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ATR Autophosphorylation as a Molecular Switch for Checkpoint Activation

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SUMMARY

The *ataxia telangiectasia*-mutated and Rad3-related (ATR) kinase is a master checkpoint regulator safeguarding the genome. Upon DNA damage, the ATR-ATRIP complex is recruited to sites of DNA damage by RPA-coated single-stranded DNA and activated by an elusive process. Here, we show that ATR is transformed into a hyperphosphorylated state after DNA damage, and that a single autophosphorylation event at Thr 1989 is crucial for ATR activation. Phosphorylation of Thr 1989 relies on RPA, ATRIP, and ATR kinase activity, but unexpectedly not on the ATR stimulator TopBP1. Recruitment of ATR-ATRIP to RPA-ssDNA leads to congregation of ATR-ATRIP complexes and promotes Thr 1989 phosphorylation in *trans*. Phosphorylated Thr 1989 is directly recognized by TopBP1 via the BRCT domains 7 and 8, enabling TopBP1 to engage ATR-ATRIP, to stimulate the ATR kinase, and to facilitate ATR substrate recognition. Thus, ATR autophosphorylation on RPA-ssDNA is a molecular switch to launch robust checkpoint response.

INTRODUCTION

ATR, *ataxia telangiectasia*-mutated (ATM), and DNA-PKcs (DNA-dependent protein kinase) are three members of the phosphoinositide-3-kinase-like protein kinase (PIKK) family and key regulators of DNA damage signaling and DNA repair. Although all these PIKKs are activated by DNA damage, their DNA damage specificities are distinct, and their functions are not identical. ATM and DNA-PKcs are activated by double-stranded DNA breaks (DSBs), whereas ATR responds to a broad spectrum of DNA damage that induces single-stranded DNA (ssDNA) (Ciccia and Elledge, 2010; Cimprich and Cortez, 2008; Flynn and Zou, 2010). This unusual versatility of ATR enables it to play a particularly important role in the cellular responses to intrinsic genomic stresses during cell proliferation. Unlike ATM and DNA-PKcs, ATR is essential for cell survival even in the absence of extrinsic genomic insults (Brown and Baltimore, 2000; Cortez et al., 2001). Elucidation of the mechanism by which ATR is activated is central to understanding how genomic integrity is maintained in humans.

In response to DNA damage, ATR, ATM, and DNA-PKcs are regulated by distinct DNA damage sensors. ATM is recruited and activated by the Mre11-Rad50-Nbs1 (MRN) complex (Berkovich et al., 2007; Lee and Paull, 2005; Uziel et al., 2003), whereas DNA-PKcs is recruited and activated by the Ku70-Ku80 heterodimer (Smith and Jackson, 1999). ATR, through ATRIP, recognizes RPA-ssDNA at sites of DNA damage or stressed replication forks (Ball et al., 2005; Costanzo et al., 2003; Namiki and Zou, 2006; Zou and Elledge, 2003). In contrast to ATM and DNA-PKcs, ATR-ATRIP is not fully activated by the sensor-DNA complex, RPA-ssDNA (MacDougall et al., 2007). The full activation of ATR-ATRIP requires additional regulators including Rad17, the Rad9-Rad1-Hus1 (9-1-1) "checkpoint clamp," and TopBP1 (Kumagai et al., 2006; Liu et al., 2006; Zou et al., 2002). Thus, ATR is a unique PIKK that is recruited and activated by different factors through a multistep process.

A major gap in our understanding of ATR activation is how the ATR-ATRIP kinase is activated on RPA-ssDNA. Even in the absence of DNA and other proteins, TopBP1 directly stimulates the ATR-ATRIP kinase in vitro (Kumagai et al., 2006). The ATR-activating domain (AAD) of TopBP1 interacts with ATR-ATRIP in vitro, but this interaction is weak and not regulated by DNA damage (Mordes et al., 2008). How the stimulation of ATR-ATRIP by TopBP1 is regulated by DNA damage in vivo is still poorly understood. TopBP1 interacts with Rad9, a 9-1-1 component, through two constitutively phosphorylated residues at its C terminus (Delacroix et al., 2007; Lee et al., 2007; Takeishi et al., 2010). In response to DNA damage, 9-1-1 is loaded onto dsDNA by a Rad17-containing, RFC-like "clamp loader" that recognizes junctions of RPA-ssDNA and double-stranded DNA (dsDNA) (Ellison and Stillman, 2003; Zou et al., 2003). The interaction between Rad9 and TopBP1 may help to recruit TopBP1 to sites

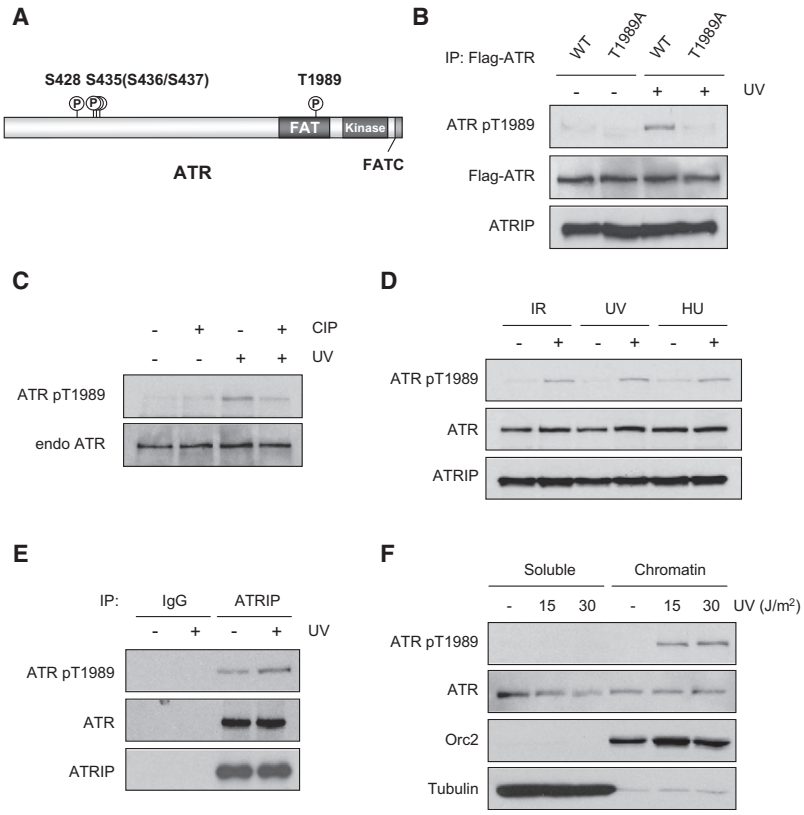

Figure 1. ATR Is Phosphorylated at Thr 1989 after DNA Damage

(A) Schematics of the phosphorylation sites of ATR. Note that mass spectrometry data only identified Ser 436 and S437 as potential phosphorylation sites.

(B) 293T cells transiently expressing Flag-ATR^{WT} or Flag-ATR^{T1989A} were treated with 50 J/m^2 of UV or left untreated, and subjected to immunoprecipitation with anti-Flag antibody in 2 hr. The levels of precipitated ATR, ATR pT1989, and ATRIP were analyzed using the indicated antibodies.

(C) Endogenous ATR was immunoprecipitated from HCT116 cell extracts that were treated with calf intestinal phosphatase (CIP) or mock treated.

(D) HCT116 cells were treated with 10 Gy of IR, 50 J/m^2 of UV, or 2 mM HU, and the phosphorylation of endogenous ATR was analyzed 2 hr after the treatment.

(E) Endogenous ATRIP was immunoprecipitated from HCT116 cell extracts. The levels of precipitated ATRIP, ATR, and ATR pT1989 were analyzed by western blot.

