TopBP1 activates ATR through ATRIP and a PIKK regulatory domain

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The ATR (ATM and Rad3-related) kinase and its regulatory partner ATRIP (ATR-interacting protein) coordinate checkpoint responses to DNA damage and replication stress. TopBP1 functions as a general activator of ATR. However, the mechanism by which TopBP1 activates ATR is unknown. Here, we show that ATRIP contains a TopBP1-interacting region that is necessary for the association of TopBP1 and ATR, for TopBP1-mediated activation of ATR, and for cells to survive and recover DNA synthesis following replication stress. We demonstrate that this region is functionally conserved in the *Saccharomyces cerevisiae* ATRIP ortholog Ddc2, suggesting a conserved mechanism of regulation. In addition, we identify a domain of ATR that is critical for its activation by TopBP1. Mutations of the ATR PRD (PIKK [phosphoinositide 3-kinase related kinase] Regulatory Domain) do not affect the basal kinase activity of ATR but prevent its activation. Cellular complementation experiments demonstrate that TopBP1-mediated ATR activation is required for checkpoint signaling and cellular viability. The PRDs of ATM and mTOR (mammalian target of rapamycin) were shown previously to regulate the activities of these kinases, and our data indicate that the DNA-PKcs (DNA-dependent protein kinase catalytic subunit) PRD is important for DNA-PKcs regulation. Therefore, divergent amino acid sequences within the PRD and a unique protein partner allow each of these PIK kinases to respond to distinct cellular events.

[*Keywords*: ATR; ATRIP; TopBP1; checkpoint; PIKK; Ddc2]

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The DNA damage response maintains genome integrity through the coordination of DNA replication, cell cycle progression, transcription, apoptosis, senescence, and DNA repair. At the apex of this pathway are two closely related kinases—ATM (ataxia-telangiectasia mutated) and ATR (ATM and Rad3-related)—that phosphorylate hundreds of proteins, including the tumor suppressor proteins BRCA1 and p53 (Kastan and Bartek 2004). ATM primarily responds to DNA double-strand breaks, while ATR is activated by replication stress and ssDNA gaps.

ATR and ATM are members of the PIKK (phosphoinositide 3-kinase related kinases) family of protein kinases, which regulate diverse biological activities. Other members of this family include mTOR (mammalian target of rapamycin), which coordinates protein synthesis and cell growth; DNA-PKcs (DNA-dependent protein kinase catalytic subunit), which promotes DNA double-strand break repair by nonhomologous end-joining; and SMG1, which regulates nonsense-mediated mRNA decay (Abraham 2004). PIKKs are large proteins (2549–4128 amino acids) with a common domain architecture. They contain dozens of N-terminal HEAT repeats that may mediate protein–protein interactions and a highly conserved C-terminal kinase domain flanked by the FAT (FRAP, ATM, TRRAP) and FATC (FAT C terminus) domains of unknown function.

Homozygous mutations in *ATM* cause ataxia-telangiectasia, which is characterized by progressive neurodegeneration and severe cancer predisposition, and people with a heterozygous *ATM* mutation have an increased risk of breast cancer (Shiloh 2003). *ATR* mutations are infrequent because ATR is essential for cell viability and embryonic development (Brown and Baltimore 2000; Cortez et al. 2001). However, hypomorphic mutations in *ATR* have been linked to rare cases of Seckel syndrome (O'Driscoll et al. 2003). This syndrome is characterized by microcephaly and developmental defects.

ATR forms a stable complex with ATRIP (ATR-interacting protein), which regulates the localization of ATR and is essential for ATR signaling in response to DNA damage and replication stress (Cortez et al. 2001; Zou and Elledge 2003; Ball et al. 2005). Many proteins in addition to ATRIP participate in the ATR pathway including TopBP1, which functions in both the initiation of DNA replication and checkpoint signaling (Garcia et al. 2005). Depletion of TopBP1 in mammalian cells does not affect the localization of ATR–ATRIP to sites of DNA damage but inhibits the damage-inducible phosphoryla-

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tion of ATR substrates (Liu et al. 2006). As first discovered by Dunphy and colleagues, the TopBP1 protein directly stimulates the kinase activity of the ATR–ATRIP complex in vitro and in cells (Kumagai et al. 2006). A region between the sixth and seventh BRCT domains of TopBP1 called the ATR Activation Domain (AAD) is sufficient to activate ATR (Kumagai et al. 2006). The mechanisms that regulate TopBP1 access to ATR–ATRIP are likely to be complex but include post-translational modifications and recruitment of TopBP1 independently of ATR–ATRIP to sites of replication stress or DNA damage (Delacroix et al. 2007; Lee et al. 2007; Yoo et al. 2007). TopBP1 binds to the phosphorylated C-terminal tail of the Rad9 protein, which is recruited to ssDNA gaps as part of a checkpoint clamp complex.

Most checkpoint proteins and activities are conserved in all eukaryotic cells. In *Saccharomyces cerevisiae*, the orthologs of ATR and ATRIP are Mec1 and Ddc2, which form a stable complex required for checkpoint signaling in response to replication stress and DNA damage. Ddc2-deficient yeast exhibit hypersensitivity to DNA damage, and Ddc2 is required for phosphorylation of Mec1 substrates in response to DNA damage (Paciotti et al. 2000; Rouse and Jackson 2000). The homolog of TopBP1 in *S. cerevisiae* is thought to be Dpb11. However, Dpb11 lacks sequence homology with the AAD of TopBP1, and it is unclear whether it is a direct activator of Mec1. In at least some circumstances, the ortholog of hRad9 (Ddc1) is capable of activating Mec1–Ddc2 complexes in vitro (Majka et al. 2006).

To gain insight into how TopBP1 activates ATR–ATRIP complexes, we examined the interaction between these proteins. We identified a conserved region of ATRIP that is necessary for the interaction of ATR–ATRIP with TopBP1 and TopBP1-dependent ATR activation. We also defined a regulatory domain within ATR that mediates TopBP1-dependent ATR kinase activation. The ATR regulatory domain maps to the region between the kinase and FATC domains. This region is important for regulation of multiple PIK kinases including mTOR, ATM, and DNA-PKcs suggesting that divergent sequences within this region provide unique regulatory opportunities for each of these kinases.

