9-1-1-mediated recruitment of TOPBP1 relies on a C-terminal tail in RAD9, which is phosphorylated at residue Ser387 (Ser373 in *X. laevis* RAD9)[38]. This phosphorylation event creates a recognition site for BRCT domains I and II in TOPBP1, thereby recruiting TOPBP1 to ATR[34,35].

TOPBP1 contains an ATR activation domain, located between BRCT domains VI and VII, that interacts with and activates ATR–ATRIP complexes *in vitro*[37]. Indeed, overexpression of this domain by itself activates ATR–ATRIP[37], and fusion of the domain to PCNA or histone

H2B bypasses the need for the RAD17 clamp loader[35]. Although it might 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 this is the primary role of the clamp in checkpoint activation. It is interesting to note, however, that the phenotype that results from the loss of ATR is more severe than the phenotype that results from the loss of HUS1 or RAD9 (REFS 1–3,39,40). This suggests that the 9-1-1 complex is not needed for every function of ATR.

The mechanism by which TOPBP1 binding activates ATR is poorly defined. The primary binding site for the activation domain of TOPBP1 on the ATR complex is within ATRIP, and mutations in this region of ATRIP block activation[41]. In addition, activation involves amino acids in ATR that are located between the ATR kinase domain and the FATC domain[41] (BOX 1). Mutations in this region, named the PIKK regulatory domain (PRD) of ATR, have no effect on the basal activity of ATR, although they prevent ATR activation by TOPBP1, cause checkpoint defects and mimic a complete deletion of ATR in human somatic cells[41]. Interestingly, this region of ATM and mammalian target of rapamycin (mTOR; another member of the PIKK family) is targeted for post-translational modifications that regulate their activity[42,43]. The PRD might be part of the conserved architecture of PIKK 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 might increase their local concentration such that a relatively weak interaction between TOPBP1 and ATR–ATRIP is promoted, thereby allowing activation (FIG. 1a).

In the case of ATM, localization of ATM to the break site by the meiotic recombination protein-11 (MRE11)–RAD50–Nijmegen breakage syndrome protein-1 (NBS1) (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 activation domain of TOPBP1 can cause phosphorylation of at least some ATR substrates[20,37] and induce cellular senescence[47], bypassing the requirement for DNA damage. Thus, the DSB and primed ssDNA structures might not be strictly required for checkpoint activation and might primarily serve as scaffolds to colocalize and concentrate the proteins that are needed for ATM or ATR activation, respectively. Indeed, RPA–ssDNA does not increase the specific activity of ATR, unlike TOPBP1 (REFS 20,48).
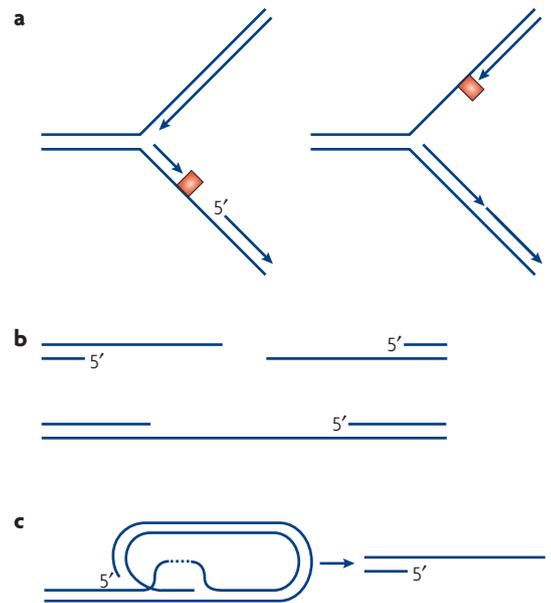
Clamp loader
A protein complex that binds and then assembles a protein clamp onto the DNA at a 3′ hydroxyl primer end for DNA replication or a 5′ phosphate primer end for checkpoint signalling.

