Analysis of Mutations That Dissociate G2 and Essential S Phase Functions of Human Ataxia Telangiectasia-mutated and Rad3-related (ATR) Protein Kinase*§

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Background: The ATR family of kinases are regulated through similar mechanisms.

Results: Mutations in three TQ amino acids separate the G2 checkpoint and essential S phase functions of ATR.

Conclusion: The ATR, ATM, and DNA-PK kinases share an important regulatory region within an inter-HEAT repeat loop.

Significance: The results indicate that the essential function of ATR for cell viability is linked to DNA replication.

ATR (ataxia telangiectasia-mutated and Rad3-related) contains 16 conserved candidate autophosphorylation sites that match its preferred S/TQ consensus. To determine whether any is functionally important, we mutated the 16 candidate residues to alanine in a single cDNA to create a 16A-ATR mutant. The 16A-ATR mutant maintains kinase and G2 checkpoint activities. However, it fails to rescue the essential function of ATR in maintaining cell viability and fails to promote replication recovery from a transient exposure to replication stress. Further analysis identified T1566A/T1578A/T1589A (3A-ATR) as critical mutations causing this separation of function activity. Secondary structure predictions indicate that these residues occur in a region between ATR HEAT repeats 31R and 32R that aligns with regions of ATM and DNA-PK containing regulatory autophosphorylation sites. Although this region is important for ATR function, the 3A-ATR residues do not appear to be sites of autophosphorylation. Nevertheless, our analysis identifies an important regulatory region of ATR that is shared among the PI3K-related protein kinase family. Furthermore, our data indicate that the essential function of ATR for cell viability is linked to its function in promoting proper replication in the context of replication stress and is independent of G2 checkpoint activity.

Three related members of the phosphoinositide 3-kinase-related kinase (PIKK) family of kinases, ataxia telangiectasia-mutated (ATM), ATM and Rad3-related (ATR), and DNA-dependent protein kinase (DNA-PK), regulate the cellular response to DNA damage. They control cell cycle transitions, DNA replication, DNA repair, and cell death. Of the three protein kinases, ATR is essential in replicating mammalian cells (1–3). In S phase, ATR regulates replication initiation, replisome stability, replication fork restart, translesion synthesis, and chromosomal fragile site expression (4–10). In G2 phase, ATR prevents premature mitotic entry in the presence of damaged DNA via the G2 checkpoint (3, 11).

Repair processing of many types of DNA lesions and replication perturbations causes the generation of single-stranded DNA (ssDNA), which becomes coated with the ssDNA-binding protein replication protein A (RPA). RPA-coated ssDNA recruits ATR and its obligate partner ATR-interacting protein (ATRIP) (3) through an interaction between ATRIP and the 70-kDa subunit of RPA (12, 13). In an independent reaction, a clamp loader containing RAD17 loads the RAD9-RAD1-HUS1 (9-1-1) complex at 5‘ recessed ssDNA/double-stranded DNA junctions (14–16) perhaps due to the interaction of RAD9 with the RPA 70N domain (17). The 9-1-1 complex binds TOPBP1 (18–20), which can activate the ATR kinase (21). Surfaces on both ATR and ATRIP bind a region of TOPBP1 termed the ATR activating domain (AAD) (22). AAD binding increases ATR substrate affinity presumably by eliciting a conformational change (23).

ATR shares domain architecture with ATM and DNA-PK. The N terminus of these PIKKs consists of an array of antiparallel helices called HEAT repeats (Huntingtin, elongation factor 3, protein phosphatase 2A, and PI3K TOR1) (24–26). The highest degree of similarity among the PIKKs occurs in the C-terminal kinase domain (27), which is flanked N-terminally by the FAT (FRAP, ATM, TRRAP) (28) and C-terminally by the PIKK regulatory domain (22) and FATC (FAT C terminus) (28) domains.