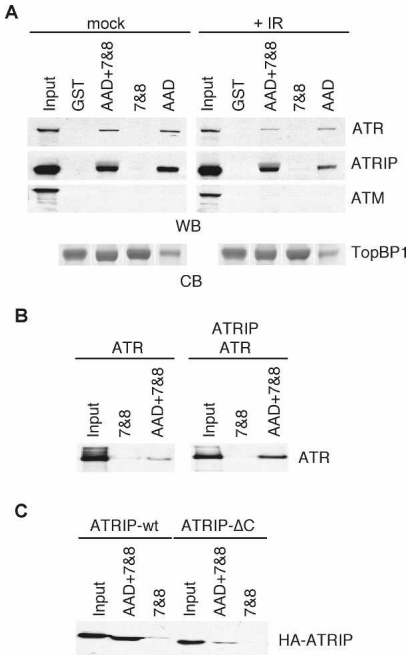

Figure 1. ATRIP promotes the association of ATR and TopBP1. (*A*) Nuclear extracts from 293T cells treated with 8 Gy of IR or mock-treated were incubated with equal amounts of recombinant GST-tagged fragments of TopBP1 fragments ([AAD] amino acids 978–1286 [Kumagai et al. 2006]; [7&8] BRCT repeats 7 and 8, amino acids 1182–1522; [AAD + 7&8] amino acids 978–1522) bound to glutathione beads. Proteins bound to the beads were eluted, separated by SDS-PAGE, and immunoblotted with antibodies to ATR, ATRIP, or ATM (WB). A duplicate gel was stained with Coomassie blue to verify equal amounts of GST-tagged TopBP1 proteins (CB). (*B*) Nuclear extracts from 293T cells transfected with a vector encoding ATR or vectors encoding ATR and ATRIP were incubated with recombinant fragments of TopBP1 bound to glutathione beads. Bound proteins were eluted, separated by SDS-PAGE, and immunoblotted with an antibody to ATR. (*C*) Nuclear extracts from U2OS cells stably expressing HA-tagged wild-type (wt) ATRIP or HA-tagged ATRIP lacking the C-terminal 32 amino acids (ΔC) were incubated with recombinant GST-tagged fragments of TopBP1 bound to glutathione beads. Proteins bound to the beads were eluted, separated by SDS-PAGE, and blotted with an anti-HA antibody. Input in all experiments is 5% of the extract added to the binding reaction.

Results

ATRIP promotes the association of ATR and TopBP1

To understand how TopBP1 activates the ATR–ATRIP complex, we sought to examine the interaction between these proteins. Nuclear extracts from mammalian cells were incubated with recombinant GST-tagged TopBP1 fragments. Endogenous ATR and ATRIP associated with TopBP1 fragments containing the AAD, but not with a C-terminal fragment of TopBP1 lacking the AAD or with GST alone (Fig. 1A). The amount of ATR and ATRIP associated with TopBP1 was not reproducibly affected by treating cells with ionizing radiation. An interaction between xATM and recombinant xTopBP1 has been demonstrated in *Xenopus* egg extracts and is potentiated by DNA templates that mimic DNA damage (Yoo et al. 2007). However, we were unable to detect an association between human ATM and TopBP1 even in the presence of DNA damage (Fig. 1A). Thus, the observed association with the TopBP1 AAD is specific to the ATR kinase.

Since TopBP1 stimulation of ATR kinase activity requires ATRIP (Kumagai et al. 2006; Ball et al. 2007), TopBP1 may interact with ATRIP, or ATRIP may stabilize an interaction between TopBP1 and ATR. Indeed, when ATR was transiently overexpressed in mammalian cells, the association of ATR with TopBP1 was significantly increased by simultaneous overexpression of ATRIP (Fig. 1B). This observation is consistent with studies performed in *Xenopus* egg extracts that indicate

that xTopBP1 depends on the presence of xATRIP to associate with xATR–xATRIP (Kumagai et al. 2006). To test if ATRIP can associate with TopBP1 independently of ATR, nuclear extracts from U2OS cells expressing wild-type ATRIP or mutant ATRIP lacking the C-terminal ATR-interacting domain (Ball et al. 2005; Falck et al. 2005) were incubated with recombinant TopBP1 fragments. The binding of this ATRIPΔC mutant to TopBP1 is severely reduced compared with wild-type ATRIP (Fig. 1C). Taken together, these data suggest that the association of the ATR–ATRIP complex with TopBP1 might involve binding surfaces on both ATR and ATRIP.

Identification of a TopBP1-interacting region of ATRIP

To search for TopBP1-binding surfaces on the ATRIP and ATR proteins, we used a yeast two-hybrid approach. Given that the TopBP1 AAD is sufficient to associate with the ATR–ATRIP complex, the TopBP1 AAD fused to the GAL4 DNA-binding domain was used as a bait to screen a library containing thousands of random fragments of ATRIP fused to the activation domain of GAL4. Sequencing of ATRIP fragments selected in the screening procedure revealed a minimal interacting region of ATRIP consisting of amino acids 203–348 (Fig. 2A). This region is adjacent to the predicted ATRIP coiled-coil domain (amino acids 108–217), which mediates homo-oligomerization (Ball and Cortez 2005; Itakura et al. 2005). Sequence conservation of this region among ATRIP homologs is low except for a section of ~30 amino acids starting at amino acid 308 (Supplemental Fig. S1A). Deletion of amino acids 301–338 of ATRIP eliminated activation of ATR by TopBP1 in vitro, but also caused reduced binding to ATR (data not shown). An amino acid substitution mutation in this region of ATRIP (LLSS332AAAA) nearly abolished the association of ATRIP with TopBP1 (Fig. 2B), yet preserved the ability of ATRIP to bind ATR (Fig. 2C) and localize to damage-induced foci (data not shown). Furthermore, the association of ATR with TopBP1 was also significantly reduced in the presence of this ATRIP-*top* mutant compared with wild-type ATRIP (Fig. 2B), which supports the idea that ATR association with TopBP1 is dependent on ATRIP.

Since the ATR–ATRIP complex containing the ATRIP-*top* mutant had decreased association with TopBP1, we expected that it would have a decreased ability to be activated by TopBP1 as well. To test this hypothesis, wild-type ATR–ATRIP and mutant ATR–ATRIP-*top* complexes were immunopurified from mammalian nuclear extracts. Addition of the TopBP1 AAD to wild-type ATR–ATRIP complexes caused a robust stimulation of ATR kinase activity toward a substrate of ATR, MCM2 (Cortez et al. 2004; Yoo et al. 2004). The ATRIP-*top* mutant severely attenuated TopBP1-dependent stimulation of ATR (Fig. 2C). Thus, a previously uncharacterized domain of ATRIP is important for an interaction between TopBP1 and the ATR–ATRIP complex and for ATR activation by TopBP1.

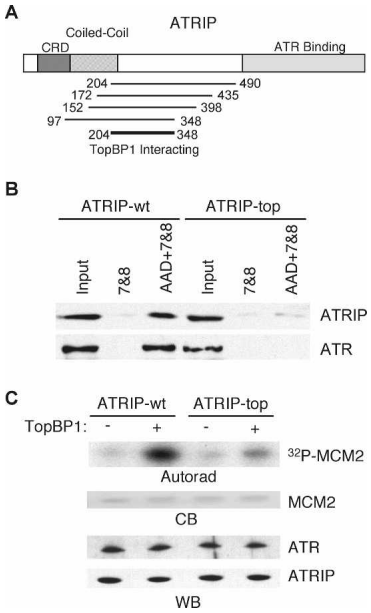

Figure 2. Identification of an ATRIP region necessary for TopBP1 association. (*A*) Schematic diagram showing the fragments of ATRIP that interacted with TopBP1 in a yeast two-hybrid assay (black lines). (CRD) Checkpoint Recruitment Domain (Ball et al. 2007). (*B*) Nuclear extracts from 293T cells transfected with vectors encoding ATR and wild-type ATRIP (wt) or ATR and ATRIP-*top* were incubated with recombinant GST-tagged fragments of TopBP1 bound to glutathione beads. Proteins bound to the beads were eluted, separated by SDS-PAGE, and blotted with antibodies to ATR or ATRIP. (*C*) Wild-type ATR–ATRIP (wt) or ATR–ATRIP-*top* complexes were isolated from transfected 293T cells and incubated with recombinant TopBP1 AAD, MCM2 substrate, and γ-^{32}PATP. Kinase reactions were separated by SDS-PAGE, stained with Coomassie blue (CB), and exposed to film (autorad). A duplicate gel was blotted and probed with anti-ATRIP and anti-ATR antibodies (WB).