End resection
The nuclease-dependent removal of base pairs at a double-strand break to leave an extended single-stranded DNA 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-Ser/Thr-interaction motif that was identified first in the C-terminal part of breast cancer-1 (BRCA1) and subsequently in several other checkpoint mediators.
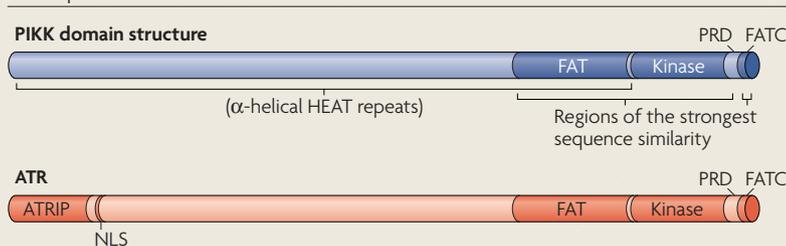
MRN complex
A double-strand-break-sensing complex that contains meiotic recombination protein-11 (MRE11), RAD50 and Nijmegen breakage syndrome protein-1 (NBS1) and that is important in the recruitment and activation of ataxia-telangiectasia mutated (ATM) kinase.

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

Figure 2 | **A common DNA structure activates ATR.** Single-stranded DNA (ssDNA) gaps with a 5′ primer end are formed during nucleic acid metabolism. **a** | Most notably, helicase–polymerase uncoupling occurs at replication forks following encounters with 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 (left). Lesions on the leading strand would require repriming to generate the gapped structure (right). **b** | End resection of double-strand breaks (DSBs) (top) and intermediates in nucleotide excision DNA repair (bottom) also form the ataxia-telangiectasia mutated and RAD3-related (ATR)-activating structure. **c** | Finally, telomere erosion, such that the normal telomere capping mechanism is removed, produces a recessed 5′ end that is adjacent to telomeric ssDNA.

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

So how does this two-man mechanism work in checkpoint activation? We can envisage at least two models. First, the requirement for two receptors might enforce a requirement for two signals. Each of the signals might have a chance of being present alone in normal DNA metabolism, but the presence of both signals simultaneously would be an unusual circumstance that would require 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

Box 1 | Functional domains of PIKKs

PIKK domain structure

(α-helical HEAT repeats)

Regions of the strongest sequence similarity

ATR

The phosphoinositide 3-kinase (PI3K)-related protein kinase (PIKK) family contains five active kinases: ataxia-telangiectasia mutated (ATM), ATM and RAD3-related (ATR), DNA-protein kinase catalytic subunit (DNA-PKcs), mammalian target of rapamycin (mTOR) and SMG1. Unfortunately, a lack of high-resolution structural information has prevented a mechanistic understanding of PIKK regulation. Low-resolution electron microscopy structures of DNA-PKcs and ATM show that these proteins fold into large globular structures, which suggests that distant regions in the primary sequence might come together in space[165–169]. However, these structures do not provide enough information to recognize where individual domains reside or how the kinases are regulated. Nonetheless, the domain architecture of the PIKKs is similar (see figure), suggesting that different regulatory inputs might 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 that is predicted to fold into an extended α-helical HEAT repeat structure[170]. The small FATC domains of at least some of the kinases are interchangeable[171].

The regions of PIKKs that lack sequence similarity among paralogues, but retain sequence similarities in orthologues, 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 ATR-interacting protein (ATRIP), Nijmegen breakage syndrome protein-1 and Raptor[54,172–174]. Furthermore, the PIKK regulatory domain (PRD) between the kinase and FATC domains is poorly conserved between family members but highly conserved within orthologues 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]. NLS, nuclear localization signal.

HEAT repeat
A tandemly repeated, 37–47-amino-acid module that forms an extended α-helical structure. It is named after four proteins: huntingtin, elongation factor-3 (EF3), protein phosphatase-2A (PP2A) and target of rapamycin-1 (TOR1).

both a 5′ junction and ssDNA. It is possible to propose some solutions to this problem. Other proteins within the replisome might 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 requires part of the same signal — RPA[13,26–29,54].

