Thr-1989 Phosphorylation Is a Marker of Active Ataxia Telangiectasia-mutated and Rad3-related (ATR) Kinase

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The DNA damage response kinases ataxia telangiectasia-mutated (ATM), DNA-dependent protein kinase (DNA-PK), and ataxia telangiectasia-mutated and Rad3-related (ATR) signal through multiple pathways to promote genome maintenance. These related kinases share similar methods of regulation, including recruitment to specific nucleic acid structures and association with protein activators. ATM and DNA-PK also are regulated via phosphorylation, which provides a convenient biomarker for their activity. Whether phosphorylation regulates ATR is unknown. Here we identify ATR Thr-1989 as a DNA damage-regulated phosphorylation site. Selective inhibition of ATR prevents Thr-1989 phosphorylation, and phosphorylation requires ATR activation. Cells engineered to express only a non-phosphorylated T1989A mutant exhibit a modest ATR functional defect. Our results suggest that, like ATM and DNA-PK, phosphorylation regulates ATR, and phospho-peptide specific antibodies to Thr-1989 provide a proximal marker of ATR activation.

The DNA damage response is an evolutionarily conserved signal transduction network that coordinates cell cycle transitions, DNA replication, DNA repair, and apoptosis to guard against genomic instability. At the apex of the DNA damage response are three related kinases belonging to the PI3K-related protein kinase (PIKKs) family, including ataxia telangiectasia-mutated (ATM), ATM and RAD3-related (ATR), and DNA-dependent protein kinase (DNA-PK). The PIKKs are large proteins that share domain architecture and regulatory mechanisms. A large portion of the PIKK proteins consists of an array of antiparallel helices called Huntingtin, elongation factor 3, protein phosphatase 2A, and PI3K TOR1 (HEAT) repeats (1). The C-terminal kinase domain of the PIKKs is flanked by the FRAP, ATM, TRRAP (FAT) domain (2); PIKK regulatory domain (PRD) (3); and FAT C terminus domain (2).

ATR is essential for viability in replicating human cells, and disruption of ATR in mice results in embryonic lethality prior to embryonic day 7.5 (4–6). ATR regulates replication fork stability, restart of collapsed forks, and late-origin firing during S-phase. ATR also activates the G2 checkpoint to prevent entry into mitosis in the presence of damaged DNA (7, 8). Clinically, hypomorphic mutations in ATR cause Seckel syndrome (9), and heterozygous ATR mutations are associated with poor prognosis of tumors with microsatellite instability (10, 11).

The ATR activation process involves recruitment of ATR and its obligate partner ATR-interacting protein (ATRIP) (4) to a DNA lesion or stalled replication fork. Single-stranded DNA coated with replication protein A often mediates this recruitment (12) because ATRIP interacts directly with the 70-kDa subunit of replication protein A (13). Independently, the RAD9-RAD1-HUS1 (9-1-1) complex is loaded onto sites of damage by the clamp loader RAD17 (14–17). This loading is specific to a 5′ recessed junction, perhaps because of the interaction of RAD9 with the 70N domain of replication protein A (18). The 9-1-1 complex binds a BRCA1 C-terminal (BRCT) repeat protein topoisomerase binding protein 1 (TOPBP1) (19, 20), which activates the ATR kinase (21). A region of TOPBP1 termed the ATR-activating domain (AAD) binds surfaces on both ATR and ATRIP and causes a conformational change that likely increases the ability of ATR to bind substrates (3, 22). Partial in vitro reconstitution of the ATR activation process has been achieved (23, 24). However, significant questions remain about ATR regulation.

ATM and DNA-PK, like ATR, are regulated by recruitment to sites of DNA damage (25) and by protein activators (26, 27). In addition, ATM and DNA-PK undergo autophosphorylation (28–31). These kinases prefer to phosphorylate serines or threonines that are immediately followed by glutamine. Phosphorylation sites on ATM and DNA-PK are functionally significant and have been used as direct markers of activation (28–31). These kinases prefer to phosphorylate serines or threonines that are immediately followed by glutamine. Phosphorylation sites on ATM and DNA-PK are functionally significant and have been used as direct markers of activation (28–31). These kinases prefer to phosphorylate serines or threonines that are immediately followed by glutamine. Phosphorylation sites on ATM and DNA-PK are functionally significant and have been used as direct markers of activation (28–31). These kinases prefer to phosphorylate serines or threonines that are immediately followed by glutamine.