The interaction between TopBP1 and ATRIP is essential for checkpoint responses to replication stress

A critical function of the ATR-dependent checkpoint signaling pathway in preserving genomic stability is to promote cell cycle recovery after replication fork arrest (Casper et al. 2002; Zachos et al. 2003). Since the ATRIP-*top* mutation selectively impairs TopBP1 association with and activation of ATR–ATRIP without impairing complex stability or localization to sites of DNA damage, this separation-of-function mutant provides a method to specifically assess the functional importance of TopBP1-dependent ATR activation. This is especially important given the possibility that RNAi depletion of TopBP1 likely interferes with many cellular processes in addition to checkpoints, such as replication control, making it difficult to unambiguously interpret phenotypic effects. To assess whether TopBP1 binding to ATRIP and activation of ATR is necessary for cells to display a normal checkpoint response to stalled replication forks, we created U2OS cell lines stably expressing siRNA-resistant wild-type ATRIP, the ATRIP-*top* mu-

tant, or empty vector as a control. Three days after transfection with siRNA targeting endogenous ATRIP, the levels of wild-type ATRIP and ATRIP-*top* were comparable (Fig. 3B). The cell cycle profile of asynchronously dividing cells expressing ATRIP-*top* is similar to cells expressing wild-type ATRIP (Fig. 3A), indicating that ATRIP-*top* did not affect normal cell cycle progression. These cell lines were treated with hydroxyurea (HU), which causes replication forks to stall and arrests cells in early S phase. After 24 h, the HU was removed, and cells were released into media containing nocodazole to prevent cell division. The ability of the *ATRIP-top* mutant to support recovery and completion of DNA replication was compared with wild-type ATRIP. Sixteen hours after release from HU, most of the cells expressing wild-type ATRIP had completed S phase (Fig. 3A). Yet, the majority of ATRIP-depleted cells expressing the ATRIP-*top* mutant or lacking any exogenous ATRIP were unable to resume DNA replication and complete S phase. Consistent with the reduced ability of ATRIP-*top* cells to recover from replication stress, these cells also displayed significantly reduced viability compared with wild-type ATRIP-expressing cells after treatment with HU (Fig. 3C). The ATRIP-*top* cells also exhibited a defect in the G2/M checkpoint after treatment with ionizing radiation (Fig. 3D). Therefore, the ability of ATR–ATRIP complexes to promote recovery from stalled replication forks and cell cycle arrest after DNA damage is dependent on an interaction between TopBP1 and ATRIP that leads to ATR kinase activation.

Conservation of the TopBP1-dependent ATRIP regulatory domain in S. cerevisiae

It is unclear whether the mechanism of activation of Mec1–Ddc2 complexes in *S. cerevisiae* is the same as ATR–ATRIP since no yeast protein has obvious sequence similarity to the AAD of TopBP1. To address whether the mechanism of regulation for Mec1–Ddc2 complexes is likely to be similar to ATR–ATRIP, we introduced a mutation into Ddc2 that is analogous to the ATRIP-*top* mutant. Despite little sequence similarity, Ddc2 and ATRIP have a common functional domain architecture: an N-terminal checkpoint recruitment domain, a predicted coiled-coil domain, and a C-terminal domain essential for interaction with Mec1 or ATR, respectively (Wakayama et al. 2001; Ball et al. 2005, 2007; Falck et al. 2005). To generate a *ddc2-top* mutation that corresponds to the *ATRIP-top* mutation, we used position and secondary structure predictions as a guide (Fig. 4A). Specifically, we mutated leucine residues that were approximately the same number (115) of amino acids C-terminal from the end of the predicted coiled-coil domain and within the third predicted α-helix (*-top*: LLLR257AAAA). For comparison, we made two additional *ddc2* alleles with mutations in the fourth predicted α-helix after the coiled-coil domain (*-A1*: LLED274AAAA and *-A2*: LIKE281AAAA). Mutant or wild-type Ddc2 were expressed on a low-copy plasmid under the endogenous *DDC2* promoter in a *ddc2*Δ strain (Paciotti et al. 2000; Rouse and Jackson 2002). The *top*, *A1*, and *A2* mutants were expressed at comparable levels as wild-type Ddc2 and are able to interact with Mec1 equivalently to wild-type Ddc2 (Fig. 4B; data not shown). The *top* mutation but not the *A1* or *A2* mutation caused marked sensitivity to HU and the alkalyating agent methyl methanesulfonate (MMS) (Fig. 4C). Furthermore, the sensitivity to DNA damage of the *ddc2-top* strain correlated with reduced phosphorylation of the Mec1 substrate Rad53 following a challenge with either HU or MMS, suggesting that disruption of this Ddc2 domain causes a defect in Mec1 activation (Fig. 4D). These data suggest that although the protein equivalent to the