(F) HCT116 cells were treated with UV or left untreated, and extracts were separated into soluble and chromatin fractions in 2 hr. The levels of ATR and ATR pT1989 in these fractions were analyzed by western blot. Orc2 and tubulin serve as markers of chromatin and soluble fractions, respectively.

of DNA damage and/or facilitates ATR-ATRIP activation (Delacroix et al., 2007; Lee and Dunphy, 2010). However, it remains elusive how the 9-1-1 and TopBP1 recruited to dsDNA engage the ATR-ATRIP on RPA-ssDNA.

A second major question on ATR activation is how ATR recognizes its substrates and transmits DNA damage signals. Several proteins implicated in ATR signaling, such as Rad17 and Chk1, are phosphorylated by ATR on chromatin (Smits et al., 2006; Zou et al., 2002), suggesting that ATR functions on damaged DNA. Furthermore, Rad17, Claspin, and Chk1 are known to associate with each other via a series of ATR-orchestrated events after phosphorylation (Kumagai and Dunphy, 2003; Wang et al., 2006). The role of these ATR substrates in signal transduction and the phosphorylation-mediated interactions among them suggest that ATR directs assembly of a dynamic signaling complex on DNA. Nonetheless, how ATR engages this signaling complex remains unknown.

To delineate the process of ATR activation, we sought to capture ATR in its active state, to molecularly define the state, and to dissect the biochemical events leading to this state. We found that during its activation, ATR, like ATM and DNA-PKcs, is transformed into a hyperphosphorylated state with multiple sites phosphorylated. Surprisingly, however, among the phosphorylation sites of ATR that we identified, only Thr 1989 is critical for robust ATR activation. The phosphorylation of Thr 1989 occurs in *trans* among the ATR-ATRIP complexes that congregate on RPA-ssDNA. Phosphorylated Thr 1989 is directly recognized by TopBP1, enabling TopBP1 to stably engage the ATR-ATRIP complex, to efficiently stimulate the kinase, and to act as a scaffold for ATR-substrate interactions. These findings reveal unexpected links among the recruitment, stimulation, and substrate recognition of ATR-ATRIP, presenting a clearer picture of how ATR is fully activated at sites of DNA damage.

RESULTS

ATR Is Phosphorylated at Thr 1989 after DNA Damage

To determine whether ATR is phosphorylated during activation, we used mass spectrometry to analyze Flag-tagged ATR purified from hydroxyurea (HU)-treated 293E cells. Our data showed that ATR was phosphorylated at Ser 428, Ser 435, Thr 1989, and possibly Ser 436 and Ser437 (Figures 1A and see Figure S1A available online). The phosphorylation of Ser 428 was previously shown by others using an antibody from Cell Signaling (http://www.cellsignal.com/products/2853.html). The phosphorylation of Ser 435 and Thr 1989 was documented by large-scale studies on protein phosphorylation (Daub et al., 2008; Dephoure et al., 2008). To date, none of these phosphorylation sites have been functionally characterized. The location of Thr 1989 in the FAT (FRAP, ATM, TRRAP) domain, a potential regulatory element conserved among PIKKs, prompted us to focus our initial analysis on this phosphorylation site.

We first asked whether the phosphorylation of T1989 is induced by DNA damage. To monitor T1989 phosphorylation in vivo, we generated phospho-specific antibodies to this site. In cells irradiated with ultraviolet (UV) light, the phospho-T1989 antibody specifically recognized Flag-tagged wild-type ATR (ATR^{WT}), but not the T1989A mutant (ATR^{T1989A}; Figure 1B).

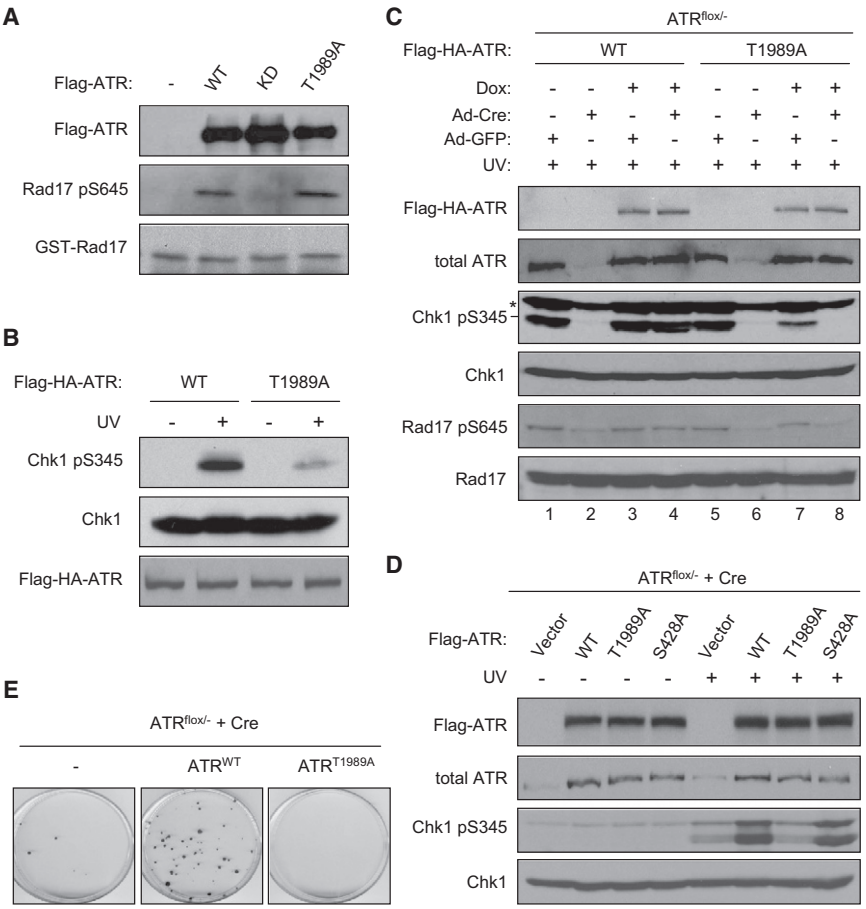

Figure 2. Thr 1989 Is Critical for ATR Function

(A) The kinase activities of Flag-ATR^{WT}, Flag-ATR^{KD}, and Flag-ATR^{T1989A} were tested in vitro using GST-Rad17 as a substrate. The phosphorylation of Rad17 S645 was analyzed using phospho-specific antibody.

(B) Flag-HA-ATR^{WT} and Flag-HA-ATR^{T1989A} were inducibly expressed in HCT116-derived stable cell lines. Cells were treated with 15 J/m^2 of UV, and the effects of ATR^{WT} and ATR^{T1989A} on Chk1 phosphorylation were analyzed in 2 hr. Flag-HA-ATR was detected with the HA antibody.

(C) ATR$^{flox/-}$-derivative lines were infected with Ad-Cre to delete endogenous ATR, or infected with Ad-GFP as controls. Where indicated, cells were treated with Dox to induce Flag-HA-ATR^{WT} or Flag-HA-ATR^{T1989A}. The phosphorylation of Chk1 and Rad17 was analyzed using phospho-specific antibodies. Total ATR includes both endogenous and exogenous ATR. *, a band cross-reacting to the phospho-Chk1 antibody.

(D) ATR$^{flox/-}$ cells were treated with Ad-Cre and transfected with plasmids expressing Flag-ATR^{WT}, Flag-ATR^{T1989A}, or Flag-ATR^{S428A}.

(E) ATR$^{flox/-}$-derivative lines were infected with Ad-Cre and treated with Dox as in (C). The ability of ATR^{WT} and ATR^{T1989A} to support colony formation was analyzed.

Endogenous ATR was also recognized by the phospho-T1989 antibody after UV irradiation in several cell lines (Figure S1B). Treatment of cell extracts with phosphatase reduced the recognition of ATR by the phospho-T1989 antibody (Figure 1C). In addition to UV, ionizing radiation (IR) and HU also induced T1989 phosphorylation (Figure 1D). Together, these results demonstrate that ATR is phosphorylated at T1989 in a DNA damage-induced manner.