Table 1 | Phosphorylation sites on ATR–ATRIP

Site	Kinase	Damage inducible?	Function	References
ATR				
Ser428	?	?	?	–
ATRIP				
Ser68/Ser72	ATR	Yes	?	187
Ser224	CDK2	No	G2–M checkpoint regulation	188
Ser239	?	No	G2–M checkpoint regulation	188,189

ATR, ataxia-telangiectasia mutated and RAD3-related kinase; ATRIP, ATR-interacting protein; CDK2, cyclin-dependent kinase-2.

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

Post-translational modifications. Post-translational modifications might regulate the activity of PIKKs as well as their localization. Indeed, activation of ATM involves autophosphorylation, an event that might help convert an inactive ATM dimer into active monomers[55]. Several phosphorylation sites on ATR and ATRIP have been mapped (TABLE 1) and, as with ATM, ATR–ATRIP might also form a higher-order oligomeric complex[56–62]. However, none of the currently identified modifications has been demonstrated to be a reliable indicator of ATR activation, and there are 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 signalling rely on the 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 that has been isolated from irradiated cells is more active than ATM that has been isolated from untreated cells[63,64]. However, there has been little success in isolating activated ATR. The Dunphy laboratory 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 that it relies on a protein activator that is lost at higher salt concentrations. It seems likely that the activation that is 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 might indicate that ATR activation is dependent on continued stimulation by TOPBP1.

Signal amplification. Another theme in kinase signalling is feedback and 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]. Mediator of DNA damage checkpoint protein-1 (MDC1), a BRCT-domain-containing protein that is needed for ATM activation, binds to γH2AX (the phosphorylated form of H2AX) through its tandem BRCT domains and brings additional ATM to the site of DNA damage (FIG. 1b).

Box 2 | **Common regulatory themes of the PIKK 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–Ku80	DSB	Ku70–Ku80 and DNA	Phosphorylation
SMG1	UPF1	mRNA	UPF2 and exon-junction complex	?
mTOR	Raptor and Rictor	–	LST8, RhebGTP	Phosphorylation

DSB, double-strand break; ssDNA, single-stranded DNA.

As all phosphoinositide 3-kinase (PI3K)-related protein kinases (PIKKs) share a similar domain architecture and sequence, it might be expected that the mechanisms that control their regulation would be similar also. Indeed, there is increasing evidence that most, if not all, of these kinases are regulated by localization, a protein activator or protein-and-nucleic-acid activator, and post-translational modifications (PTMs; see table).

DNA-dependent protein kinase catalytic subunit (DNA-PKcs), ataxia-telangiectasia mutated (ATM) and ATM and RAD3-related (ATR) localize to DNA through their interactions with Ku70–Ku80, Nijmegen breakage syndrome protein-1 (NBS1) and ATR-interacting protein (ATRIP), respectively[13,54,175–179]. SMG1 localizes to mRNAs with premature termination codons to mediate nonsense-mediated mRNA decay at least in part through an interaction with its major substrate, up-frameshift suppressor-1 (UPF1)[180]. Mammalian target of rapamycin (mTOR) also binds one of two protein cofactors (Raptor and Rictor), to form the two signalling complexes mTORC1 and mTORC2, respectively[174,181,182]. mTOR might signal from intracellular membranes, but it is unclear whether Raptor and Rictor regulate this localization. Each of the PIKKs are also regulated by a protein activator or protein-and-nucleic-acid activator (meiotic recombination protein-11 (MRE11)–RAD50 plus DNA for ATM[183], Ku plus DNA for DNA-PKcs[175,176], topoisomerase-binding protein-1 (TOPBP1) for ATR[37], UPF2 and the exon-junction complex for SMG1 (REF. 180), and mammalian LST8 and RhebGTP for mTORC1 (REFS 184,185)). PTMs, including phosphorylation and acetylation, have been described for 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 five PIKKs as well as the related transformation/transcription domain-associated protein (TRRAP). Telomere length regulation protein-2 (TEL2) binds all six proteins and regulates their stability[186]. The existence of common regulatory mechanisms for the PIKKs suggests that advances in understanding the regulation of one of the kinases might be informative for the other family members.