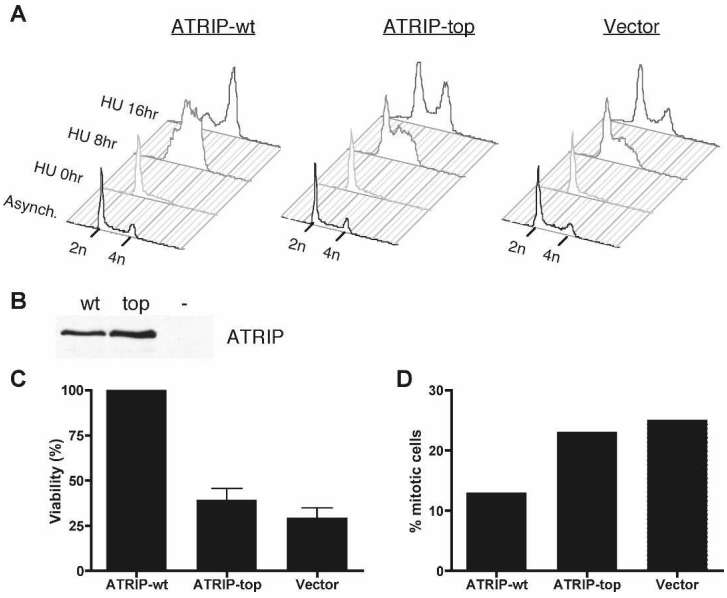

Figure 3. ATRIP association with TopBP1 is essential for cellular recovery from replication stress. (*A–C*) U2OS cells stably expressing siRNA-resistant wild-type ATRIP (wt), ATRIP-*top*, or an empty vector (vector) were transfected with siRNA targeting ATRIP to deplete endogenous ATRIP. Three days later, cells were exposed to 1 mM HU for 24 h. (*A*) Cells were collected immediately (0 hr) or rinsed and released into media containing 1 µg/mL nocodazole for either 8 h (8 hr) or 16 h (16 hr). Cells were fixed and stained with propidium iodine and processed for FACS analysis. (Asynch) Asynchronous cells that were not exposed to HU. (*B*) Immunoblot showing ATRIP levels from U2OS cells stably expressing wild-type ATRIP (wt), ATRIP-*top* (*top*), or empty vector (vt). (*C*) Twenty-four hours after release from HU, cellular viability was measured using a colorimetric assay. Viability was normalized to cells expressing exogenous wild-type ATRIP. Error bars indicate standard error, $n = 6$. (*D*) Three days after siRNA transfection, cells were treated with 4 Gy of IR, and 1 µg/mL nocodazole was added to the media. Sixteen hours later, the percentage of mitotic cells were determined by propidium iodine and antiphosphohistone H3 staining followed by flow cytometry.

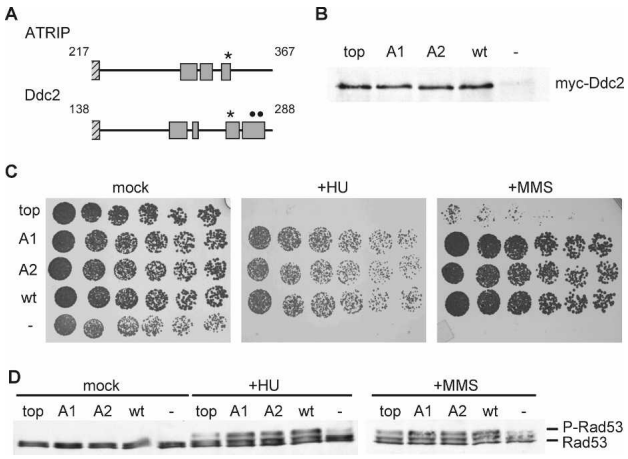

Figure 4. An *S. cerevisiae ddc2-top* mutant is defective in checkpoint signaling. (*A*) Secondary structure prediction of the TopBP1-interacting region of ATRIP and the equivalent region of *S. cerevisiae* Ddc2. Hashed boxes denote the C-terminal ends of the coiled-coil domains. Solid boxes indicate predicted α-helices. The asterisk denotes the location of the *ATRIP-top* mutation or the *ddc2-top* (LLLR257AAAA) mutation. The adjacent dots in Ddc2 denote the location of the *A1* (LLED274AAAA) and *A2* (LIKE281AAAA) mutations. (*B*) Immunoblot showing Ddc2 levels in yeast strains expressing Ddc2 mutants, wild-type Ddc2, or empty vector. (*C*) Serial dilutions of the indicated yeast strains grown on YPD with no drug (mock), 150 mM HU (+HU), or 0.008% MMS (+MMS). (*D*) Exponentially growing yeast were treated with no drug (mock), 150 mM HU (+HU), or 0.015% MMS (+MMS) for 90 min. Extracts were prepared, separated by SDS-PAGE, and immunoblotted with an antibody against Rad53. The *top* bands are phosphorylated forms of Rad53.

TopBP1 ATR activator in budding yeast is unclear, a common mechanism of regulation dependent on the ATRIP (or Ddc2) protein is likely to exist for vertebrate and yeast ATR kinases.

Regulation of ATR activation via a PIKK regulatory domain

Since the ATR-binding-defective ATRIPΔC protein does not bind as well to TopBP1 as wild-type ATRIP (Fig. 1), we hypothesized that regions of ATR contribute to the interaction between TopBP1 and the ATR–ATRIP complex. ATR has similar domain architecture to other members of the PIKK protein kinase family. It contains dozens of N-terminal HEAT repeats and a kinase domain flanked by FAT and FATC domains (Fig. 5A). The N-terminal heat repeats provide a binding surface for ATRIP (Ball et al. 2005; Chen et al. 2007). Aside from this, little is known about the structure of ATR. We again used the TopBP1 AAD as bait in a yeast two-hybrid approach to screen a library of thousands of ATR fragments. All of the interacting fragments recovered contained amino acids 2483–2597 of ATR (Fig. 5A). These residues span the C-terminal end of the kinase domain and an uncharacterized region of ATR between the kinase and FATC domains, which we named the PIKK Regulatory Domain (PRD) for reasons discussed below.

Although the kinase and FATC domains share a high degree of sequence similarity among all PIKK family members, the sequence of the PRD is highly divergent in these paralogs (Supplemental Fig. S1B). However, the PRD has a high degree of sequence identity within orthologous ATR proteins from different organisms. Deletion of the entire ATR PRD abolished all kinase activity (data not shown), probably as a result of disrupting the folding of the adjacent kinase domain. Scanning mutagenesis of the ATR PRD identified two mutations (K2589E and HVL2591AAA) that largely eliminated TopBP1-activation of ATR kinase activity without changing the basal activity of the kinase in the absence of TopBP1 (Fig. 5B; Supplemental Fig. S2). Several other mutations in this region including a small deletion Δ2569–2576 and a charge reversal of a lysine one amino acid separated from K2589 (K2587E) had no significant effect on either the basal or TopBP1-activated ATR kinase activity (Fig. 5B,C; Supplemental Fig. S2). None of these mutations had any effect on the ability of ATR to bind ATRIP. Mutation of K2589 to alanine did not impair ATR activation (Supplemental Fig. S2A), indicating that a post-translation modification at this site is not necessary for ATR activation by TopBP1.

Since ATR K2589E has equivalent basal kinase activity toward itself, ATRIP, and a substrate as wild-type ATR (Fig. 5D), the mutation does not alter the catalytic activity of ATR in the unactivated state but specifically affects the formation of the active ATR protein. Additionally, this mutation does not affect the ability of ATR to form homo-oligomeric complexes (Supplemental Fig. S3).

Next, we tested the ability of ATR–ATRIP complexes containing ATR K2589E to associate with TopBP1. The ATR K2589E mutant modestly decreased the association with TopBP1 (approximately twofold) in pull-down assays compared with wild-type ATR–ATRIP complexes (Fig. 5E). The remaining association is likely mediated by the ATRIP–TopBP1-binding interface.

Regulation of PIK kinases via the PIK regulatory and FATC domains

A recent study suggested that the region of ATM equivalent to the ATR PRD is targeted for acetylation by the Tip60 histone acetylase (Sun et al. 2007). The DNA-damaged inducible acetylation of a specific lysine in the ATM PRD is necessary for the increased kinase activity of ATM after DNA damage. To test whether acetylation of the ATR PRD might regulate its activation by TopBP1, we made lysine-to-arginine mutations in the PRD. None of these mutations, even when combined into a single ATR protein, had any effect on TopBP1-mediated activation of ATR in vitro (Supplemental Fig. S2B). Also, siRNA depletion of Tip60 did not affect the ability of cells to recover from HU (data not shown). Hence, the PRD of ATR is likely not regulated through acetylation in the same manner as the PRD of ATM.