EXPERIMENTAL PROCEDURES

Cell Culture and Genotyping—HCT116 ATRflox/−TR cells were generated as described in Ref. 3 and maintained in McCoy’s 5A medium containing 10% FBS and 10 μg/ml blasticidin. Clonal ATR stable cell lines expressing tetracycline-in-
ducible FLAG-HA3 epitope-tagged ATR cDNAs were generated as in Ref. 3 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 with adenovirus encoding the Cre recombinase to excise the floxed allele. Cre-mediated excision was verified by PCR as described previously (4). ATRdel/− TR cell lines were maintained in McCoy’s 5A medium containing 10% FBS and 1 μg/ml tetracycline. HEK293T and HeLa cells were maintained in DMEM containing 10% FBS. Adenovirus infections were carried out as described (4).

Drugs were added to cells at the following concentrations: 2 mM HU, 50 J/m² UV radiation, 5 Gray IR radiation, 50 mM HU, 50 J/m² UV radiation, 5 mM caffeine, 10 μM ATM inhibitor (KU55933) (32), 1 μM DNA-PK inhibitor (NU7441) (33), 50 μM roscovitine, 100 nM CHK1 inhibitor (AZD7762) (34), and 3 μM ATR inhibitor (AZ20). AZ20 is a selective inhibitor of ATR provided by AstraZeneca.3

DNA Constructs and siRNA—FLAG-HA3-WT-ATR was subcloned into the BamHI site of a modified pCDNA5/TO (Invitrogen). The BamHI-Xhol fragment (9668–9724) of the multiple cloning site was deleted, making the NotI and BstXI sites of the ATR cDNA unique. ATR mutants were generated using the QuikChange site-directed mutagenesis (Agilent Technologies) method. Mutagenesis reactions were carried out on fragments of the ATR cDNA in pBSKII(−), subcloned back into the full-length cDNA, and verified by sequencing. In some cases, the FLAG-HA3 epitope was replaced with a single FLAG epitope to facilitate purification of the HA-ATR/FLAG-ATR complexes by HA immunoprecipitation. siGENOME SMARTpool TOPBP1 siRNAs were purchased from Dharmacon. Plasmid and siRNA transfections were carried out using Lipofectamine 2000 using the manufacturer’s protocol.

Colonies were then incubated with λ phosphatase (New England Biolabs) for 30 min at 37 °C. Antibodies used included ATR-N19 (Santa Cruz Biotechnology, Inc.), CHK1-G4 (Santa Cruz Biotechnology, Inc.), phosphorylated Ser-317 CHK1 (Cell Signaling Technology, Inc.), phosphorylated Ser-345 CHK1 (Cell Signaling Technology, Inc.), phosphorylated Ser-296 CHK1 (Cell Signaling Technology, Inc.), and phosphorylated Ser-428 CHK1 (Cell Signaling Technology, Inc.). A phospho-ATR antibody was generated by Bethyl Laboratories, Inc. using a 13-amino acid human ATR peptide antigen with phosphorylated Thr-1589 at position 7. A cysteine was added to the N terminus to facilitate conjugation to a carrier. All immunoblotting was performed with infrared-conjugated secondary antibodies (LiCor) and quantified using an Odyssey system. Cells were processed for immunofluorescence as described previously (38). Immunoprecipitations were performed overnight at 4 °C using 5 μg of antibody and protein G-agarose (Santa Cruz Biotechnology, Inc.), washed three times with Nonidet P-40 buffer, and separated by SDS-PAGE prior to immunoblotting.

**Kinase Reactions**—Kinase reactions were performed as described previously (3, 13).

**RESULTS**

**ATR Thr-1989 is a DNA Damage-induced Phosphorylation Site**—To identify phosphorylation sites on ATR, we purified endogenous ATR from HU-treated HeLa cells using a single-step immunoprecipitation. We separated the ATR protein from associated proteins by gel electrophoresis and subjected it to in-gel digestion followed by liquid chromatography-coupled tandem mass spectrometry. This method identified several phosphorylation sites, including Ser-428 and Thr-1989 (Fig. 1A and data not shown). A phosphopeptide-specific antibody to

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3 AstraZeneca, unpublished data.
ATR Ser-428 is available commercially (Cell Signaling Technology, Inc.). However, no data has been published to indicate how this site is regulated, what kinase is responsible, or whether it is functionally significant. Our data indicate that it is not a useful marker for active ATR because it does not correlate with ATR activity (supplemental Fig. 1).