In the ATR pathway, the interaction between ATR and TOPBP1 might provide a point for signal auto-amplification. Evidence for this comes from an experiment carried out in *X. laevis* egg extracts using DNA structures containing dsDNA 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 Ser1131, an event that promotes the interaction of TOPBP1 with ATR–ATRIP. Although 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 DSBs that have been induced by ionizing radiation (IR) and other agents. This induced interaction between TOPBP1 and ATR–ATRIP might reflect an important component of the ATR signalling response that helps to enhance ATR activation after replication stress. Residue Ser1131 of *X. laevis* TOPBP1 is phosphorylated after replication stress, presumably by ATR. Under physiological conditions or low levels of replication stress, this might provide positive feedback that enhances ATR activation.

Common themes and outstanding questions. Common regulatory themes for all of the PIKKs continue to emerge, suggesting that we should try to use discoveries regarding the regulation of one of the kinases in our research on the others (BOX 2). Although a good model

for ATR activation has emerged from the current body of work, the true picture is undoubtedly more complex. For example, ATR might also respond to stalled transcriptional complexes[67,68]. Similarly, our understanding of TOPBP1 regulation and function is incomplete. BRCT domains I and II of TOPBP1 bind phosphorylated RAD9 (REFS 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 might 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–TOPBP1 interaction is not needed for every ATR-dependent phosphorylation event[71].

In addition, some experimental observations do not fit the model that multiple lesions produce a single ATR-activating DNA structure. Several results indicate that there might be other means of activating ATR–ATRIP that are secondary to, or function together 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 signalling[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 mostly proficient in supporting the replication checkpoint[30].

Replisome
A multiprotein complex at the junction of the DNA replication fork that contains all of the enzymes that are required for DNA replication.

Nonsense-mediated mRNA decay
A pathway that eliminates mRNAs that bear premature stop codons.

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

Additional RPA–ssDNA interaction motifs on ATRIP[72], direct DNA interactions, or even additional proteins might help explain these results. Indeed, in *Schizosaccharomyces pombe*, cell-division control protein-18 (Cdc18; the orthologue of human CDC6, a protein that is required for the 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 signalling[73], and mismatch repair proteins might recruit ATR to some DNA adducts using an RPA-independent mechanism[19]. Even the lesion itself might 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, including the Ser/Thr protein phosphatase-5 (PP5), p18 and Smad nuclear-interacting protein-1 (SNIP1), that have been implicated in ATR regulation through undefined mechanisms[75–77]. Some of these regulatory activities could help produce or maintain the checkpoint-activating nucleic acid structure[78]. Others might 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 signalling

Once an active ATR complex is assembled at a DNA lesion or stalled replication fork, signalling to coordinate cell cycle, repair and replication can begin. The list of ATR substrates is rapidly expanding owing to large-scale proteomic profiling methodologies[79–82]. However, the best studied is the Ser/Thr kinase checkpoint kinase-1 (CHK1).

ATR activation of CHK1. CHK1 activation requires phosphorylation by ATR on Ser317 and Ser345, which seems 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 that is found at the replication fork, is crucial 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 (Ser864 and Ser895 in *X. laevis*)[87]. Although phosphorylation is ATR dependent, the responsible kinase might not be ATR itself because neither Ser residue 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 claspin is required for the phosphorylation of any other ATR substrates[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]. As RAD17 phosphorylation is ATR dependent[90], this might provide another mechanism for signal amplification.

In addition to claspin, a second replication-fork-associated complex that is composed of timeless and timeless-interacting protein might also mediate the 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 phosphorylation of cyclin-dependent kinases (CDKs). 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 signalling through CHK1 is also crucial for regulating replication. ATR signalling 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 crucial to reduce the rate of DNA synthesis under DNA-damaging conditions[100–106]. The precise mechanism by which ATR signalling regulates replication origins is not clear, but this might occur, at least partly, through the regulation of CDK2–cyclin E kinases by the CHK1–CDC25 pathway. There is also evidence that in mammalian cell and *Schizosaccharomyces pombe* systems ATR signalling slows replication-fork elongation[91,107], although the precise mechanism by which this might occur is unknown.