The phosphorylation of ATR at T1989 occurs rapidly after DNA damage. Like the ATR-mediated Chk1 phosphorylation, T1989 phosphorylation was detected within 0.5 hr after UV treatment (Figure S1C). Unlike Chk1 phosphorylation, which declined after 2 hr, T1989 phosphorylation persisted until 12 hr post UV treatment. The UV-induced T1989 phosphorylation is dose dependent. T1989 phosphorylation was readily detected in cells treated with 5 J/m^2 of UV, and was maximally induced by 50 J/m^2 of UV (Figure S1D). Phosphorylated ATR was coimmunoprecipitated by ATRIP (Figure 1E), showing that T1989 is phosphorylated in the ATR-ATRIP complex. Furthermore, T1989 phosphorylation was detected only in the chromatin fractions, but not in the soluble fractions (Figure 1F), suggesting that ATR is phosphorylated on chromatin. These features of T1989 phosphorylation are consistent with a potential role in ATR activation.

T1989 Phosphorylation Is Critical for ATR Function

We next used the ATR^{T1989A} mutant to investigate whether T1989 phosphorylation is implicated in ATR activation. Like ATR^{WT}, ATR^{T1989A} was able to phosphorylate a Rad17-derivative substrate (GST-Rad17) in vitro (Figure 2A), showing that the T1989A mutation does not significantly alter the kinase domain. When inducibly expressed in stable cell lines, ATR^{T1989A}, but not ATR^{WT}, attenuated the ATR-mediated Chk1 phosphorylation after UV treatment (Figure 2B; cell-cycle distributions shown in Figure S2A). Moreover, even in the absence of UV, induction of ATR^{T1989A} elicited H2AX phosphorylation in a large fraction of cells (Figures S2B and S2C), indicating an increase in genomic instability. These results suggest that although ATR^{T1989A} possesses an intact kinase domain, it interferes with the function of endogenous ATR.

To directly determine whether ATR^{T1989A} is functional, we established *ATR$^{flox/-}$*-derivative cell lines (Cortez et al., 2001) allowing inducible expression of Flag-HA-tagged ATR^{WT} or ATR^{T1989A} in cells devoid of endogenous ATR. Both ATR^{WT} and ATR^{T1989A} were expressed at levels similar to that of endogenous ATR in these cell lines (Figure 2C). As expected, the ATR^{T1989A} mutant expressed in the cell line was detected by ATR antibodies but not the phospho-T1989 antibody after UV irradiation, and it retained the ability to phosphorylate GST-Rad17 in vitro (Figures S2D and S2E). In cells lacking endogenous ATR, Chk1 and Rad17 were efficiently phosphorylated by ATR^{WT} but not ATR^{T1989A} after UV treatment (Figure 2C; lanes 4 and 8). To rule out the possibility that the compromised

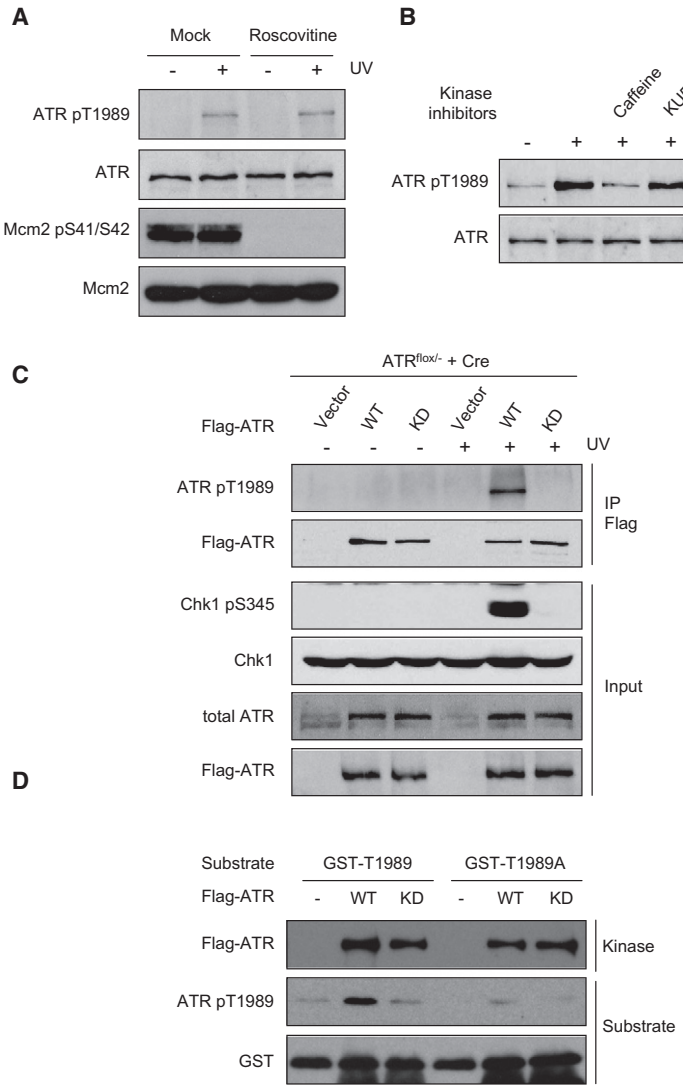

Figure 3. ATR Autophosphorylates Thr 1989

(A) HCT116 cells were treated with 50 μM roscovitine for 12 hr and then irradiated with 15 J/m^2 UV. The phosphorylation of ATR and Mcm2 was analyzed using phospho-specific antibodies 2 hr after UV treatment.

(B) HCT116 cells were treated with the indicated kinase inhibitors (4 mM caffeine, 10 μM KU55933, 10 μM NU7026, 1 μM UCN-01) for 1 hr and then irradiated with 15 J/m^2 UV. The phosphorylation of ATR was analyzed 2 hr after UV treatment.

(C) ATR$^{flox/-}$ cells were treated with Ad-Cre and transfected with plasmids expressing Flag-ATR^{WT} and Flag-ATR^{KD}. The phosphorylation of Flag-ATR and Chk1 was analyzed using phospho-specific antibodies.

(D) Purified GST-T1989 or GST-T1989A was incubated with Flag-ATR^{WT} or Flag-ATR^{KD} in the presence of ATP. The levels of ATR and substrates were monitored using anti-Flag and anti-GST antibodies. The phosphorylation of substrates was analyzed using the phospho-T1989 antibody.

checkpoint response in ATR^{T1989A} expressing cells is due to unexpected events during cell line generation, we tested additional independently generated cell lines that express ATR^{WT} or ATR^{T1989A}. Consistent with the experiment above, all ATR^{T1989A}-expressing cell lines displayed defective Chk1 activation (Figure S2F). Furthermore, similar results were obtained using *ATR$^{flox/-}$* cells infected with Cre-expressing adenovirus (Ad-Cre) and transfected with plasmids encoding Flag-ATR^{WT} or Flag-ATR^{T1989A} (Figure 2D). Together, these results demonstrate that the ATR^{T1989A} mutant is compromised in its ability to initiate robust checkpoint signaling.

In marked contrast to ATR^{T1989A}, neither ATR^{S428A} nor ATR$^{S435/436/437A}$ failed to activate Chk1 (Figure 2D and Figure S2G), showing that among the phosphorylation sites of ATR that we identified, T1989 is the only one critical for ATR activation. Since ATR is critical for genomic stability in cycling cells, deletion of *ATR* from *ATR$^{flox/-}$* cells resulted in loss of cell viability (Cortez et al., 2001). Expression of ATR^{WT}, but not ATR^{T1989A}, suppressed the growth defects of Ad-Cre-infected *ATR$^{flox/-}$* cells in both cell proliferation and colony formation assays (Figure 2E and Figure S2H). These results suggest that T1989 is critical not only for the activation of ATR by extrinsic DNA damage but also for its essential function in cycling cells.