Phosphoproteomic screens also previously identified Thr-1989 as a phosphorylated residue (39, 40). Thr-1989 is within the ATR FAT domain, which is composed of HEAT repeats and is generally highly conserved among ATR orthologues. However, sequence alignment revealed that Thr-1989 is only conserved in primates, changed to a serine in rodents, and not conserved in frogs or flies (Fig. 1B). The amino acids flanking Thr-1989 are also poorly conserved.

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To characterize phosphorylation of Thr-1989 further, we generated a phosphopeptide-specific antibody (pATR). This antibody recognizes an ATR peptide phosphorylated at position Thr-1989 but not the corresponding unmodified peptide (supplemental Fig. 2). The antibody recognizes ATR on immunoblots following immunoprecipitation (Fig. 2A). It is specific to ATR because excision of the floxed allele from ATRflox/− TR cells eliminated reactivity. Attempts to detect phosphorylated ATR by immunoblotting without prior immunoprecipitation were unsuccessful because of a cross-reacting protein near the same molecular weight as ATR.

ATR phosphorylation in cells treated with HU for 1 h (Fig. 2D). A commonly used marker of ATR activation is phosphorylation of the ATR substrate CHK1. Although CHK1 Ser-317 and ATR Thr-1989 are both phosphorylated within 1 h of HU, UV, or IR exposure, CHK1 phosphorylation did not substantially increase at later times. In contrast, Thr-1989 phosphorylation continued to increase at least until 3 h after treatment (Fig. 2C). Importantly, λ phosphatase abolished detection of DNA damage-induced Thr-1989 phosphorylation (Fig. 2E).

To determine whether the pATR antibody is specific to phosphorylated Thr-1989, we assessed its recognition of an ATR T1989A mutant protein. In contrast to wild-type ATR, the antibody did not recognize an HU-inducible phosphorylation on the ATR T1989A protein (Fig. 2F). We did note some residual antibody recognition of a protein at the molecular weight of ATR after immunoprecipitation of FLAG-ATR WT or T1989A were expressed in 293T cells. FLAG immunoprecipitates were mock-treated or treated with phosphatase prior to immunoblotting. G, FLAG immunoprecipitates from ATRflox/− TR (Ctl), FLAG-HA-ATR WT, or FLAG-HA-ATR T1989A expressing ATRflox/− TR cells were immunoblotted.
can autophosphorylate in vitro (4). Most characterized ATM, DNA-PK, and ATR phosphorylation sites are Ser or Thr followed by Gln. Thr-1989 does not fit this consensus. However, there are several characterized ATM and DNA-PK autophosphorylation sites that also fail to conform to this consensus sequence (41, 42). Therefore, we tested whether Thr-1989 phosphorylation in cells depends on ATR kinase activity. First, we assayed whether a kinase-dead ATR mutant can be phosphorylated on Thr-1989. Unlike wild-type ATR, the kinase-dead mutant did not exhibit HU-inducible Thr-1989 phosphorylation (Fig. 3A). We next used small molecule inhibitors to the PIKK family of kinases. Selective ATM and DNA-PK inhibitors did not reduce Thr-1989 phosphorylation following HU treatment (Fig. 3B). However, the non-selective ATR and ATM inhibitor caffeine significantly decreased Thr-1989 phosphorylation in HU-treated cells and inhibited ATR signaling as measured by CHK1 phosphorylation. Finally, an agent that selectively inhibits ATR (AZ20) but not DNA-PK or ATM also abrogates HU-induced Thr-1989 phosphorylation on both exogenous (Fig. 3C) and endogenous ATR (D). These results indicate that Thr-1989 phosphorylation in cells requires ATR kinase activity.