To determine if the PRD is necessary for the regulation of other PIKK family members besides ATR and

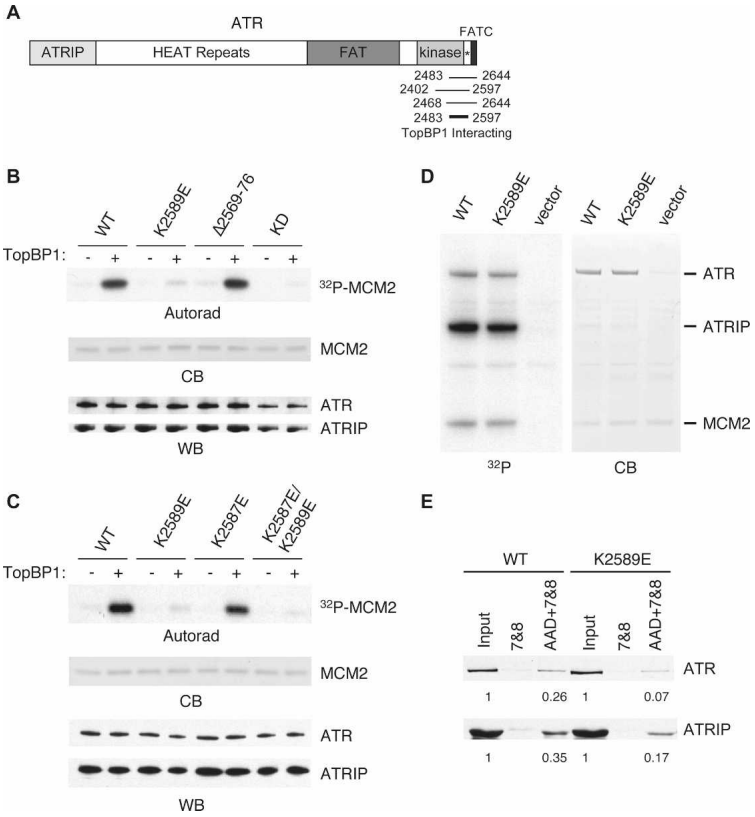

Figure 5. An ATR regulatory region between the kinase and FATC domains is critical for TopBP1-dependent activation of ATR in vitro. (*A*) Schematic diagram showing the domains of ATR and the ATR fragments that interact with TopBP1 in a yeast two-hybrid assay (black lines). (*B*) Wild-type ATR (WT), ATR K2589E, ATR Δ2569–2576, or ATR kinase dead (KD) proteins complexed with wild-type ATRIP were isolated from transfected 293T cells and incubated with MCM2 substrate, γ-^{32}P-ATP, and recombinant TopBP1 AAD where indicated. Kinase reactions were separated by SDS-PAGE, stained with Coomassie blue (CB), and exposed to film (autorad). A duplicate gel was immunoblotted with anti-ATRIP and anti-ATR antibodies (WB). (*C*) Kinase reactions to measure TopBP1-dependent activation of wild-type ATR (WT), ATR K2589E, ATR K2587E, or ATR K2587E/K2589E were performed as in *B*. (*D*) To measure basal kinase activity plasmids encoding ATRIP and Flag-tagged wild-type ATR (WT), ATR K2589E, or empty vector were expressed in 293T cells and immunoprecipitated with anti-Flag antibodies. Complexes were incubated with MCM2 substrate and γ-^{32}PATP. Kinase reactions were separated by SDS-PAGE, stained with Coomassie blue (CB), and exposed to film (autorad). (*E*) Nuclear extracts from 293T cells transfected with vectors encoding wild-type ATR (wt) and ATRIP or ATR K2589E and ATRIP were incubated with recombinant fragments of TopBP1 bound to glutathione beads. Bound proteins were eluted, separated by SDS-PAGE, and immunoblotted with ATR or ATRIP antibodies. Input equals 5% of the extract used in the binding reactions. Quantification of the immunoblot signal normalized to the input is shown.

ATM, we examined the PRD of DNA-PKcs. Activation of DNA-PKcs requires the Ku70/80 heterodimer and DNA ends (Smith and Jackson 1999). Electron microscopy structural studies of the DNA-PK complex demonstrated an interaction between the Ku70/80 heterodimer and a region immediately C-terminal to the kinase domain, suggesting that Ku70/80 binding could be mediated in part by the DNA-PKcs PRD (Spagnolo et al. 2006). Therefore, we tested if the PRD is necessary for the activation of DNA-PKcs. In response to dsDNA breaks, DNA-PKcs is autophosphorylated at Ser 2056, an event that is required for nonhomologous end-joining-mediated DNA double-strand break repair (Chen et al. 2005). Wild-type DNA-PKcs and DNA-PKcs PRD mutants (M1: K4043E/K4048E/R4049E/K4050E, M2: D4062K/E4063K/E4069K, M3: K4075E/R4082E/R4085E/R4090E) were expressed in DNA-PKcs-defective cells. Cells were exposed to ionizing radiation and the phosphorylation status of Ser 2056 was assessed. Although wild-type DNA-PKcs and the M2 DNA-PKcs PRD mutant exhibited Ser 2056 phosphorylation, the M1 and M3 DNA-PKcs PRD mutants did not (Supplemental Fig. S4). Thus, specific residues within the PRD of DNA-PKcs are required for DNA-PKcs autophosphorylation, suggesting that the PRD is important for the regulation of DNA-PKcs as well.

Since the FATC domain amino acid sequence is highly conserved among PIK kinases and adjacent to the PRD, we tested if it is also important for TopBP1 to activate ATR. Deletion of part of or the entire FATC domain abolished even the basal kinase activity of ATR (Supplemental Fig. S5). Since the FATC domain of ATR can substitute for the FATC domain of ATM (Jiang et al. 2006), we performed the reciprocal experiment. Replacement of the FATC domain of ATR with that of ATM also resulted in a kinase-dead mutant, indicating that it cannot substitute (Supplemental Fig. S5). Thus, the FATC domain of ATR is essential for even basal ATR kinase activity.