ATR substrates at the replication fork. Whereas ATR phosphorylation of CHK1 helps to spread the damage signal, many of the crucial functions of ATR are on chromatin. More specifically, they are at the site of the stalled replication fork (FIG. 3). These activities promote replication-fork stability and the 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 Pol ε and PCNA, dissociate from stalled forks in the absence of ATR signalling[103,108]. ATR substrates at the fork include the replication factor C complex, RPA1 and RPA2, the minichromosome maintenance (MCM)2–7 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 *X. laevis* 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 Ser108 and on the equivalent site in *X. laevis* MCM2, Ser92 (REFS 111,112). MCM2 phosphorylation creates a docking site for Polo-like kinase-1 (PLK1). PLK1 recruitment then promotes the recovery of DNA replication in extracts that have been treated with replication inhibitors[115]. PLK1 activity near a stalled replication fork might promote neighbouring origins to fire by regulating the chromatin association of CDC45 — this association is a prerequisite for unwinding and recruitment of DNA

MCM2–7 complex
A complex of 6 minichromosome maintenance proteins (MCM2–7) that functions, together with accessory proteins, as the replicative helicase that unwinds double-stranded DNA.

Polo-like kinase
An evolutionarily conserved Ser/Thr kinase with functions in mitosis and checkpoint signalling.
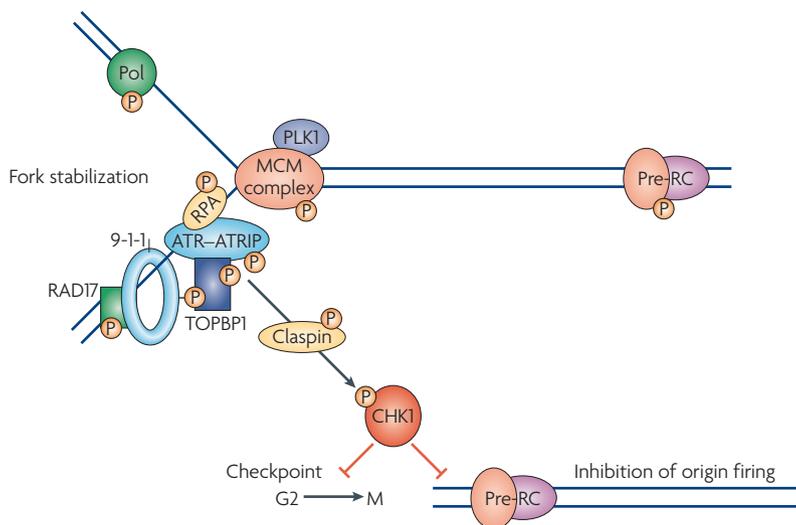
Figure 3 | **ATR phosphorylates numerous substrates to regulate replication and cell-cycle transitions.** A major ataxia-telangiectasia mutated and RAD3-related (ATR) kinase substrate is checkpoint kinase-1 (CHK1). Phosphorylation of CHK1 releases it from chromatin and increases its kinase activity. CHK1 has numerous substrates, some of which regulate cell-cycle transitions and replication-origin firing. Many ATR substrates, including minichromosome maintenance protein-2 (MCM2), MCM3, replication protein A (RPA), RAD9 (which is part of the 9-1-1 complex), topoisomerase-binding protein-1 (TOPBP1), RAD17 and CHK1, are at the replication fork. In most cases the consequences of phosphorylation are still unknown, but probably contribute to fork stabilization. As discussed in the main text, ATR-dependent MCM2 phosphorylation regulates binding to Polo-like kinase-1 (PLK1), which might promote the completion of DNA replication near the stalled fork. ATRIP, ATR-interacting protein; Pol, polymerase; Pre-RC, pre-replicative complex.

Bloom syndrome protein (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 might be to limit the types and frequencies of crossover events. ATR also targets the Fanconi-anaemia protein FANCD2 to regulate inter-strand crosslink repair. ATR-dependent FANCD2 phosphorylation promotes its monoubiquitylation and localization to damage foci[123]. Finally, ATR phosphorylates the nucleotide-excision repair protein XPA to regulate its intracellular localization[124]. Thus, ATR signalling promotes the repair of a variety of DNA lesions.