ATR, ATM, and DNA-PK also share regulatory mechanisms (29). Like ATR, recruitment to sites of DNA damage and protein activators controls ATM and DNA-PK activity (30, 31). In addition, autophosphorylation regulates ATM and DNA-PK (32–36). ATR, ATM, and DNA-PK prefer to phosphorylate serines or threonines that are immediately followed by a glutamine.
(S/TQ) (37–39). In this study we employed a candidate approach to characterize potential ATR autophosphorylation sites. Mutation of three sites (T1566A/T1578A/T1589A) impairs the S phase and essential functions but not G2 checkpoint activities of ATR. Mass spectrometry, in vitro kinase assays, and two-dimensional phosphopeptide mapping experiments suggest that these residues are not phosphorylated. Nevertheless, secondary structure analysis indicates that the residues reside in a conserved regulatory region among DNA-damaged PIKKs. Our analysis of this region in ATR provides the first example of a separation of function mutant in human ATR and indicates that the essential activity of ATR for cell viability is linked to DNA replication and not its ability to maintain the G2 checkpoint in response to DNA damage.

EXPERIMENTAL PROCEDURES

Cell Lines—HCT116 ATRflox/− TR cells were generated as described in Ref. 22 and maintained in McCoy’s medium containing 10% fetal bovine serum (FBS) and 10 μg/ml blasticidin. Clonal ATR stable cell lines expressing tetracycline-inducible FLAG-HA3-epitope-tagged ATR cDNAs were generated as described in Ref. 22 and maintained in McCoy’s medium containing 10% FBS, 300 μg/ml hygromycin B, and 10 μg/ml blasticidin. ATR expression was induced with 1 μg/ml tetracycline. ATRdel/− TR cell lines were created by infecting ATRflox/− TR cell lines expressing the respective cDNAs with adenovirus encoding the Cre recombinase to excise the flox allele. Cre-mediated excision was verified by PCR. ATRdel/− TR cell lines were maintained in McCoy’s 5A medium containing 10% FBS and 1 μg/ml tetracycline. HEK293T were maintained in DMEM containing 10% FBS. Adenovirus infections were carried out as described (3).

DNA Constructs—FLAG-HA3-ATR mutants were generated as described (22). In some cases the FLAG-HA3 epitope was replaced with a single FLAG epitope. Plasmid transfection was carried out with Lipofectamine2000 using the manufacturer’s protocol (Invitrogen).

Colony Formation, Cell Cycle, Immunoblotting, Immunofluorescence, and Immunoprecipitations—Colony formation assays, cell cycle analysis, and immunoblotting were conducted as described in Ref. 22. Cell proliferation was measured using the WST-1 assay (Roche Applied Science). Cells were mock treated, treated with 2 mM hydroxyurea (HU), and 100 μM cycloheximide where indicated, and lysates were prepared using Nonidet P-40 buffer (1% Nonidet P-40, 200 mM NaCl, 50 mM Tris, pH 8.0). ATRN19 (Santa Cruz Biotechnology), CHK1-G4 (Santa Cruz Biotechnology), phosphorylated Ser-317 CHK1 (Cell Signaling), phosphorylated Ser-966 SMC1 (Bethyl), HA (Covance), FLAG-M2 (Sigma), and GAPDH (Santa Cruz Biotechnology) primary antibodies were used for immunoblotting. Infrared-conjugated secondary antibodies (LiCor) were used, and blots were scanned and quantified using an Odyssey system. Immunofluorescence was carried out on control and HU-treated cells using HA (Covance) and ATRIP-403 (3) antibodies as described in Ref. 40. Co-immunoprecipitations examining ATR oligomerization were conducted as described (41).