ATR regulation through the PRD is essential for checkpoint signaling and cell viability

The ATR-PRD mutation provides a second separation of function mutant useful for examining the functional importance of TopBP1-mediated ATR activation. To determine if regulation of ATR by TopBP1 through the PRD is necessary for cellular responses to replication stress, we created ATR$^{flox/-}$ cell lines expressing a Tet-inducible form of either wild-type ATR or mutant K2589E ATR. The cell lines were treated with tetracycline to induce expression of the exogenous ATR prior to deletion of the floxed ATR allele with the Cre recombinase. Expression levels of the wild-type or K2589E ATR protein were similar and near the endogenous amount of ATR expression (Fig. 6A). Phosphorylation of the essential kinase

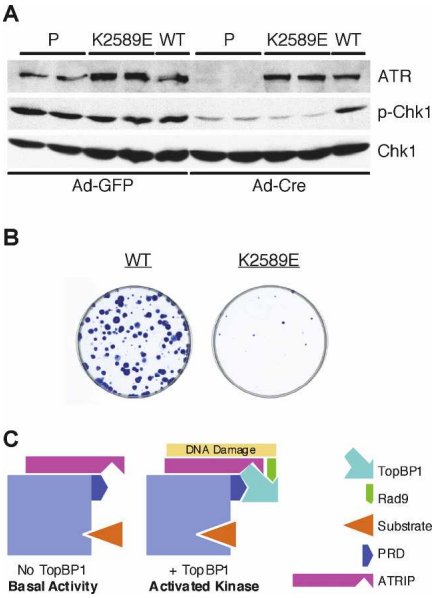

Figure 6. ATR K2589E does not support checkpoint signaling. (*A*) ATR$^{flox/-}$ cell lines were created that contained an inducible form of either ATR K2589E or ATR wild-type (WT). P1 and P2 denote ATR$^{flox/-}$ parental cell lines lacking any exogenous ATR. The cells were induced to express exogenous ATR with tetracycline and were treated with adenovirus encoding the Cre recombinase (Ad-Cre) to delete the endogenous ATR or adenovirus expressing GFP (Ad-GFP) as a control. Four days after infection, cells were treated with 1 mM HU for 6 h. Cell lysates were separated by SDS-PAGE and blotted with the indicated antibodies to ATR, Chk1, or phosphorylated Chk1. (*B*) Equal numbers of Cre-infected ATR$^{flox/-}$ cells expressing wild-type ATR or ATR K2589E were plated. Seventeen days after plating, the surviving colonies were stained with methylene blue. (*C*) Schematic model for ATR activation. In the absence of TopBP1, ATR exhibits a low basal kinase activity. In response to genotoxic stress, ATRIP recruits ATR to sites of DNA damage. Loading of the Rad9–Hus1–Rad1 complex allows Rad9 to recruit TopBP1. TopBP1 makes contact with both ATRIP and the ATR PRD. The interaction between TopBP1 and ATR–ATRIP greatly stimulates ATR kinase activity, perhaps due to a conformational change in the ATR kinase domain that facilitates the ability of ATR to interact with its substrates.

Chk1, an ATR substrate (Liu et al. 2000), was used to assess ATR signaling in cells. Robust Chk1 phosphorylation following HU treatment is observed in all the cell lines prior to deletion of the endogenous ATR allele (Fig. 6A). However, the K2589E ATR protein failed to support Chk1 phosphorylation after HU treatment, yielding phospho-Chk1 levels comparable with cells not expressing any exogenous ATR. In contrast, wild-type, exogenous ATR could support Chk1 phosphorylation in cells lacking endogenous ATR (Fig. 6A, last lane). Two K2589E ATR-expressing clonal cell lines yielded identical results. Thus, ATR K2589E is defective in checkpoint signaling after replication stress.

ATR is essential for the viability of proliferating cells, but its essential function is not clear (Brown and Baltimore 2000; Cortez et al. 2001). Given that the K2589E mutant ATR could not support checkpoint signaling, we wanted to assay whether this mutant would be capable of supporting cellular viability even in the absence of exogenously added genotoxic agents. The ATR$^{flox/-}$ cells lines stably expressing wild-type or mutant K2589E ATR were treated with adenovirus expressing Cre recombinase and plated at low density. After 17 d, surviving cell colonies were stained. The colony formation assay revealed a dramatic difference in the number of colonies between the two cell lines (Fig. 6B). Only a few colonies grew from the mutant cell line. PCR genotyping of these colonies from a duplicate sample indicated that all the surviving colonies expressing the K2589E ATR had not undergone Cre-mediated recombination to delete the endogenous ATR allele; whereas, all of the colonies expressing wild-type ATR underwent Cre-mediated recombination and lacked the endogenous ATR allele (Supplemental Fig. S6). Thus, although we could obtain an ATR$^{-/-}$ cell line rescued by a wild-type ATR cDNA, we were not able to obtain an ATR$^{-/-}$ cell line expressing only the K2589E ATR mutant. This suggests that TopBP1-mediated activation of ATR is essential for the viability of, at least, this human cell type in culture.

Discussion

The PIK kinases regulate many cellular responses including nutrient sensing and the DNA damage response. Therefore, their activities impact many human diseases. Unfortunately, their large size and atypical kinase domains have made understanding their activation mechanisms difficult. In this study, we define how the TopBP1 activator protein binds to the ATR–ATRIP complex and identify critical regulatory regions within both the ATRIP and ATR proteins. Importantly, we provide evidence that the ATRIP regulatory region is conserved functionally in the yeast ATRIP protein Ddc2, and the ATR regulatory domain is a common site for regulation of most, if not all, of the PIK kinases.

Our data support the ATR activation model shown in Figure 6C. In the absence of DNA damage or replication stress, ATR has basal kinase activity. Following a challenge to the genome that exposes ssDNA gaps, ATRIP and the 9–1–1 checkpoint clamp are recruited independently (Melo et al. 2001; Zou et al. 2002). ATRIP brings ATR and Rad9 brings TopBP1. The assembly and concentration of these components at sites of DNA damage facilitates an interaction between TopBP1 and interacting surfaces on both ATRIP and ATR. This interaction then promotes ATR activation.

Additional regulatory steps are clearly important. For example, the ATR–ATRIP complex, 9–1–1 checkpoint clamp, and TopBP1 are phosphorylated, suggesting that post-translational modifications may fine-tune ATR activation (Roos-Mattjus et al. 2003; Myers et al. 2007; Venere et al. 2007; Yoo et al. 2007). Furthermore, specific ATR substrates (such as Chk1) require additional protein cofactors (such as Claspin) to be efficiently phosphorylated (Kumagai and Dunphy 2000; Liu et al. 2006). Exactly how TopBP1 binding increases ATR kinase activity

will require a structural description of these proteins. One possibility is that it alters the conformation of the ATR kinase domain such that substrates can access ATR more easily. Consistent with this interpretation, we found that TopBP1 binding to ATR decreases the apparent K_m of ATR for substrates (D.A. Mordes, unpubl.).

ATRIP provides several functions to regulate ATR activation

ATR function is dependent on its binding partner ATRIP. ATRIP provides at least four activities that promote ATR signaling. First, it stabilizes ATR (Cortez et al. 2001). Second, it promotes the localization of ATR to sites of DNA damage or replication stress (Zou and Elledge 2003; Ball et al. 2005, 2007). Third, ATRIP post-translational modifications regulate ATR signaling (Myers et al. 2007; Venere et al. 2007). Finally, as demonstrated in this report, ATRIP binds directly to TopBP1, and this binding is essential for ATR activation.