PIKK 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 insufficient? A simple but incomplete answer to this question is that ATM and ATR respond to different types of DNA damage: ATM responds to DSBs and ATR responds to replication stress. More specifically, the kinases sense different DNA structures: ATM senses DNA ends and ATR senses ssDNA. 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 type of DNA damage to be converted into another, the kinetics of the ATM-checkpoint compared with the ATR-checkpoint responses, and crosstalk between the pathways suggest 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 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 DSB 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 the recruitment of ATR[125–128]. A second level of crosstalk might 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 importance 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
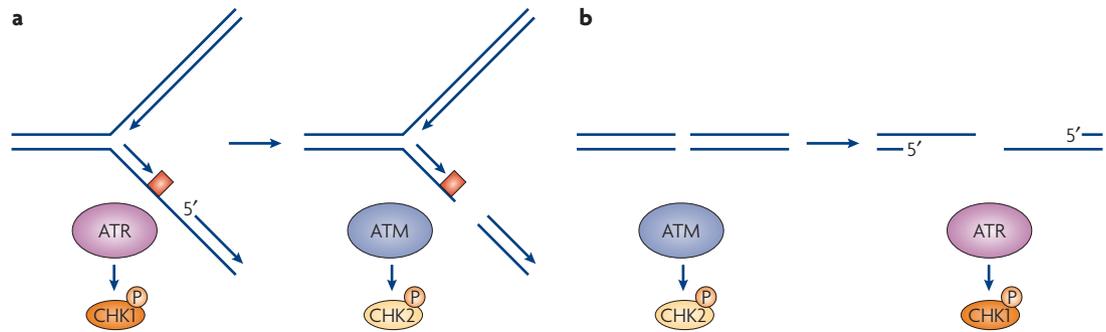
polymerase. By promoting origin firing, PLK1 overrides the ATR–CHK1-dependent inhibition of origin firing that is the major cause of decreased replication rates in damaged cells.

Why would ATR signalling both inhibit and promote origin firing? One possibility is suggested by the observation that dormant origins fire under conditions of replication stress as a way of ensuring complete replication[116,117]. It is possible that the ATR–MCM2–PLK1 mechanism acts locally to promote replication near the stalled fork, whereas the ATR–CHK1 signalling pathway operates throughout the nucleus to slow overall rates of DNA synthesis. Indeed, it is interesting to note that PLK1 suppresses CHK1 activation[115]. It might 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 hypothesis is required, such a model would reconcile the apparently contradictory effects of ATR on origin firing and would allow the cell to make use of both the global and the local components of ATR signalling.

ATR and DNA repair. A final class of ATR substrates comprises those that regulate DNA repair. Specifically, ATR signalling might regulate recombination at stalled and collapsed replication forks. ATR phosphorylates several proteins that regulate recombination, including BRCA1, Werner syndrome ATP-dependent helicase (WRN) and

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

Figure 4 | **Interconversion of ATR- and ATM-activating DNA lesions. a** | Stalled replication forks activate the ataxia-telangiectasia mutated and RAD3-related (ATR) kinase. Nucleases can cleave stalled forks, causing double-strand breaks (DSBs) to form and activate ataxia-telangiectasia mutated (ATM). The rate at which DSBs form at stalled forks is greatly increased in cells with defective ATR signalling. **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 DSBs occurs in S- and G2-phase cells. Checkpoint kinase-1 (CHK1) and CHK2 are primarily ATR and ATM substrates, respectively.

of this interdependency is that ATM-deficient cells have severe cell-cycle checkpoint defects in response to IR exposure. The importance for active ATR signalling 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 as 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 signalling at a DSB site, then the obvious question to ask is: does ATM function 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 signalling 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, and so the question of whether ATM functions at forks that stall without generating a DSB remains unanswered.