Immunopurified ATR-ATRIP complexes failed to phosphorylate a small, recombinant ATR fragment containing Thr-1989 purified from Escherichia coli. We also failed to observe a significant increase in Thr-1989 phosphorylation on purified full-length ATR subjected to in vitro conditions that allow autophosphorylation (data not shown). Thus, it remains possible that the in vivo dependence on ATR kinase activity is due to an ATR-activated kinase rather than autophosphorylation. However, treating cells with a selective CHK1 inhibitor did not significantly impair HU-induced Thr-1989 phosphorylation (supplemental Fig. 3) indicating if another kinase is involved, it is not CHK1-dependent. Unlike ATRIP Ser-224 phosphorylation (35), treatment with the cyclin-dependent kinase inhibitor roscovitine also had no effect on Thr-1989 phosphorylation (supplemental Fig. 3).

Thr-1989 Phosphorylation Requires ATR Activation—ATM and DNA-PK activation and autophosphorylation require their protein activators MRE11-RAD50-NBS1 and KU70/80, respectively (43). Thus, we first sought to determine whether Thr-1989 phosphorylation requires the ATR activator TOPBP1 (21). Transfection with TOPBP1 siRNA alone caused increased Thr-1989 phosphorylation even in the absence of a DNA damaging agent. This level of phosphorylation did not change upon addition of HU and remained below the level seen in HU-treated cells transfected with a control non-targeting siRNA (supplemental Fig. 4). We were unable to achieve complete knockdown of TOPBP1 in these experiments and could still detect significant CHK1 phosphorylation in HU-treated TOPBP1 siRNA cells (supplemental Fig. 4). The incomplete silencing of TOPBP1 expression and increased basal Thr-1989 phosphorylation but lack of HU-inducible phosphorylation makes this siRNA experiment difficult to interpret. As an alternative approach, we examined Thr-1989 phosphorylation on the ATR PRD mutant, which contains the point mutation K2589E. This mutation disrupts the ability of ATR to interact with and be activated by TOPBP1 while retaining basal ATR kinase activity (3). The ATR-PRD mutant is not phosphorylated on Thr-1989 in response to HU (Fig. 4A), suggesting that Thr-1989 phosphorylation depends on activation of ATR.

Because Thr-1989 phosphorylation depends on ATR activation, phosphorylation is unlikely to be required for the activation process, and an ATR T1989A mutant should still be activated by TOPBP1 in vitro. To test this hypothesis, we assayed whether the AAD of TOPBP1 could activate immunopurified wild-type, T1989A, and T1989E ATR (WT) or ATR(ΔC-terminal) TR cells expressing FLAG-HA-ATR were treated with 2 mM HU and 5 mM AZ20 for 6 h, as indicated. D, 293T cells were treated with HU and 3 μM AZ20 for 6 h.
T1989A-ATR\textsuperscript{flox/−}/TR cell lines. ATR cDNAs also contain an N-terminal FLAG-HA\textsubscript{3} epitope tag to differentiate exogenous and endogenous proteins. After selection, we screened stable integrants for inducible expression of the ATR protein and selected only clones expressing similar protein levels for further analysis. In addition, we verified that the percentage of cells expressing tagged ATR in each clone was similar using immunofluorescence (Fig. 5\textit{A}). We also confirmed that the tagged protein correctly localized to the nucleus and can localize to stalled replication forks in response to HU-treatment (Fig. 5\textit{B}). This analysis confirms that the T1989A mutation does not interfere with the ATR-ATRIP complex, because ATRIP association is required for both the stability and localization of ATR (4, 44). In subsequent experiments, we characterized two independent mutant cell lines to account for any possible clonal differences.

Infecting the parental ATR\textsuperscript{flox/−}/TR cells with an adenovirus encoding the Cre recombinase nearly ablated ATR protein expression within 4 days post-infection (Fig. 5\textit{C}). Tetracycline-induced expression of the exogenous ATR cDNAs restored ATR expression with both WT-ATR and T1989A-ATR expressed at similar levels (Fig. 5\textit{C}). To compare the signaling activities of the T1989A and WT ATR proteins, we deleted the floxed allele, expressed the WT or T1989A mutant, and treated the cells with HU. Deletion of ATR\textsuperscript{flox} in the parental cell line attenuates CHK1 phosphorylation (Fig. 5\textit{C}, compare \textit{lanes 5 and 13}). WT-ATR restored CHK1 phosphorylation (Fig. 5\textit{C}, \textit{lane 14}). Similarly, both T1989A-ATR clones also supported ATR-dependent CHK1 phosphorylation (Fig. 5\textit{C}, \textit{lanes 15 and 16}), suggesting that Thr-1989 phosphorylation is not required for ATR signaling in response to replication stress.