Kinase Reactions and Two-dimensional TLC Phosphopeptide Analysis—Kinase reactions were performed as described previously (22). For in vitro phosphopeptide analysis, ATR-ATRIP complexes were immunopurified, subjected to kinase reactions, and prepared for phosphopeptide mapping as described previously (42). HEK293T cells overexpressing FLAG-HA3-ATR constructs were 32P metabolically labeled and treated with HU. HA-ATR immunoprecipitates were prepared for phosphopeptide mapping similar to in vitro peptides. Tryptic phosphopeptides were resuspended in electrophoresis buffer, pH 1.9, spotted onto thin layer cellulose-coated glass plates, and electrophoresed in pH 1.9 buffer on a Hunter Box at 1000 V for 30 min. Plates were allowed to dry and then separated by chromatography using phosphochromatography buffer overnight (42).

Sequence Alignments—Conservation of S/TQ residues was determined by sequence alignment of ATR orthologs using ClustalW v.1.83. Secondary structure alignments by HEAT repeats is described in Ref. 26. DNA-PK secondary structure predictions were determined by Jpred3 (43).

RESULTS

Identification of an ATR Separation of Function Mutant—Human ATR contains 19 putative autophosphorylation consensus sites (S/TQ). Primary sequence alignment of ATR orthologs revealed conservation of 16 of these residues in mouse ATR with several conserved in the Xenopus laevis and Saccharomyces cerevisiae ATR proteins (Fig. IA). To examine their functional significance, we mutated the 16 conserved residues to alanine within a single cDNA, generating a 16A-ATR mutant.

We characterized the 16A-ATR mutant in a cellular complementation assay (see Ref. 44 and supplemental Fig. 1). This assay utilizes the parental cell line ATRflox/− TR, which harbors a conditional ATR allele, a second ATR allele disrupted by a neomycin cassette, and a stably integrated construct expressing the tetracycline repressor (TR) (22). We then integrated either wild-type (WT) or 16A-ATR constructs with a tetracycline response promoter, creating WT-ATRflox/− TR and 16A-ATRflox/− TR cell lines. ATR cDNAs also contain an N-terminal FLAG-HA3 epitope tag to differentiate exogenous and endogenous proteins. We screened stable integrants for equal protein levels and verified that the percentage of cells expressing tagged ATR were similar among clones. We characterized at least two independent mutant clones in subsequent experiments to account for possible clonal differences.

We induced expression of integrated ATR alleles and infected cell lines with adenovirus expressing Cre recombinase (AdCre) to delete the ATRflox allele or GFP (AdGFP) as a control. 96 h after infection, excision of ATRflox in parental ATRflox/− TR cells caused a substantial reduction in ATR protein levels (Fig. 1B, lanes 1 versus 6). The integrated WT-ATR or 16A-ATR cDNAs express at comparable levels (Fig. 1B, lanes 8–10), which is similar to the amount expressed from the endogenous locus in the heterozygous ATR+/− cells lacking a flox allele.

To test the proficiency of 16A-ATR to prevent progression into mitosis after DNA damage, we performed a G2 checkpoint assay. After tetracycline induction and adenovirus infection, we treated uncomplemented parental, WT-ATR, and 16A-ATR
cells with ionizing radiation and cultured cells in nucodazoide to block cells in mitosis. We determined the mitotic index by quantification of Ser(P)-10 histone H3-positive cells using flow cytometry. Following deletion of the ATR^{flox} allele, 37% of uncomplemented parental cells progressed into mitosis after ionizing radiation treatment. However, only 13% of WT-ATR integrants and 15 and 13% of the two 16A-ATR cell lines were irradiated with 8 Gy of ionizing radiation and incubated with nucodazolote for 8 h. Cells were collected, stained with propidium iodide, and anti-Ser(P)-10 histone 3, and examined by flow cytometry to determine DNA content and percentage of mitotic cells. Shown are the results from three independent experiments (**, p < 0.01, n ≥3). Representative methylene blue-stained colonies from AdCre-infected WT and 16A-ATR^{flox}–TR cells are also shown. E, PCR was performed to monitor excision of the flox allele.