Data indicate that ATM and the ATM-activating MRN complex have important activities at replication forks. The most convincing data are from *X. laevis* egg extracts. *X. laevis* ATM and MRE11 are important to prevent DSBs 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 are less convincing, although MRN components do localize to replication forks, especially in response to agents that cause fork stalling[131]. It is 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 is limited to when DSBs form at a collapsed fork. It is also

possible that MRE11 has functions at replication forks that are independent of ATM. In contrast to ATM, MRE11, as with ATR, is essential for life, and every known MRE11 mutation is hypomorphic[132–134]. The MRN complex has been found in some ATR–ATRIP complexes and might function upstream of ATR-signalling 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 one report states that ATM might be directly phosphorylated by ATR[137]. Interestingly, ATR has also been reported to phosphorylate another PIKK, DNA-dependent protein kinase catalytic subunit (DNA-PKcs), in response to UV-induced replication stress[138]. Clearly, the role of ATR upstream of ATM and other PIKKs 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 might be exclusively ATR and ATM substrates, respectively. Indications of crosstalk at the level of the CHK kinases (such as ATM-dependent CHK1 phosphorylation after exposure to IR[142]) are probably due to interconversion of the DNA lesions. End resection converts DSBs into ssDNA structures that activate ATR, whereas nucleases can cleave ssDNA to yield DSBs that activate ATM (FIG. 4).

Essential function of ATR

Although ATM and ATR might 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 who lack functional ATM can live for decades. So what is the essential function of ATR? As already detailed, ATR responds primarily to primed ssDNA, implying that the formation of this primed ssDNA structure in unperturbed cell cycles is at the heart of the matter. One can thus envision at least two answers to the above 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 might be repeatedly produced. This activation might 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 the essential function of Mec1/ATR 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 owing 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, the essential function of ATR might be to respond to DNA damage or replication stress, just as it does when cells are stressed by a DNA-damaging agent or a replication inhibitor. 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]. As Mec1/ATR regulates deoxynucleotide production by regulating ribonucleotide reductase, the loss of Mec1/ATR might 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 that are used in the laboratory to study signalling. 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 before repair. In addition, some DNA, DNA–RNA and DNA–protein structures might stall replication, even in the absence of any change in nucleic acid chemistry[147–153]. In some of these cases the helicase might not uncouple from the polymerase, and 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 *Escherichia coli* genome, it is estimated that nearly all of the replication forks encounter lesions that must be repaired during every round of replication, even in normal growth conditions[154]. For mammals, sister chromatid exchange rates suggest that ~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 might be a major cause of genetic instability. Stalled forks are usually not problematic in eukaryotic cells with multiple replication origins, as a fork that converges from the other direction can complete replication. In the absence of ATR, however, a stalled fork will collapse and DSBs accumulate[156]. Chromosomal fragile sites might 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 in 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 signalling[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 makes experimentation difficult. However, concerted efforts in multiple biological systems have 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 signalling 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. It will be especially crucial to gain 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 might continue to foil attempts at crystallizing the entire complex, but domain mapping and mutagenesis studies have matured and might enable smaller subdomain structures to be solved and interpreted.

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

Finally, the relevance of ATR signalling to human disease will be an increasing focus of research. Although homozygous loss-of-function mutations in ATR are probably 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, because mouse studies suggest that ATR is a gene-dosage-dependent tumour suppressor in specific genetic backgrounds[162]. The observation that the DNA-damage response is activated in premalignant lesions, at least in part by replication stress[163,164], makes the understanding of how ATR promotes genome maintenance an important research goal. ATR signalling might 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 might 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 signalling into the clinic.

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DATABASES
Interpro: http://www.ebi.ac.uk/interpro
BRCT | FAT | FATC | OB-fold
OMIM: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM
ataxia-telangiectasia | Bloom syndrome | Werner syndrome |
UniProtKB: http://ca.expasy.org/sprot
ATM | ATR | ATRIP | BLM | BRCA1 | CDK2 | CEP164 | CHK1 | claspin | Ddc1 | Ddc2 | HUS1 | MCM2 | MCM10 | p18 | PCNA | PI3K | PLK1 | PP5 | RAD1 | RAD9 | RPA1 | RPA2 | SNIP1 | TOPBP1 | WRN

FURTHER INFORMATION
David Cortez's homepage: http://www.mc.vanderbilt.edu/root/vumc.php?site=cortezlab
Karlene Cimprich's homepage: http://cimprich.stanford.edu

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