Mutations of the TopBP1-interacting region of ATRIP impair the ability of cells to recover from replication stress and arrest the cell cycle after DNA damage. Mutations of the same region in *S. cerevisiae* ATRIP (Ddc2) cause sensitivity to replication stress and impair activation of Mec1 in response to DNA damage. This suggests that the mechanism of activation for ATR may be conserved throughout evolution. Although Mec1 and Ddc2 are clear orthologs of ATR and ATRIP, respectively, no obvious *S. cerevisiae* ortholog exists for TopBP1. Two checkpoint proteins, Dpb11 and Ddc1, are candidates for protein activators of Mec1, but neither share sequence identity to the TopBP1 AAD. Like TopBP1, Dpb11 is a BRCT-repeat-containing protein that has essential roles in replication and checkpoint signaling. Yet, Dpb11 has not been reported to activate Mec1 directly. In vertebrates, TopBP1 is recruited to gapped ssDNA regions through an interaction with the Rad9 subunit of the checkpoint clamp (Delacroix et al. 2007; Lee et al. 2007). Ddc1 is the yeast ortholog of Rad9 and also binds to Dpb11 (Wang and Elledge 2002). The C-terminal tail of Ddc1 stimulates the kinase activity of the Mec1–Ddc2 complex in vitro under low-salt conditions (Majka et al. 2006). However, overexpression of this portion of Ddc1 is not sufficient to cause activation of Mec1. In contrast, overexpression of the TopBP1 AAD is sufficient to cause pan-nuclear activation of ATR in mammalian cells (Kumagai et al. 2006; Ball et al. 2007). It is possible that both proteins may act as activators of Mec1 in response to different types of DNA damage or they may form an activator complex. In any case, our results predict that a Mec1 activator is likely to bind to a surface on Ddc2 encoded just C-terminal of the coiled-coil domain containing amino acids 257–260.

PIK kinase regulation

PIKKs are key regulators of many critical signaling pathways, including cell growth, cell cycle checkpoints, and DNA damage. Despite their diverse functions, emerging data suggest many similarities among them. PIKKs associate with an interacting partner (ATRIP for ATR, NBS1 for ATM, Ku70/80 for DNA-PKcs, and Rictor and Raptor for mTOR) that is critical for their function and, in the case of ATRIP, NBS1, and Ku80, their localization to sites of DNA damage (Cortez et al. 2001; Hara et al. 2002; Sarbassov et al. 2004; Falck et al. 2005). PIKKs also require an interaction with another regulatory protein or protein complex for their activation. GβL (mLST8) binds to the kinase domain of mTOR and stimulates its catalytic activity (Kim et al. 2003; Wullschleger et al. 2005). Rheb also activates mTOR–Raptor complexes (Long et al. 2005). The Mre11/Rad50 complex makes multiple contacts with ATM and is sufficient to stimulate its kinase activity especially in the presence of DNA (Lee and Paull 2004). The Ku70/Ku80 complex stimulates DNA-PKcs in the presence of DNA ends (Smith and Jackson 1999). Finally, TopBP1 stimulates the kinase activity of ATR in an ATRIP-dependent manner (Kumagai et al. 2006). There are apparently even common regulators of most, if not all, the PIK kinases. Recently, Tel2 has been demonstrated to interact with all PIKKs and regulate their stability (Takai et al. 2007).

Given the similarity of sequence and structure of the PIK kinases, it might be expected that the mechanisms controlling their activation would be similar (although responsive to different inputs). Indeed, the FATC domain is highly conserved in sequence among PIKKs and is functionally interchangeable in some instances (Jiang et al. 2006). The FATC domain is required for the kinase activity of ATM, DNA-PKcs, mTOR, and SMG-1 (Banin et al. 1998; Priestley et al. 1998; Takahashi et al. 2000; Morita et al. 2007). Our data indicate that the FATC domain of ATR is essential for its kinase activity as well. We noticed that mutation of the FATC domain of ATR caused a reduction in expression levels, and a similar observation has been made for ATM (Jiang et al. 2006). Since all kinase activity is lost when the FATC domain is mutated, we suspect that the FATC domain may be important for the stability of PIKKs or for proper folding of the kinase domain. One possibility is that the FATC domain makes critical contacts within the kinase or FAT domains that stabilize the kinase domain.

In contrast to FATC domain alterations, mutations in the PRD (the region between the kinase and FATC domains) of ATM, ATR (this study), mTOR, and SMG-1 do not abolish PIK kinase activity (Sekulic et al. 2000; Morita et al. 2007; Sun et al. 2007). However, these mutations do impair kinase regulation. This region does not exhibit sequence similarity among PIKKs, suggesting that it could be sensitive to different regulatory inputs for each of the kinases. The PRD domain of mTOR contains an Akt phosphorylation site that regulates its activity (Sekulic et al. 2000). The Tip60 histone acetyltransferase complex acetylates ATM within the ATM PRD, and this acetylation is important for the activation of ATM kinase activity after DNA damage (Sun et al. 2005, 2007). We now show that the ATR PRD is critical for TopBP1-dependent activation of ATR and the PRD of

DNA-PKcs is necessary for its autophosphorylation. Despite the similarity of ATM and ATR, we have no evidence that the ATR PRD is acetylated. In fact, mutations of all the lysines in this region to arginines did not impair TopBP1-dependent activation.

We propose that the PRD has evolved to allow distinct regulation of the kinase activity of the PIKK family members. Differences in the PRD and the PIK-binding partner provide specificity for the types of cellular events that can activate each PIKK. It will be interesting to determine whether the ATM PRD or mTOR PRD is important for Mre11/Rad50-dependent or GβL/Rheb-dependent ATM or mTOR activation, respectively. Also, it will be important to examine the impact of ATM PRD acetylation on Mre11/Rad50-dependent regulation.

Defects in cellular responses to replication stress in cells lacking TopBP1-dependent ATR activation

Previous studies have demonstrated critical roles for ATR signaling and the TopBP1 protein for replication stress-induced checkpoints. Furthermore, the C-terminal half of TopBP1 was shown to be essential for checkpoint signaling in *Xenopus laevis* egg extracts (Hashimoto et al. 2006; Kumagai et al. 2006; Yan et al. 2006). However, the interpretation of genetic experiments on TopBP1 in human cells is problematic given that it forms complexes with many other proteins and regulates both DNA replication and checkpoints. The analysis of the separation-of-function mutants that we created in both ATR and ATRIP allows us to unambiguously define the cellular requirements for TopBP1-dependent ATR activation. Our analysis reveals that not only is TopBP1-dependent ATR activation essential for checkpoint responses to replication stress, but it is also required for the essential function of ATR in promoting cellular viability.

Conclusions

Our data suggest both similarities and differences among the regulation of the PIKK family of protein kinases. The ability of TopBP1 to uniquely stimulate ATR is explained, in part, by the requirement of an ATRIP surface for the binding of TopBP1 to the ATR–ATRIP complex. The PRD of ATR and the other PIKKs is a second important regulatory determinant. The exact mechanism by which the PRD functions in PIKK activation remains to be determined, but given its position between the kinase and FATC domains, it seems likely that it mediates a conformational change that may allow greater kinase activity.