Because ATR is essential for cellular viability in mammalian cells even in the absence of DNA damage, we examined the ability of 16A-ATR to support viability using a colony formation assay. We induced expression of integrated ATR constructs, infected cells with AdGFP or AdCre, and allowed single colonies to form over a period of 14 days. 32% of the parental cells formed colonies upon ATR^{flox} deletion (Fig. 1D) whereas expression of WT-ATR significantly improved viability with 72% of cells forming colonies. As previously reported, the surviving colonies of the parental cell line are due to incomplete Cre excision of the flox allele (Fig. 1E) (3). Unlike the WT-ATR cells, only 25 and 30% of cells in the two 16A-ATR cell lines formed colonies (Fig. 1D).

Only one in eight of the surviving 16A-ATR colonies from AdCre-infected cells actually successfully deleted the ATR^{flox} allele (Fig. 1E). Thus, most of the surviving colonies of 16A-ATR cells grew because they maintained expression of the endogenous wild-type ATR allele. In contrast, five of five colonies tested from the AdCre-infected WT-ATR cell line contained a deleted flox allele and only express the integrated WT-ATR cDNA. Thus, the 16A-ATR protein has a significantly diminished capacity to maintain cell viability after deletion of the endogenous ATR alleles. These data together with G2 checkpoint results indicate that 16A-ATR acts as a separation of function mutant.

16A-ATR Mutations Result in Replication Stress Response Defects—The mec1-100 allele of S. cerevisiae ATR is a separation of function mutant defective in S but not G2, checkpoint functions (45). Thus, we hypothesized that reduced viability but G2 checkpoint proficiency of 16A-ATR may be due to a separation of human ATR functions in S and G2 phases. To examine S phase functions, we investigated whether 16A-ATR could support resumption of DNA synthesis following a transient exposure to replication stress. We treated WT- and 16A-ATR^{del}–TR cells (see nomenclature explanation in supplemental Fig. 1) with HU for 24 h, removed the HU, and monitored DNA content. 16 h after release from HU, WT-ATR cells proceeded through S phase and accumulated with 4N DNA content because we added nucodazoide to the release medium. However, a large portion of 16A-ATR cells did not complete replication upon release (Fig. 2A), demonstrating 16A-ATR has diminished capacity to support the completion of DNA synthesis after a transient replication block.

In agreement with a replication stress response defect, 16A-ATR has a reduced ability to phosphorylate CHK1 in HU-treated cells (Fig. 2B). Quantification of three independent experiments confirmed a 50% reduction that is reproducible and statistically significant (Fig. 2C). Importantly, this 16A-
A 16A-ATR signaling defect was not due to a difference in the number of cells in S phase because BrdU incorporation indicated that 55% of WT-ATR and 53% of 16A-ATR cells were in S phase at the time of HU addition. These observations indicate that the 16A mutation separates G2 and S phase functions of ATR.

The inability of 16A-ATR to support viability and to respond to replication stress is not due to a difference in protein stability, basal kinase activity, association with ATRIP, oligomerization, or the ability to localize to stalled replication forks (supplemental Fig. 2). The 16A-ATR mutations actually reproducibly increased the TOPBP1-stimulated in vitro ATR kinase activity compared with WT-ATR (Fig. 2D). We do not know the cause of this increased in vitro activation. However, this phenotype does not segregate with the minimal ATR mutations causing viability and replication stress response defects (see 3A-ATR below), and we have not yet determined the specific mutation(s) that cause this effect. These results indicate that the functional defects of the 16A-ATR protein in cells are not due to some general defect in protein folding, stability, or interaction with well characterized protein partners.

**3A-ATR Phenocopies 16A-ATR Replication Stress Response Defects**—To determine which of the 16 mutations within 16A-ATR cause the functional defects, we analyzed mutants containing a subset of the 16A mutations in the cellular complementation assay. First, we generated two mutants containing either the 4 N-terminal sites (Fig. 1A; 4N-ATR) or the remaining 12 C-terminal sites mutated to alanine (Fig. 1A; 12C-ATR) and integrated the mutant cDNAs into the ATRlox-TR cell line. We screened mutant cell lines as before and determined the protein levels to be at least equal to wild-type-expressing cells (Fig. 3A). Colony formation assays revealed that two 4N-ATR-expressing clones complemented the essential function of ATR similar to WT-ATR (61 and 51% viable 4N-ATR colonies compared with 64% WT). However, both 12C-ATR-expressing clones exhibited significantly reduced viability (26 and 9% in the colony formation assay (Fig. 3B)).