While the ATR^{T1989A} mutant is defective for checkpoint response, the phosphomimetic ATR^{T1989D} mutant is fully functional in Chk1 activation after DNA damage (Figure S2I). Furthermore, we note that ATR^{T1989D} did not induce Chk1 phosphorylation in the absence of DNA damage, suggesting that T1989 phosphorylation is necessary but not sufficient for initiating robust checkpoint signaling. As described below, phosphorylated ATR functions in concert with other DNA damage sensors and TopBP1 to activate checkpoint response.

ATR Autophosphorylates T1989

We next investigated which kinase is responsible for T1989 phosphorylation. T1989 is followed by a Pro residue, raising the possibility that it is a substrate of CDKs. However, inhibitors of various CDKs did not affect T1989 phosphorylation after UV (Figure S3A). Treatment of cells with 50 μM of roscovitine for 14 hr completely abolished the CDK-dependent Mcm2 phosphorylation (Montagnoli et al., 2006) but did not alter the UV-induced T1989 phosphorylation (Figure 3A). In marked contrast, T1989 phosphorylation was clearly diminished by caffeine, a pan-inhibitor of ATR and ATM (Figure 3B). To pinpoint the PIKK responsible for T1989 phosphorylation, we tested the effects of specific ATM and DNA-PKcs inhibitors. Even when used in combination at high concentrations, ATM and DNA-PKcs inhibitors did not eliminate T1989 phosphorylation (Figure 3B and Figure S3B). These results suggest that ATR, rather

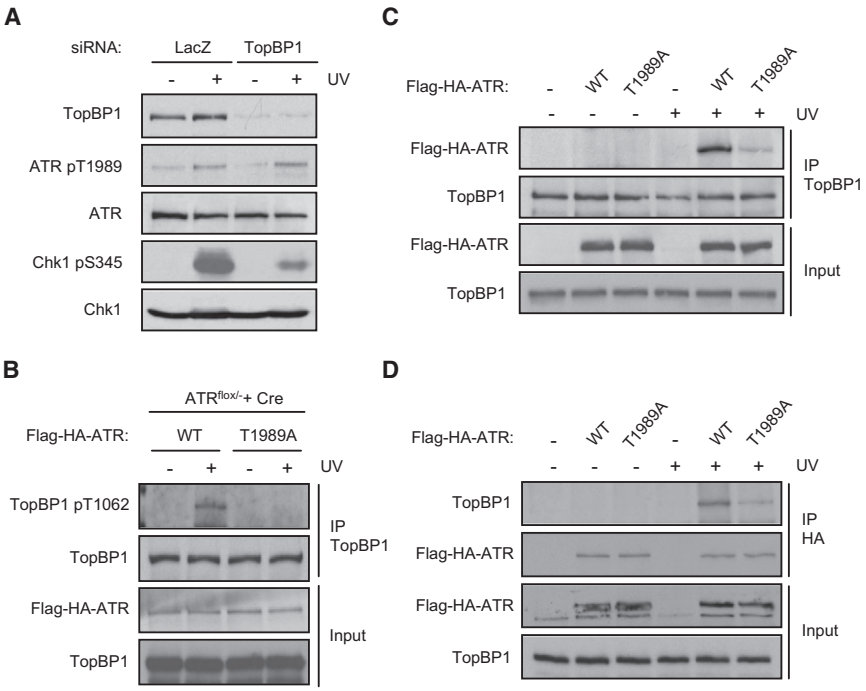

Figure 4. Phosphorylated T1989 Functions Upstream of TopBP1

(A) HCT116 cells were transfected with control or TopBP1 siRNA. The phosphorylation of ATR and Chk1 was analyzed using phospho-specific antibodies.

(B) ATR$^{flox/-}$-derivative cell lines were infected with Ad-Cre and treated with Dox to induce Flag-HA-ATR^{WT} or Flag-HA-ATR^{T1989A}. The phosphorylation of TopBP1 was analyzed using phospho-specific antibody.

(C and D) Flag-HA-ATR^{WT} and Flag-HA-ATR^{T1989A} were inducibly expressed in ATR$^{flox/-}$-derivative stable cell lines. Flag-HA-ATR and endogenous TopBP1 were immunoprecipitated from the chromatin fractions. The TopBP1 that coprecipitated with ATR and the ATR that coprecipitated with TopBP1 were analyzed by western blot.

than ATM and DNA-PKcs, is likely responsible for the UV-induced T1989 phosphorylation.

If T1989 is autophosphorylated by ATR, one would expect that the kinase-deficient ATR mutant (ATR^{KD}) is not phosphorylated at T1989. To test this possibility, we transiently expressed Flag-ATR^{WT} and Flag-ATR^{KD} in cells lacking endogenous ATR (Figure 3C). In the absence of endogenous ATR, Flag-ATR^{WT} but not Flag-ATR^{KD} was phosphorylated at T1989 after UV treatment, showing that T1989 phosphorylation is dependent upon ATR activity. Inhibition of Chk1 did not alter T1989 phosphorylation, suggesting a direct role of ATR in this phosphorylation event (Figure 3B).

To test whether T1989 is directly phosphorylated by ATR, we generated a GST-fusion protein that contains a peptide encompassing T1989 and its surrounding residues (GST-T1989). Like GST-Rad17, GST-T1989 was significantly phosphorylated by ATR (Figure 3D). This phosphorylation of T1989 by ATR was specific because the kinase did not phosphorylate GST-T1989A, and the phosphorylation of T1989 was compromised when ATR^{KD} was used. Together, these results suggest that T1989 is a direct substrate of ATR in vitro.

TopBP1 Engages ATR-ATRIP via Phosphorylated T1989

To pinpoint the role of T1989 phosphorylation in ATR activation, we asked if this event is dependent upon TopBP1. Knockdown of TopBP1 with siRNA dramatically reduced UV-induced Chk1 phosphorylation but did not affect T1989 phosphorylation (Figure 4A). On the other hand, in the absence of endogenous ATR and the presence of ATR^{T1989A}, UV-induced TopBP1 phosphorylation at T1062 was compromised (Figure 4B). Together these results show that T1989 phosphorylation is independent of TopBP1 but that the phosphorylation of TopBP1 requires T1989 phosphorylation. Thus, T1989 phosphorylation likely functions upstream of TopBP1 during ATR activation.

In response to UV damage, ATR was coimmunoprecipitated by TopBP1 from the chromatin fractions (Figure 4C). This damage-induced interaction of ATR and TopBP1 was abolished by phosphatase treatment of extracts (see Figure 5A), showing its dependence on phosphorylation. In cells expressing ATR^{WT} or ATR^{T1989A}, only ATR^{WT} but not ATR^{T1989A} was efficiently coprecipitated by TopBP1 (Figure 4C). Furthermore, TopBP1 was efficiently coprecipitated by ATR^{WT}, but not ATR^{T1989A} (Figure 4D). These results suggest that T1989 phosphorylation is important for the interaction between ATR and TopBP1 after DNA damage.

To understand how TopBP1 interacts with phosphorylated T1989 (Figures 4C, 4D, and 5A), we generated two biotinylated peptides that contain phosphorylated or unphosphorylated T1989 and its surrounding residues. Only the phospho-T1989 peptide, but not the unphosphopeptide, captured endogenous TopBP1 from extracts (Figure 5B). Using this binding assay and Flag-tagged TopBP1 fragments, we mapped the phospho-T1989-binding motif of TopBP1 to its BRCT domains 7 and 8 (Figures 5C and 5D and Figure S4A), which are required for activation of the ATR pathway (Gong et al., 2010). When overexpressed in human cells, a TopBP1 fragment containing only BRCT 7-8 associated with endogenous ATR (Figure S4B). Furthermore, TopBP1 fragments lacking BRCT 1-6, expressed and purified from *E. coli*, directly bound to the phospho-T1989 peptide (Figure 5E and Figure S4C). These results demonstrate that TopBP1 directly engages phosphorylated ATR via BRCT 7-8.