ATR promotes completion of DNA synthesis following an HU challenge (8). To test whether Thr-1989 phosphorylation regulates this activity, we monitored completion of DNA replication following a transient exposure to HU. After Cre-deletion of the floxed allele, both WT-ATR and T1989A-ATR cells have

![FIGURE 4. Thr-1989 phosphorylation requires ATR activation via the PRD. A, empty vector (Ctl), FLAG-ATR-WT, or FLAG-ATR-PRD expression vectors were transfected into 293T cells. Cells were HU-treated or untreated for 6 h, lysed, and FLAG-immunoprecipitates (IP) immunoblotted to detect phosphorylated and total ATR. B and C, wild-type ATR (WT), ATR T1989A, or ATR T1989E proteins complexed with ATRIP were isolated from transfected 293T cells and incubated with MCM2 substrate, [\gamma\textsuperscript{32P}]ATP, and increasing concentrations of recombinant GST-TOPBP1-AAD or GST (B) or GST-TOPBP1-BRCT7&8 or GST-TOPBP1-AAD+BRCT7&8 (C), as indicated. Kinase reactions were separated by SDS-PAGE and detected by phosphorimaging ([\gamma\textsuperscript{32P}]ATR and [\gamma\textsuperscript{32P}]MCM2). The amount of ATR, ATRIP, TOPBP1, MCM2, and GST proteins in each reaction was detected by staining with Coomassie blue.]

![FIGURE 5. ATR Thr-1989 phosphorylation is dispensable for cellular recovery from replication stress. A, ATR\textsuperscript{flox/−}/TR cells with an integrated tetracycline-responsive expression vector encoding FLAG-HA-ATR (WT) or FLAG-HA-T1989A proteins were analyzed by immunofluorescence after a transient induction of protein expression. B, localization of wild-type or T1989A ATR in HU-treated cells. C and D, ATR\textsuperscript{flox/−}/TR, WT-ATR\textsuperscript{flox/−}/TR, or two independent clones of T1989A-ATR\textsuperscript{flox/−}/TR cells were cultured in tetracycline media and infected with control (AdGFP) or Cre-expressing adenovirus (AdCre) to delete the floxed ATR allele. C, four days after infection, cells were treated with 0 or 2 mM HU for 6 h. Lysates were separated by SDS-PAGE and immunoblotted with the indicated antibodies. D, AdCre-infected cells were treated with HU for 24 h, washed once, and released into growth media containing nocodazole for 0, 4, or 10 h. Cells were fixed, stained with propidium iodide, and examined for DNA content by flow cytometry. Untreated (Asyn) cells were also analyzed.]

![DNA Content](https://example.com/dna-content-graph.png)

![Cell Number](https://example.com/cell-number-graph.png)
similar distributions of cells in each cell cycle phase (Fig. 5D). HU synchronizes the cells in early S-phase. Cre-infected parental ATR\textsuperscript{floxed}/H11002 TR cells are unable to complete S-phase when released from the HU arrest. In contrast, both WT-ATR\textsuperscript{floxed}/H11002 TR and T1989A-ATR\textsuperscript{floxed}/H11002 TR cells complete replication with similar kinetics (Fig. 5D).

ATR is an essential gene required for human cell viability even in the absence of exogenous DNA damage (4). To determine whether Thr-1989 phosphorylation is required for the essential function of ATR, we grew ATR\textsuperscript{floxed}/H11002 TR, WT-ATR\textsuperscript{floxed}/H11002 TR, and T1989A-ATR\textsuperscript{floxed}/H11002 TR cells in the presence of tetracycline, infected with adenovirus encoding either GFP or Cre, and allowed single colonies to form over a period of 14 days. We visualized surviving colonies by methylene blue staining and counted colonies to determine survival fractions. Upon ATR deletion, 5% of AdCre-infected parental ATR\textsuperscript{floxed}/H11002 TR cells were able to form colonies (Fig. 6, A and B). Wild-type ATR complements this viability defect with 82% of AdCre-infected WT-ATR\textsuperscript{floxed}/H11002 TR cells forming colonies. In contrast, T1989A-ATR does not fully complement the viability defect caused by ATR\textsuperscript{floxed} deletion with only 29 and 25% of AdCre infected T1989A-1 and T1989A-2-ATR\textsuperscript{floxed}/H11002 TR cells forming colonies, respectively.