Further analysis identified a triple mutant, T1566A/T1578A/T1881A (3A-ATR), that phenocopies the reduced viability observed in the 16A-ATR mutant. Only 21 and 25% of cells from two independent 3A-ATR cell lines formed colonies after excision of the flox allele compared with 80% of WT-ATR cells despite similar protein expression (Fig. 3, C and D). Slight differences in colony formation by the parental cell line in Fig. 3, B and D, are likely due to differences in the adenovirus batch used to delete the floxed allele. However, within each group of experiments, the colony formation efficiencies were highly reproducible. PCR genotyping confirmed the reduced viability of the 3A-ATR clones after Cre-mediated excision of the ATRlox allele. 12 of 14 colonies from Cre-infected WT-ATR colonies contained a deleted flox allele compared with only 6 of 21 3A-ATR-expressing clones.

We next examined whether 3A-ATR also recapitulates 16A-ATR replication stress response defects. Three independent clones of 3A-ATR cells exhibited reduced CHK1 and SMC1 phosphorylation after HU treatment compared with WT (Fig. 4A). BrdU incorporation assays showed that this difference in phosphorylation cannot be explained by differences in the percentage of S phase cells among clones at the time of the experiment (supplemental Fig. 3A).
3A-ATR mutations also compromised completion of DNA synthesis following a transient replication block. Only 22%, 21%, and 22% of WT-ATR cells in three independent cell lines did not complete DNA synthesis 16 h after HU removal whereas 36%, 49%, and 39% of the 3A-ATR cells failed to complete replication at the same time point (Fig. 4B). In agreement with the 16A-ATR results, the G2 checkpoint remained intact in the 3A-ATR mutant (Fig. 4C). The lack of a G2 checkpoint defect in these cell lines is not due to differences in cell cycle distribution or doubling times (supplemental Fig. 3, B and C). Together, these data confirm that the reduced viability observed in 3A-ATR cells correlates with a defect in S phase but not G2 checkpoint functions of ATR similar to the 16A-ATR phenotype. We also measured TOPBP1-mediated activation of 3A-ATR and found that it was similar to WT-ATR (Fig. 4D). Therefore, the increased in vitro ATR activation observed with the 16A-ATR mutations does not segregate with the viability and S phase defects.

A Conserved Extended Inter-HEAT Repeat Loop Is Important for ATR Function—Due to the functional defects observed with 3A-ATR, we were curious about the domain architecture surrounding the mutated residues. According to the HEAT repeat alignments designated by Perry and Kleckner (26), 3A-ATR residues occur in the inter-HEAT repeat loop between ATR HEAT repeats 31R and 32R (amino acids 1557–1628) and are just N-terminal to the FAT domain (amino acid 1640). Notably although inter-HEAT repeat loops are typically short 5–20 amino acid linkers, the loop between 31R and 32R is 71 amino acids long and predicted to be largely unstructured except for a short predicted helix (Fig. 5A). ATR HEAT repeats 31R and 32R align with ATM HEAT repeats 34M and 35M (26) (Fig. 5A). Similar to ATR, the inter-HEAT repeat loop between 34M and 35M (ATM amino acids 1875–1927) is longer than a typical inter-HEAT repeat loop (52 amino acids), predicted to be unstructured except for a single helix and is just adjacent to the FAT domain. Importantly, this region contains the regulatory ATM autophosphorylation site Ser-1893 whose mutation to alanine results in radiosensitivity and loss of checkpoint activity (35). Secondary structure predictions of DNA-PK also reveal the presence of an extensive 158-amino acid inter-HEAT repeat loop (amino acids 2566–2723) N-terminal to the DNA-PK FAT domain that contains the regulatory ABCDE (Thr-2609/Ser-2612/Thr-2638/Thr-2647) autophosphorylation cluster (Fig. 5A) (32, 33). These similarities suggest that the region between ATR 31R and 32R is a conserved regulatory region among the DNA damage PIKKs, and several of these sites may be targeted for autophosphorylation.
We analyzed purified ATR from undamaged and HU-treated cells for phosphorylation by mass spectrometry. We detected phosphorylation on T1989 (44) but were unable to detect a modified peptide containing T1566, T1578, or T1589 despite multiple attempts. This mass spectrometry analysis was limited by our ability to detect peptides spanning this region. We next assayed autophosphorylation of WT and 3A-ATR in vitro. We immunoprecipitated ATR from cells expressing FLAG-WT or FLAG-3A-ATR and allowed ATR to autophosphorylate. 