To confirm the specificity of the interaction between BRCT 7-8 and phospho-T1989, we characterized the interaction using point mutants of both binding partners. When the phosphate-binding pocket of BRCT 7-8 was disrupted by the S1273A, R1280Q, or K1317M mutations (Leung et al., 2011), the interaction between purified BRCT 7-8 and phospho-T1989 was compromised (Figure S4C). We recently showed that the binding

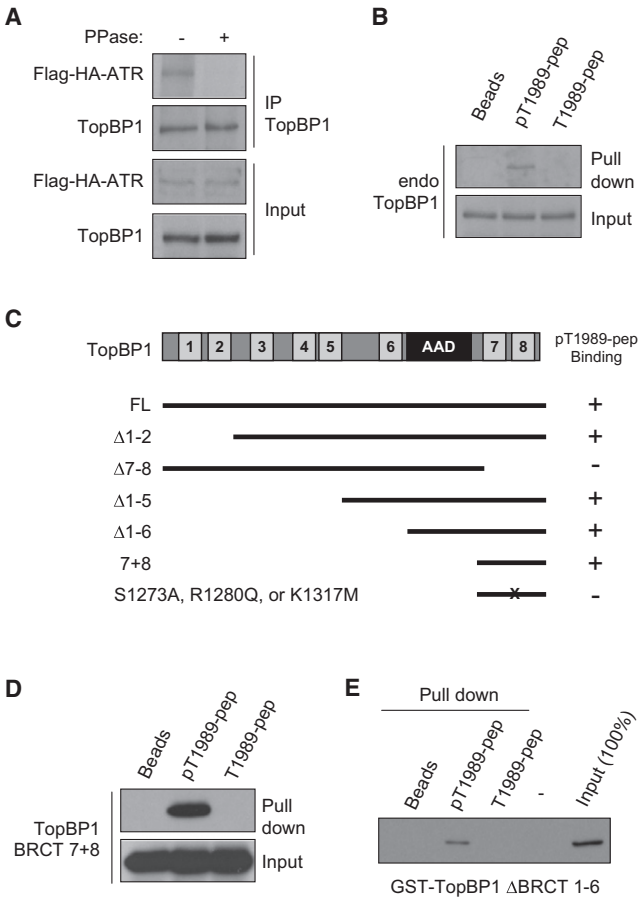

Figure 5. TopBP1 Directly Engages Phospho-T1989 via BRCT 7-8

(A) ATR$^{flox/-}$-derivative cells expressing Flag-HA-ATR^{WT} were irradiated with UV. The chromatin fractions of cells were solubilized with benzonase, treated with phosphatase or mock treated, and then subjected to TopBP1 immunoprecipitation. The Flag-HA-ATR^{WT} coprecipitated with TopBP1 was analyzed by western blot.

(B) Endogenous TopBP1 in cell extracts specifically bound to the phospho-T1989 peptide but not the unphosphopeptide.

(C) Flag-tagged TopBP1 fragments lacking various BRCT domains were transiently expressed in 293T cells. The ability of the indicated TopBP1 fragments to bind the phospho-T1989 peptide was summarized.

(D) Transiently expressed TopBP1 BRCT7+8 specifically bound to the phospho-T1989 peptide in cell extracts.

(E) TopBP1 BRCT Δ1-6 purified from *E. coli* directly bound to the phospho-T1989 peptide.

of phospho-T1133 of BACH1 to BRCT 7-8 depends on its neighboring residues at the +3/+4 positions (Leung et al., 2011). Similarly, Ala substitutions of the +3 Glu and +5 Lys residues of T1989, which are highly conserved among the ATR orthologs in mammals (see Figure S6D), significantly reduced the binding of phospho-T1989 to BRCT 7-8 (Figure S4D). These results suggest that both phospho-T1989 and the +3/+5 residues contribute to the specific binding to BRCT 7-8.

ATR Phosphorylates T1989 In *trans*

T1989 phosphorylation is induced by DNA damage, and it occurs on chromatin. ATR^{T1989A} colocalized with RPA at DNA damage-induced foci (Figure S5), suggesting that T1989 is not required for the localization of ATR to sites of DNA damage. In cells treated with ATRIP or RPA1 siRNA, UV-induced T1989 phosphorylation was diminished (Figures 6A and 6B). In contrast, knockdown of Rad17, a regulator of ATR that does not affect the recruitment of ATR-ATRIP to RPA-ssDNA (Zou et al., 2002), did not alter T1989 phosphorylation (Figure 6C). These results suggest that T1989 phosphorylation may be directly regulated by the recruitment of ATR-ATRIP to RPA-ssDNA.

The recruitment of ATR-ATRIP by RPA-ssDNA may bring multiple ATR-ATRIP complexes together and promote ATR autophosphorylation in *trans*. Consistent with this possibility, purified Flag-tagged ATR-ATRIP pulled down increased amounts of GFP-ATR from extracts in the presence of RPA-ssDNA (Figure 6D), suggesting that multiple ATR-ATRIP complexes congregate on RPA-ssDNA. To test whether ATR can phosphorylate T1989 in *trans*, we generated an ATR mutant lacking the kinase domain at the C terminus (ATR$^{\Delta C}$). In the presence of endogenous ATR, ATR$^{\Delta C}$ was efficiently phosphorylated at T1989 after UV treatment (Figure 6E). This result, although it does not exclude the possibility of ATR *cis* autophosphorylation, demonstrates that ATR can indeed autophosphorylate T1989 in *trans* after DNA damage.

The crosstalk among ATR molecules on RPA-ssDNA raised the possibility that the defect of ATR^{T1989A} might be complemented in *trans*. To assess this possibility, we generated an ATR^{KD,T1989D} double mutant. Since ATR^{KD,T1989D} is inactive as a kinase, it does not directly contribute to ATR substrate phosphorylation. However, the phosphomimetic mutation of ATR^{KD,T1989D} may allow it to bring in TopBP1 and facilitate activation of neighboring ATR^{T1989A} molecules on RPA-ssDNA. Indeed, coexpression of ATR^{KD,T1989D} and ATR^{T1989A} in cells lacking endogenous ATR partially rescued UV-induced Chk1 activation (Figure 6F). This result strongly suggests that the defect of ATR^{T1989A} stems from its compromised ability to interact with TopBP1, and this defect can be partially complemented in *trans* by the phosphomimetic ATR^{KD,T1989D} mutant.

Engagement of TopBP1 to ATR-ATRIP Stimulates the ATR Kinase and Facilitates ATR Substrate Recognition

The results above suggest that T1989 phosphorylation is a crucial event linking ATR-ATRIP recruitment to TopBP1-mediated ATR-ATRIP activation. To directly test whether T1989 is important for stimulation of the specific kinase activity of ATR-ATRIP, we purified ATR^{WT}-ATRIP and ATR^{T1989A}-ATRIP complexes from 293E cells and GST-tagged TopBP1 from *E. coli*. To ensure that the in vitro kinase assay measures the effect of TopBP1 on ATR-ATRIP activity rather than substrate binding, we used GST-Rad17, which only contains a short peptide from Rad17, as substrate. Compared to ATR^{WT}-ATRIP, ATR^{T1989A}-ATRIP was stimulated by full-length TopBP1 (TopBP1^{WT}) less efficiently (Figure 7A). Furthermore, a TopBP1 fragment lacking BRCT 7-8 (TopBP1$^{\Delta BRCT7-8}$) stimulated ATR^{WT}-ATRIP less efficiently than TopBP1^{WT} (Figure S6A). Thus, the efficient stimulation of ATR-ATRIP by TopBP1 relies on the ATR-TopBP1 interaction mediated by the phospho-T1989 of ATR and the BRCT 7-8 of TopBP1.