We further confirmed the colony formation results by monitoring excision of the flox allele in colonies from AdCre-infected cells by PCR genotyping. As expected, 100% of colonies (9 of 9) obtained from the parental ATR\textsuperscript{floxed}/H11002 TR cell line contained an intact flox allele (Fig. 6C). Thus, they survived only because Cre-catalyzed excision was not complete. In contrast, PCR genotyping showed excision of the floxed allele in every colony obtained from T1989A-ATR\textsuperscript{floxed}/H11002 TR cells (53 of 55). These results suggest that Thr-1989 phosphorylation may be important but not essential for ATR to support cell viability.

Because the T1989A-ATR protein yielded a modest colony formation defect, we considered the possibility that a mild checkpoint signaling defect may not have been observed in the Cre infection experiments (Fig. 5D) because of residual endogenous ATR protein expression following Cre-mediated excision of the flox allele. To address this possibility, WT and T1989A AdCre-infected colonies determined to have an excised flox allele by PCR genotyping were expanded, creating WT-ATR\textsuperscript{del}/H11002 TR and T1989A-ATR\textsuperscript{del}/H11002 TR cell lines that have no intact ATR genes at the endogenous loci. We confirmed that the only ATR expressed in these cell lines comes from the integrated ATR wild-type or T1989A cDNAs (supplemental Fig. 6).
and that the percentage of S-phase cells was similar among cell lines (supplemental Fig. 7). We examined CHK1 phosphorylation in two WT-ATRdel−/− TR and six T1989A-ATRdel−/− TR clones with varying levels of ATR expression. T1989A ATR expression varied between 37% (T1989A2.2) and 122% (T1989A1.3) of the ATR level in the parental ATRFlox−/− TR cells. For comparison, WT1.1 and WT1.2 expressed ATR at 80 and 64% of the ATRFlox−/− TR expression level. Both WT clones supported CHK1 phosphorylation equally (Fig. 6D). The amount of CHK1 phosphorylation varied in the T1989A-ATRdel−/− TR cell lines (Fig. 6D) and correlated with the amount of T1989A protein expressed (Fig. 6E). The cell lines expressing the least amount of T1989A protein exhibited ~60% of wild-type CHK1 phosphorylation, whereas cell lines expressing the most T1989A had 150% of wild-type CHK1 phosphorylation levels. Thus, we conclude that the T1989A-ATR protein signals to phosphorylate CHK1 as efficiently as wild-type ATR when expressed at similar levels.

**DISCUSSION**

A challenge to studying ATR function has been the lack of a proximal biomarker to detect an active ATR kinase. Thus, researchers use CHK1 phosphorylation as a surrogate. However, ATR-dependent CHK1 phosphorylation requires proteins like CLASPIN that are not necessary for ATR to phosphorylate other substrates (45). There may also be instances in which CHK1 is phosphorylated by other kinases like ATM. Our data on Thr-1989 phosphorylation suggests that it is a good biomarker for active ATR. Thr-1989 phosphorylation is DNA damage-regulated, depends on ATR kinase activity, appears concurrently with ATR-dependent phosphorylation of CHK1, and requires activation of the ATR kinase. Functionally, Thr-1989 phosphorylation is not essential for the ATR-CHK1 signaling axis following replication stress but has some function in supporting cellular viability.

Our cellular data indicate that Thr-1989 requires ATR kinase activity, suggesting that it may be an autophosphorylation site. Although Thr-1989 does not conform to the typical ATR S/TQ phosphorylation consensus, there is precedence for non-consensus ATM and DNA-PK autophosphorylation. Several DNA-PK autophosphorylation residues in the PQR cluster are tyrosine-directed (46), and the ATM autophosphorylation sites Ser-1893 (29) and Ser-2996 (42) are glutamic and aspartic acid-directed, respectively. We were unable to obtain evidence that Thr-1989 is phosphorylated by ATR in vivo. However, these experiments are done in conditions that do not fully reconstitute the ATR activation mechanism, and two-dimensional phosphopeptide maps of *in vivo* and *in vitro* ATR phosphopeptides have minimal similarities. Thus, it is unclear whether the *in vitro* ATR kinase analysis mimics what happens *in vivo*. The other possibility is that an ATR-dependent kinase phosphorylates Thr-1989. Only CHK1 is a well-defined ATR-dependent kinase, but our experiments indicate that it is not required for Thr-1989 phosphorylation. Further analyses will be necessary to unambiguously determine whether Thr-1989 is truly an autophosphorylation site or phosphorylated by an ATR-activated kinase in an autoregulatory circuit.