Comparison of WT and 3A-ATR phosphopeptides from GST maps did not reveal loss of a detectable phosphopeptide (Fig. 5C, compare top panels), although there is small variability in intensity of some phosphopeptides. Upon GST-AAD addition, one relatively low abundance phosphopeptide appeared (Fig. 5C, asterisk in bottom panels). GST-AAD addition to 3A-ATR also induced this phosphopeptide. Therefore under these conditions, 3A-ATR mutations fail to eliminate a discernible phosphopeptide.

We also examined in vivo ATR phosphopeptides obtained from HU-treated, 32P metabolically labeled cells transiently overexpressing FLAG-HA3-WT or FLAG-HA3-16A-ATR and transiently coexpressing HA-ATRIP. These experiments showed that the phosphopeptide pattern is similar to that seen in vitro. However, the intensity of some phosphopeptides is decreased in vivo compared to in vitro.
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did not observe loss of a phosphopeptide with 16A-ATR mutations (Fig. 5D). Thus, most ATR phosphorylation must not occur on sites that match the S/TQ consensus. Of note, the in vivo and in vitro WT-ATR phosphopeptide maps are dissimilar (compare maps in C and D). The in vivo maps will include phosphorylation sites catalyzed by other kinases, which likely explains some of the difference. However, these results also indicate that in vitro and in vivo autophosphorylation may be different. We attempted to examine phosphopeptide maps of endogenous ATR protein but were unable to purify sufficient labeled protein for this analysis. In summary, none of our attempts to demonstrate ATR autophosphorylation on Thr-1566, Thr-1578, or Thr-1589 has been successful. Our phosphopeptide maps do indicate that there is considerable complexity in ATR phosphorylation.

DISCUSSION

Our data demonstrate that the S and G2 phase functions of ATR can be separated by mutation of three candidate ATR autophosphorylation sites within an S/TQ cluster. However, our data suggest these residues are unlikely to be autophosphorylated by ATR. Nonetheless, the 3A-ATR residues occur in a region of ATR that aligns to regulatory regions of ATM and DNA-PK containing functional autophosphorylation sites. Mutation of 3A-ATR residues perturbs cell viability and replication stress responses but not the G2 checkpoint. This is the first identification and characterization of a separation of function mutant in human ATR that dissociates S and G2 phase activities. The correlation between cell viability and S phase defects suggests that the essential function of ATR is to promote completion of DNA replication.

Incomplete peptide coverage of ATR hindered detection of phosphorylation by mass spectrometry. We also did not detect loss of phosphorylation in phosphopeptide maps. These maps were generated using transiently overexpressed ATR, which may not be regulated the same as the endogenous protein. Indeed, Sancar and colleagues reported that they could only reconstitute ATR signaling in vitro by purifying endogenous, not overexpressed ATR complexes (46). We attempted to analyze phosphopeptides of endogenous ATR but were unable to obtain sufficient quantities of purified labeled protein. Our phosphopeptide maps do suggest that there are multiple regulatory phosphorylation sites on ATR. We recently found that Thr-1989 is one of these sites (44). However, Thr-1989 is not evolutionarily conserved, is not followed by a glutamine as is typical of ATR phosphorylation sites, and its mutation has minimal functional consequences.