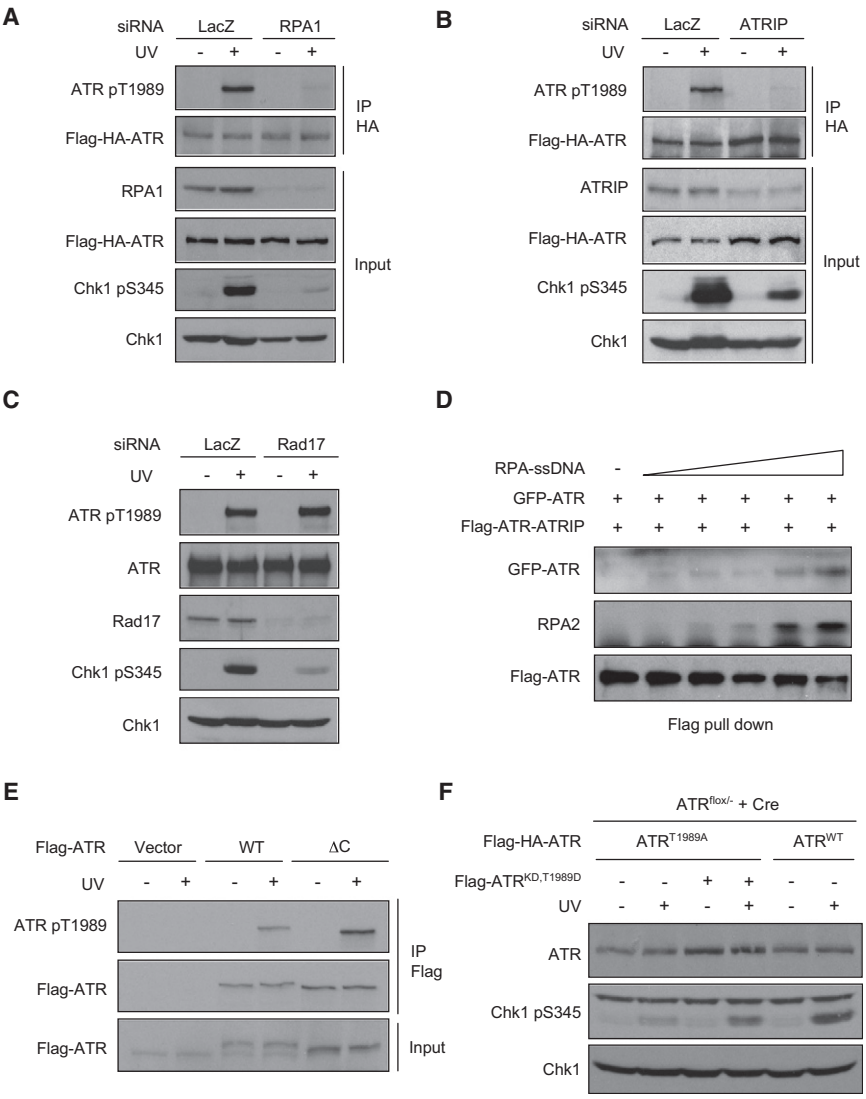

Figure 6. ATR Phosphorylates Thr 1989 In *trans* and Promotes the TopBP1-ATR Interaction

(A and B) ATR$^{flox/-}$-derivative cells were treated with control, ATRIP, or RPA1 siRNA and induced to express Flag-HA-ATR^{WT}. The UV-induced phosphorylation of Chk1 and Flag-HA-ATR^{WT} was analyzed using phospho-specific antibodies.

(C) HCT116 cells were transfected with control or Rad17 siRNA. The phosphorylation of endogenous Chk1 and ATR was analyzed using phospho-specific antibodies.

(D) Purified Flag-tagged ATR-ATRIP was incubated with increasing amounts of RPA-ssDNA and constant amounts of extracts containing GFP-ATR. The levels of GFP-ATR and RPA2 pulled down by Flag-ATR were analyzed.

(E) HCT116 cells expressing Flag-ATR^{WT} or Flag-ATR$^{\Delta C}$ were treated with UV or left untreated. Flag-ATR^{WT} and Flag-ATR$^{\Delta C}$ were precipitated, and the phosphorylation of T1989 was analyzed using phospho-specific antibodies.

(F) ATR$^{flox/-}$-derivative cells infected with Ad-Cre were treated with Dox to induce Flag-HA-ATR^{WT} or Flag-HA-ATR^{T1989A}. Cells expressing ATR^{T1989A} were transfected with a control plasmid or a plasmid encoding Flag-ATR^{KD,T1989D}. The UV-induced Chk1 phosphorylation was analyzed in the indicated cell populations.

A number of proteins participating in ATR signaling are phosphorylated after DNA damage. One example of these proteins is Rad9 (Roos-Mattjus et al., 2003), which binds to TopBP1 via its BRCT 1-2. The ability of TopBP1 to bind ATR-ATRIP and Rad9 through distinct BRCT domains raised the possibility that TopBP1 might function as a scaffold to facilitate substrate phosphorylation. To test this possibility, we performed in vitro ATR-ATRIP kinase assays in the absence of DNA and the presence of TopBP1^{WT} or TopBP1$^{\Delta BRCT1-2}$ (a TopBP1 fragment lacking BRCT 1-2). Purified 9-1-1 complex and GST-Rad17 were used as TopBP1-bound and free substrates, respectively (Figure S6B) (Yang and Zou, 2006; Zou et al., 2003). Both TopBP1^{WT} and TopBP1$^{\Delta BRCT1-2}$ stimulated the phosphorylation of GST-Rad17 by ATR-ATRIP (Figure 7B), suggesting that the function of BRCT 1-2 in recruiting TopBP1 to DNA is bypassed in vitro. In contrast, only TopBP1^{WT} but not TopBP1$^{\Delta BRCT1-2}$ enhanced the phosphorylation of Rad9 by ATR-ATRIP (Figure 7C), showing that the BRCT 1-2 of TopBP1 promote Rad9 phosphorylation independently of ATR-ATRIP stimulation. Together, these results suggest that in addition to the ability to stimulate ATR-ATRIP, a distinct scaffolding function of TopBP1 is also needed for efficient Rad9 phosphorylation.

DISCUSSION

In this study, we identified ATR autophosphorylation at T1989 as a hallmark of the active state of ATR. Furthermore, we elucidated how this phosphorylation event functions as a molecular switch for checkpoint activation.

Recruitment of ATR-ATRIP by RPA-ssDNA Drives Full ATR Activation

The recruitment of ATR-ATRIP by RPA-ssDNA plays a crucial role in the spatial regulation of ATR. It has been proposed that the colocalization of ATR-ATRIP and its substrates at sites of DNA damage facilitates phosphorylation of ATR substrates (Zou and Elledge, 2003). Whether RPA-ssDNA has a direct role in activating the kinase is not known. In contrast to RPA-ssDNA, TopBP1 directly stimulates the activity of ATR-ATRIP (Kumagai et al., 2006). While TopBP1 is clearly important for ATR signaling, how TopBP1 is regulated by DNA damage and whether it is needed for the phosphorylation of all ATR substrates in humans is not addressed. This study suggests that the recruitment and the stimulation of ATR-ATRIP are, in fact, two mechanistically coupled events. RPA-ssDNA not only spatially regulates