Inhibition of ATR sensitizes cancer cells to multiple DNA-damaging agents (Wilsker and Bunz 2007). Thus far, specific inhibitors of ATM and DNA-PKcs kinases have been developed and are being studied as potential therapeutic agents (Lord et al. 2006); however, inhibitors of the ATR kinase have not been isolated. Targeting the interaction between ATRIP and TopBP1 or the ATR PRD may provide a novel means for developing an agent to disrupt ATR signaling.

Materials and methods

Cell lines

HEK 293T and U2OS cell lines were maintained in DMEM + 7.5% fetal bovine serum. The V3 CHO cell line was maintained in αMEM supplemented with 10% fetal bovine serum, 10 µg/mL ciprofloxacin (Mediatech), 50 U/mL penicillin, and 50 µg/mL streptomycin (Invitrogen). Construction of ATRIP stable cell lines and use of ATRIP siRNA were as described previously (Ball et al. 2005), except transfections of siRNAs at 10 nM were performed with HiPerFect (Qiagen). Plasmid transfections of HEK 293T cells were performed with Lipofectamine 2000 (Invitrogen). Plasmid transfections of V3 CHO cells were performed with TransIT-CHO (Mirus Bio). HCT116 $ATR^{flox/-}$ cells were maintained in McCoy's medium + 7.5% fetal bovine serum. Clonal ATR stable lines were made by transfecting HA-tagged ATR tetracycline-inducible vectors into $ATR^{flox/-}$-TetR cells, and selecting with 300 µg/mL Hygromycin B (Invitrogen) for single colonies. ATR expression was induced with 1 µg/mL tetracycline (Invitrogen). Deletion of the ATR gene and PCR genotype analysis for the ATR allele was performed as described previously (Cortez et al. 2001). For the colony formation assays, equal numbers of cells were plated onto 60 mM tissue culture dishes and incubated for 17 d in the presence of 300 µg/mL Hygromycin B and 1 µg/mL tetracycline. Media was changed every 3 d. Colonies were stained with methylene blue (Sigma).

Yeast

Ddc2 mutations were made in the pNML1 centromeric plasmid encoding myc-Ddc2 under the endogeonous Ddc2 promoter (Rouse and Jackson 2002) and were expressed in strain DMP2995/1B: *MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 sml1*Δ::*KanMX4 ddc2*Δ::*KanMX4* (Paciotti et al. 2000). Yeast protein samples were prepared using TCA precipitation as described (Longhese et al. 1997).

DNA constructs

PCR site-directed mutagenesis was performed using the QuickChange method and PfuUltra DNA polymerase (Stratagene). All constructs generated using PCR were confirmed by sequencing. The DNA-PKcs expression vector was kindly provided by Dr. Kathryn Meek. Cloning details for any construct are available upon request.

Protein interactions

TopBP1-binding assay: Recombinant GST-tagged TopBP1 fragments were purified from *Escherichia coli* with glutathione Sepharose 4B beads (GE Healthcare). Nuclear extracts were prepared as described (Kumagai et al. 2006) and incubated with the indicated GST-tagged proteins bound to glutathione Sepharose beads overnight at 4°C. Beads were washed three times in low-salt buffer (20 mM HEPES-KOH at pH 7.9, 175 mM NaCl, 20% glycerol, 0.05% Tween 20), and proteins were eluted and separated by SDS-PAGE prior to immunoblotting.

Two-hybrid experiments were performed using the TopBP1 AAD (amino acids 978–1286) cloned into pDAB1 containing the DNA-binding domain of Gal4. This bait was used to screen the

pACT-ATRIP and pACT-ATR cDNA fragment libraries that we described previously (Ball et al. 2005) using the PJ694A yeast strain (James et al. 1996).

Kinase assays were performed largely as described previously (Cortez et al. 2001; Ball et al. 2007). Assays measuring the basal ATR activity were performed by immunoprecipitating Flag-ATR protein using anti-Flag M2 agarose beads (Sigma) from TGN buffer (50 mM Tris, 150 mM NaCl, 10% glycerol, 1% Tween 20, 5 µg/mL aprotinin, 5 µg/mL leupeptin, 1 mM NaF, 50 mM β-glycerol phosphate, 1 mM sodium vanadate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) cell lysates. Immunoprecipitates were washed three times in TGN buffer, once in TGN + 500 mM LiCl, and twice in kinase buffer (10 mM HEPES at pH 7.5, 50 mM NaCl, 50 mM β-glycerol phosphate, 10 mM $MgCl_2$, 1 mM diothiothreitol) prior to performing kinase reactions. Assays measuring TopBP1 stimulation of ATR activity were performed by immunoprecipitating Flag-ATR/HA-ATRIP complexes using anti-HA agarose beads (Sigma). Immunoprecipitates were washed three times in TGN buffer, once in TGN + 500 mM LiCl, and twice in kinase buffer prior to performing kinase reactions. GST-TopBP1 AAD was added to the reactions prior to adding ATP and substrate. All reactions were stopped within the linear range of the assay and analyzed by SDS-PAGE and autoradiography.

For experiments to assess ATR oligomerization, 293T cells transiently expressing the indicated plasmid were lysed in CHAPS lysis buffer as described (Ball and Cortez 2005).

Antibodies

The ATRIP-N antibody has been described previously (Cortez et al. 2001). The following antibodies were purchased: ATM (Novus), ATR and Chk1 (Santa Cruz Biotechnologies), Chk1 P317 (Cell Signaling), DNA-PKcs (Serotec), DNA-PKcs pS2056 (Abcam), HA.11 and Myc9E10 (Covance), and Flag M2 (Sigma). The Rad53 antibody was a gift from Stephen Elledge.

Bioinformatics

Protein sequence alignments were performed using ClustalW, Boxshade, and Coils (http://www.ch.embnet.org). Protein secondary structure predictions were generated by PsiPred (http://bioinf.cs.ucl.ac.uk/psipred).

HU recovery, viability, and checkpoint assays

Three days after siRNA transfection, cells were incubated in media with or without HU for 24 h. Cells were released into fresh media for 24 h, and cell viability was assessed using the WST-1 reagent assay (Roche), which is a colorimetric assay based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases. The percentage viability was calculated as the ratio of the 450 nM absorbance of the HU-treated cells to the untreated cells. The HU recovery assays were performed using the same protocol with the following differences: Nocodazole was added to the media when the HU was removed, and cells were harvested at 0, 8, or 16 h after release. Harvested cells were fixed in ethanol, stained with propidium iodide, and analyzed on a BD Biosciences FACSCalibur. A stock solution of HU (Sigma) was prepared in water at 1 M and stored frozen. Nocodazole (Acros) was dissolved in dimethylsulfoxide at 10 mg/mL. For the G2/M checkpoint assay, harvested cells were fixed in ethanol, permeabilized, stained with an antibody against H3 phospho-S10 antibody and an anti-FITC secondary antibody, propidium iodide, and then analyzed by FACS. Cells with 4n DNA content and H3 phospho-S10-positive were counted as mitotic cells.

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