The in vitro kinase activity of 3A-ATR is indistinguishable from WT-ATR; however, 3A-ATR does not phosphorylate CHK1 or SMC1 as well as the wild-type protein in cells. This observation is similar to the S1981A-ATM autophosphorylation mutant, which has in vivo but not in vitro substrate phosphorylation defects (34). Likewise, DNA-PK autophosphorylation mutants also do not show in vitro phosphorylation defects (47).

3A-ATR mutations cause defects in viability but only a 50% reduction in signaling to CHK1 and SMC1. A 50% reduction in phosphorylation may be sufficient to cause viability defects especially considering other ATR substrates are likely to be affected as well. Gene dosage is known to be important for ATR function. ATR is a haploinsufficient tumor suppressor in certain genetic backgrounds (48, 49), and reducing ATR protein levels can sensitize tumor cell lines to chemotherapies (50). Additionally, a hypomorphic allele of ATR found in Seckel syndrome patients results in diminished ATR protein levels due to aberrant splicing (51, 52).

The mec1-100 allele of the budding yeast ATR ortholog MEC1 dissociates S and G2 phase functions, suggesting that ATR signaling in different cell cycle phases may have distinct requirements (45). This allele contains the point mutations F1179S and N1700S, which correspond to Leu-1405 and Ala-1934, respectively, in human ATR (45). Neither amino acid is close to 3A-ATR residues in primary sequence, although we do not know their relative proximity in three-dimensional space. Work by Navadgi-Patti and Burgers supports that there are distinct protein activators for Mec1 in G1, S, and G2 phases (53). Dpb11, the yeast homolog of TOPBP1, activates Mec1 in G2 whereas the RAD9 homolog Ddc1 functions in both G1 and G2 phases (53–55). A double ddc1Δdpb11-1 mutant does not fully abolish S phase activation of Mec1, suggesting an undefined S phase activator of Mec1. The 3A-ATR mutant is stimulated by TOPBP1 and retains G2 but not S phase functions. Therefore, it is possible that these mutations specifically disrupt regulation of ATR by an undefined protein activator in S phase.

Cryo-EM and x-ray crystal structures of DNA-PK reveal a “crown” structure that consists of the FAT, kinase, and FATC domains (56–59). The crown structure sits atop two “arms” consisting of the N-terminal HEAT repeats. Juxtaposed between the crown and arm structures are irregular regions in the DNA-PK structure predicted to be flexible hinges. Autophosphorylation produces a conformational shift in the DNA-PK structure possibly due to a shift in this flexible region. Notably, the regulatory ABCDE DNA-PK autophosphorylation (Thr-2609/Ser-2612/Thr-2638/Thr-2647) cluster occurs just before the DNA-PK FAT domain (begins at amino acid 2881) and may reside in this predicted flexible region.

Indeed, by secondary structure prediction, the ABCDE cluster occurs between HEAT repeats in a 158-amino acid inter-HEAT repeat loop (amino acids 2566–2723) predicted to be unstructured except for a single short helix. Examining HEAT repeat alignments by Perry and Kleckner (26), an analogous loop between HEAT repeat units occurs just before the ATR and ATM FAT domains. ATM is autophosphorylated in the linker at Ser-1893, and 3A-ATR residues occur in the region as well. Furthermore, alignment of ATR and DNA-PK by secondary structure places ATR Thr-1589 within 1–2 amino acids of DNA-PK autophosphorylation sites Thr-2609 and Ser-2612. Although ATR may not be phosphorylated in this region, these similarities among ATR, ATM, and DNA-PK suggest that these uncommonly long inter-HEAT repeat loops are an important regulatory region involved in controlling PIKK activity.

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