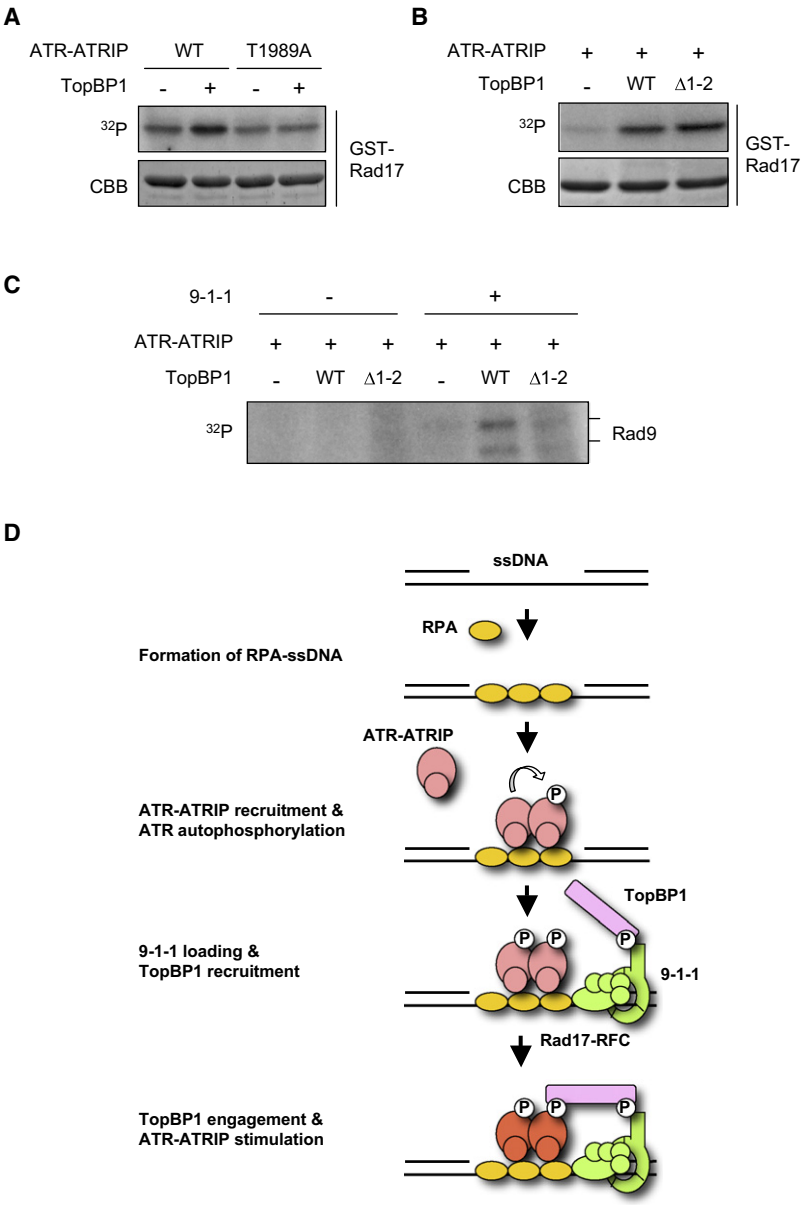

Figure 7. TopBP1 Engages ATR-ATRIP to Stimulate the Kinase and Facilitate Substrate Recognition

(A) Purified ATR^{WT}-ATRIP and ATR^{T1989A}-ATRIP complexes were incubated with GST-Rad17 in the presence of ^{32}P-labeled ATP. Where indicated, purified full-length TopBP1 was included in the reactions.

(B) Purified ATR^{WT}-ATRIP was incubated with GST-Rad17, ^{32}P-labeled ATP, and $TopBP1^{WT}$ or $TopBP1^{\Delta BRCT1-2}$. $TopBP1^{WT}$ and $TopBP1^{\Delta BRCT1-2}$ were normalized to give similar stimulation of ATR-ATRIP.

(C) The in vitro kinase assays were performed as in (C) except that the 9-1-1 complex was used as substrate.

(D) A model for the role of ATR autophosphorylation in checkpoint activation. The change of color of ATR-ATRIP from light red to dark red indicates stimulation of the ATR-ATRIP kinase by TopBP1.

ATR-ATRIP but also directly contributes to its activation by promoting ATR autophosphorylation at T1989, which directs TopBP1 to stimulate ATR-ATRIP (Figure 7D). Since T1989 phosphorylation is independent of TopBP1, ATR, through its basal activity, plays a surprising role in promoting its own activation. Together, our results suggest that the full activation of ATR by DNA damage is sequentially driven by RPA-ssDNA, ATR autophosphorylation, and TopBP1, thus unifying the previous studies that demonstrated the dependence of ATR signaling on RPA-ssDNA and TopBP1 (Ciccia and Elledge, 2010; Cimprich and Cortez, 2008; Flynn and Zou, 2010).

The length of ssDNA induced by DNA damage is an important determinant for ATR activation (Byun et al., 2005; MacDougall et al., 2007; Sartori et al., 2007; Shiotani and Zou, 2009). In vitro, ATR-ATRIP binds to RPA-ssDNA in a length-dependent manner (Zou and Elledge, 2003). We suggest that the binding of ATR-ATRIP to RPA-ssDNA leads to congregation of ATR-ATRIP complexes and T1989 phosphorylation in *trans* (Figure 7D). In this model, RPA-ssDNA is not only a quantitative signal for ATR-ATRIP recruitment but also a length-dependent platform that promotes ATR autophosphorylation. The coupling of ATR-ATRIP recruitment and one of the initial events of ATR activation may be the key mechanism ensuring the quantitative regulation of ATR signaling by ssDNA.

A Critical Link between ATR-ATRIP and the DNA Damage Sensors on dsDNA

In addition to the length of ssDNA, the number of ssDNA-dsDNA junctions is another important determinant for the strength of ATR signaling (MacDougall et al., 2007; Van et al., 2010), suggesting that the DNA damage sensors on dsDNA also quantitatively regulate ATR activation. Nonetheless, how exactly the ATR-ATRIP on ssDNA is regulated by the DNA damage sensors on dsDNA remains elusive. Independently of ATR, the Rad17-RFC complex recognizes ssDNA-dsDNA junctions and loads 9-1-1 onto dsDNA (Ellison and Stillman, 2003; Zou et al., 2003). Rad9 in the 9-1-1 complex interacts with TopBP1 (Delacroix et al., 2007; Lee et al., 2007; Makiniemi et al., 2001), thereby recruiting TopBP1 to sites of DNA damage. Although 9-1-1 brings TopBP1 to the vicinity of ATR-ATRIP, the TopBP1 and 9-1-1 on dsDNA are physically separate from the ATR-ATRIP on ssDNA. Our data showing that TopBP1 stably engages phosphorylated ATR via the BRCT domains 7 and 8 reveals a previously unknown step during ATR activation (Figure 7D). The TopBP1 recruited by 9-1-1 needs to be redirected to ATR-ATRIP by this phosphorylation-dependent interaction, allowing the DNA damage sensors on ssDNA and dsDNA to act jointly to fully activate ATR-ATRIP. The interaction between BRCT7-8 and phospho-T1989 may poise the AAD of TopBP1 to stimulate ATR-ATRIP (Mordes et al., 2008).

Additionally, as indicated by the *trans* complementation of ATR^{T1989A}, phospho-ATR may tether TopBP1 to stimulate multiple ATR-ATRIP complexes on RPA-ssDNA (Choi et al., 2010).

TopBP1 as a Scaffold for ATR Signaling

In addition to stimulating the ATR-ATRIP kinase, the interaction between TopBP1 and ATR may facilitate ATR to recognize its substrates. In fission yeast, Rad4/Cut5, the homolog of TopBP1, interacts with Rad3/ATR substrates Rad9, Crb2, and Chk1 (Furuya et al., 2004; Mochida et al., 2004; Saka et al., 1997). In vertebrates, TopBP1 interacts with Rad9 through its N terminal BRCT domains 1 and 2 (Delacroix et al., 2007; Lee et al., 2007). *Xenopus* TopBP1 remains associated with 9-1-1 and Rad17 after DNA damage (Lee and Dunphy, 2010). When phosphorylated by ATR in human cells, Rad17 interacts with the mediator protein Claspin (Wang et al., 2006). ATR-dependent phosphorylation of Claspin enables it to associate with Chk1, promoting Chk1 phosphorylation by ATR and stimulating Chk1 activity (Kumagai et al., 2004; Lindsey-Boltz et al., 2009). Together, these findings suggest that a checkpoint signaling complex is physically linked to the N terminus of TopBP1. Our result that the BRCT 1-2 of TopBP1 promote Rad9 phosphorylation independently of their function in TopBP1 recruitment provides an example of how TopBP1 functions as a scaffold to bring ATR to its substrates (Figure S6C). Interestingly, Dpb11, the budding yeast homolog of TopBP1, is known to associate with phosphorylated Sld3 and Sld2 through its BRCT repeats at the N and C termini (Tanaka et al., 2007; Zegerman and Diffley, 2007). During the initiation of DNA replication, these interactions allow Dpb11 to function as a scaffold and recruit the GINS complex to the MCM-Cdc45 complex at replication origins (Muramatsu et al., 2010). It is tempting to speculate that human TopBP1 functions analogously as a scaffold of phosphorylation-mediated protein complexes during the initiation of DNA replication and activation of the ATR checkpoint.