We noted that CHK1 phosphorylation remains constant after reaching a high level 1 h after HU treatment, whereas ATR Thr-1989 phosphorylation continued to increase throughout the HU time course. The source of the difference is presently unclear but could be explained if different phosphatases act on these sites to cause the dynamic differences. Alternatively, it may take longer for most ATR molecules in the cell to be activated compared with CHK1 molecules.

Mutation of the ATR PRD, which prevents TOPBP1-dependent activation (3), abolishes DNA damage-induced Thr-1989 phosphorylation. Thus, Thr-1989 phosphorylation requires ATR activation and suggests that this site is downstream of TOPBP1 function. Likewise, autophosphorylation of ATM and DNA-PK also requires activation by their protein activators (30, 47, 48). Although the only known function of the ATR PRD is to facilitate ATR activation by binding TOPBP1, we cannot exclude the possibility that proteins other than TOPBP1 function through the PRD to promote Thr-1989 phosphorylation.

Ablation of the ATM autophosphorylation site Ser-1981 does not impact MRN-mediated activation of ATM (49). Similarly, mutation of Thr-1989 to an alanine does not perturb TOPBP1-mediated activation of ATR *in vitro*. Also in agreement with a lack of an ATR activation defect is the absence of strong signaling and functional defects in cells expressing only the unphosphorylatable T1989A ATR protein. ATR mutations that disrupt the activation process, such as the K2589E PRD mutant, exhibit severe cellular defects because of their inability to be activated (3).

CHK1 phosphorylation in multiple T1989A-ATRdel−/− TR cell lines showed a strong correlation with ATR protein levels. When T1989A ATR is expressed at levels similar to those found in ATRFlox−/− TR cells, it signals to CHK1 similar to wild-type ATR. ATR protein levels in the ATRFlox−/− TR cells is ~30−40% of that found in the ATR+/+ cell line because of the deletion of one ATR allele and the insertion of the loxP sites in the remaining expressed allele. Thus, the lack of an obvious ATR signaling defect in the T1989A mutant cell lines is not due to overexpression of this protein.

Despite the lack of a signaling defect, we observed a significant albeit partial decrease in the ability of T1989A ATR to maintain cell viability when challenged in a colony formation assay. A possible explanation is that T1989A ATR is expressed at variable levels in the clonal population. Low expression levels may not support viability because signaling under these circumstances is attenuated compared with cells with wild-type expression levels. The viability assay likely presents a stringent requirement for ATR function because it requires colony growth from a single cell. However, once the T1989A-ATRdel−/− TR cells that survived were expanded, they showed no evidence of a continued survival or proliferation deficit, indicating Thr-1989 is not essential for ATR function. The low conservation of Thr-1989 even among vertebrate ATR orthologues and the lack of salient activation and signaling defects corroborate this conclusion.

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4 E. Nam and D. Cortez, unpublished data.
ATR Is Phosphorylated on T1989

The modest phenotypic consequence of the T1989A mutation is reminiscent of single mutants of DNA-PK autophasphorylation clusters (50). Mutation of multiple sites across clusters (51) or of a single cluster in entirety (50) is required to observe strong defects. Disruption of additional ATR phosphorylation sites may be needed to produce a significant biological defect. The functional significance of Thr-1989 phosphorylation may also depend on specific cellular contexts. For instance, mutation of ATM Ser–1981 to an alanine results in functional defects in human cells (28), yet there is no functional consequence of mutating this phosphorylation site in mouse cells (52).

Importantly, because Thr-1989 is a damage-regulated phosphorylation site that requires activation of ATR, antibodies to this phosphorylation site will be useful to directly monitor an active ATR kinase in cells. In addition to facilitating future research on ATR function, monitoring ATR Thr-1989 phosphorylation may also prove to be a useful biomarker for cancer treatment with agents such as ATR-selective inhibitors or as a measurement of oncogene-induced replication stress.

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