Reminiscent to the assembly of the replication complex at origins, ATR, TopBP1 and other proteins are assembled into a signaling complex at sites of DNA damage. The phosphorylation-mediated ATR-TopBP1 interaction may be one of the key events for the assembly of this signaling complex. Consistent with the model in which ATR functions in concert with Rad17, 9-1-1, and TopBP1 on damaged DNA, phospho-ATR is unable to phosphorylate Chk1 after DNA damage in the absence of Rad17 or TopBP1 (Figures 4A and 6C), and the phosphomimetic ATR^{T1989D} mutant cannot induce Chk1 activation in the absence of DNA damage (Figure S2I). While the interaction between phospho-ATR and BRCT 7-8 is specific, it is weak and only captured under mild binding conditions (data not shown), which may explain why phospho-ATR is not sufficient to efficiently recruit TopBP1 in the absence of Rad17. These findings emphasize the requirement of both ssDNA and dsDNA for the assembly of the ATR signaling complex. It is interesting to note the phosphorylation of ATR at T1989 persists much longer than Chk1 phosphorylation after DNA damage (Figure S1D), suggesting that the ATR signaling complex is dynamically regulated during the course of DNA damage response.

A Conserved Mechanism for ATR Activation?

Like ATR, ATM and DNA-PKcs undergo autophosphorylation in or near the FAT domain during activation, indicating that this is a common feature of PIKKs (Bakkenist and Kastan, 2003; Chan et al., 2002; Kozlov et al., 2006). However, the autophosphorylation of each of the PIKKs appears to be functionally distinct (Bakkenist and Kastan, 2003; Berkovich et al., 2007; Daniel et al., 2008; Ding et al., 2003; Lee and Paull, 2005; Pellegrini et al., 2006; So et al., 2009; Uematsu et al., 2007), suggesting that these modifications may have adapted to different regulatory roles during evolution. Interestingly, the number of BRCT domains of TopBP1 has risen during evolution, suggesting that it may also have acquired new regulatory roles (Garcia et al., 2005). Among the three PIKKs activated by DNA damage, ATR is the only one that is recruited and activated by different factors, and the autophosphorylation of ATR plays a key role in integrating the functions of these factors. We note that the sequence conservation of T1989 is only apparent in mammals (Figure S6D). It will be interesting to elucidate whether the homologs of human ATR in other organisms are autophosphorylated during activation, and whether the role of ATR autophosphorylation is conserved.

EXPERIMENTAL PROCEDURES

Plasmids

Plasmids expressing Flag-ATR^{WT} and Flag-ATR^{KD} were previously described (Tibbetts et al., 2000). Plasmids encoding Flag-ATR^{T1989A}, Flag-ATR^{S428A}, Flag-$ATR^{S435/436/437A}$, and Flag-$ATR^{\Delta C}$ (amino acids 2350–2644 deleted) were derived from Flag-ATR^{WT} using site-directed mutagenesis. The pNZ2-ATR^{WT} plasmid encoding FLAG-HA-ATR^{WT} was provided by Dr. David Cortez (Mordes and Cortez, 2008). The pNZ2-ATR^{T1989A} plasmid was derived from pNZ2-ATR^{WT} using site-directed mutagenesis. The entire coding sequence of ATR in the plasmids encoding ATR^{T1989A} was confirmed by DNA sequencing. For purification of the ATR-ATRIP complex from 293E cells, Flag-ATR and His-ATRIP were cloned into the pTT3 vector. The Flag-CMV2-TopBP1 plasmid is a gift from Dr. Jiri Lukas. Plasmids expressing Flag-TopBP1Δ1-2, Flag-TopBP1Δ1-5, Flag-TopBP1Δ1-6, Flag-TopBP1Δ7-8, Flag-TopBP1-BRCT7+8, and GST-TopBP1Δ1-6 were generated either by direct cloning or with the pUNI system (Liu et al., 1998).

Antibodies

The phospho-specific antibodies to ATR pT1989 were generated by Cell Signaling and Bethyl against the peptide NH_2-Cys-FPENE(pT)PPEGK-COOH. ATR, TopBP1, phospho-Rad17, phospho-TopBP1, and phospho-Mcm2 antibodies are from Bethyl. ATRIP antibodies were previously described (Cortez et al., 2001). Rad17 and Chk1 antibodies are from Santa Cruz. Phospho-Chk1 antibody is from Cell Signaling. Phospho-H2AX antibody is from Millipore.

Immunoprecipitation

To precipitate ATR and TopBP1 from the chromatin fractions, cell extracts were first fractionated as previously described (Zou et al., 2002). To release chromatin-bound proteins, chromatin-enriched pellet (P3) were sonicated and digested with benzonase in binding buffer (100 mM Na_2HPO_4, 2 mM K_2HPO_4, 137 mM NaCl, 2.7 mM KCl). The resulting lysates were centrifuged at 14,000 rpm for 10 min, and the supernatants were collected and precleared with protein G/A Sepharose. The solubilized chromatin fractions were subsequently incubated with primary antibodies and 20 μl protein G/A beads, and the beads were collected and washed four times with binding buffer.

Peptide Binding

The pT1989 (CFPENEpTPPEGKNML) and the corresponding unphosphorylated peptides were synthesized by the Tufts University Core Facility. In pull-down assays, biotinylated peptides were attached to streptavidin-coated Dynalbeads and incubated with nuclear extracts or purified proteins. The

beads were subsequently retrieved and extensively washed with PBS containing 0.12%–0.15% Triton X-100, and bound proteins were subjected to SDS-PAGE and immunoblotting.

In Vitro Kinase Assays

The ATR kinase assays were performed essentially as previously described (Canman et al., 1998) with the following modifications. HEK293E cells were transfected with Flag-ATR- and His-ATRIP-expressing plasmids, and Flag-ATR was immunoprecipitated with anti-Flag M2 antibody in the TGN buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 50 mM phosphoglycerol, 10% glycerol, 1% Tween 20, 1 mM PMSF, 1 mM NaF, 1 mM Na_3VO_4, 1 mM DTT, and protease inhibitors). The precipitates were washed twice with the TGN buffer, once with the TGN buffer supplemented with 0.5 M LiCl, and twice with the reaction buffer (10 mM HEPES [pH 7.5], 50 mM NaCl, 10 mM $MgCl_2$, 50 mM glycerophosphate, 1 mM DTT, and protease inhibitor) without ATP. The in vitro kinase reactions were conducted in the presence of 5 μM ATP and purified GST-T1989 (GST-PENETPPETPPEGK) or GST-T1989A as substrates. The phosphorylation of T1989 was monitored using the phospho-T1989 antibody.

To analyze the stimulation of ATR-ATRIP by TopBP1, the ATR-ATRIP complex was purified using a two-step protocol. The ATR-ATRIP complex, which contains Flag-ATR and His-ATRIP, was first purified using Ni beads and eluted with imidazole. The proteins eluted from Ni beads were subsequently incubated with anti-Flag M2 beads, and the ATR-ATRIP complex was eluted with 200 μg/ml 3× Flag peptide. The kinase reactions were conducted with purified ATR-ATRIP complex, GST-Rad17, and 5 μCi [γ-^{32}P] ATP in the presence or absence of GST-TopBP1.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at doi:10.1016/j.molcel.2011.06.019.

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