Supporting Information

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SI Text

SI Materials and Methods. ***Antibodies and preparation of checkpoint proteins.*** Chk1 phospho-S345 antibodies were purchased from Cell Signaling Technology, RPA2 antibodies were from Calbiochem, Flag M2 antibodies were from Sigma, His antibodies were from Abgent, TopBP1 antibodies were from Millipore, DNA PK, ATM, ATR, ATRIP and RPA1, Chk1, and GST antibodies were purchased from Santa Cruz Biotechnology. GST-TopBP1-His, GST-TopBP1 fragments, HisFlag-ATRIP, and His-Chk1 kinase dead (Chk1-kd) were all purified as previously described (1–4). RPA (5), aRPA (6), RPA1-t11 and RPA1-ΔN168 (7), *Saccharomyces cerevisiae* (sc) RPA (8), HisFlag-ATRIP (3), and human SSB1 (9) were purified as described. *Escherichia coli* SSB1 was obtained from J. D. Griffith (University of North Carolina, Chapel Hill, NC).

Purification of ATR-ATRIP from HeLa cells. Whole cell extract was prepared from 33 L of HeLa cells by the method of Manley et al. with some modifications (10). Briefly, the cell pellet from 33 L of HeLa cells (0.52×10^6 cells/mL, National Cell Culture Center) was resuspended in four packed-cell volumes of Buffer I (10 mM Tris-HCl, pH 7.9, 1 mM EDTA, 5 mM DTT) and incubated on ice for 20 min. The cells were dounced with a Wheaton B homogenizer 10 times and mixed with four packed-cell volumes of Buffer II (50 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 2 mM DTT, 25% sucrose, 50% glycerol). One packed-cell volume of saturated $(NH_4)_2SO_4$ was slowly added to the mixture with gentle stirring (10% saturation at 0 °C). The mixture was stirred for 30 min and centrifuged at 40,000 rpm in a Beckman Ti 45 rotor for 3 h at 4 °C. The supernatant was collected, and then solid $(NH_4)_2SO_4$ was added to 35% saturation (35 saturated ammonium sulfate (SAS)) (0.14 g/mL), which is commonly used as a first step in ammonium sulfate fractionation of proteins, and centrifuged at 40,000 rpm in a Beckman Ti 45 rotor for 1 h at 4 °C. To obtain the 35%–55% cut, the supernatant was recollected, and solid $(NH_4)_2SO_4$ was added to 55% saturation (55SAS) (0.12 g/mL) and centrifuged as before. The pellets were resuspended in 1/10th the original volume with a buffer containing 25 mM Hepes, pH 7.9, 100 mM KCl, 12 mM $MgCl_2$, 0.5 mM EDTA, 2 mM DTT, 12.5% glycerol and dialyzed against 4 L of 100 mM KCl-Buffer D (20 mM Hepes, pH 7.9, 20% glycerol, 0.2 mM EDTA, 0.5 mM DTT). The dialyzed extract (34 mL, 6.5 mg/mL) of 35% cut was applied onto a 20 mL phosphocellulose column (Whatman), which had been preequilibrated with 100 mM KCl-Buffer D. The column was washed with 100 mL of the same buffer and eluted with a 100 mL gradient of 100–400 mM KCl (Buffer D). The fractions (15 mL) were analyzed by SDS-PAGE/silver stain and Western blotting. All of the remaining ATM from the 35% SAS was in the flow-through of this column. ATR-ATRIP eluted at ∼230 mM KCl (Buffer D). The ATR-containing fractions were pooled, diluted with Buffer D to 100 mM KCl, and loaded onto a 20 mL Mono Q column (GE Healthcare) and then eluted with a 200 mL gradient of 100–500 mM KCl (Buffer D). The column fractions were analyzed by SDS-PAGE/silver stain and Western blotting. ATR-ATRIP eluted at ∼220 mM KCl. The active fractions were frozen in dry ice and stored at −80 °C.

Preparation of DNA substrates. Single- and double-stranded forms of ϕX174 DNA were purchased from New England Biolabs. Benzo[a]pyrene diol epoxide (BPDE)-damaged DNA (10 adducts/plasmid) was prepared as previously described (1, 4). For various sizes of ssDNA fragments, PCR was performed with ϕX174 DNA as a template, and ssDNA was prepared by heating the dsDNA (>10 ng/μL) for 5 min at 100 °C and rapid cooling on ice.

ATR kinase assay. Kinase reactions, unless stated otherwise, contains 1 fmol ATR-ATRIP, 50 fmol GST-TopBP1, and 100 fmol His-Chk1 (kd) in 10 μL of 15 mM Hepes, pH 7.9, 35 mM NaCl, 3 mM $MgCl_2$, 1 mM ATP, 0.5 mM DTT, 1 μM microcystin, 5% glycerol, 1% polyethylene glycol (6000). Where indicated, different forms of ϕX174 DNA were mixed with RPA and incubated on ice for 10 min before addition of the kinase reaction mixture. Kinase reactions were incubated at 30 °C for 20 min, terminated by the addition of SDS-PAGE loading buffer, and the proteins were separated by SDS-PAGE. Chk1 phosphorylation was detected by immunoblotting using phospho-S345 antibodies. The level of phosphorylation was quantified using ImageQuant 5.2 software after scanning immunoblots. The highest level of phosphorylation in each experiment was set equal to 100, and the levels of phosphorylation in the other lanes were expressed relative to this value. Averages from at least three independent experiments were graphed and presented as mean ± standard error (SEM).

DNA pull-down assays. Biotinylated 80-mer oligonucleotide (1 pmol) (3) was bound to streptavidin-beads (Dynal) according to the manufacturer's directions and then incubated with the indicated amounts of RPA, aRPA, or RPA mutants in 50 μL of Binding Buffer (10 mM Tris-Cl, pH7.5, 100 mM NaCl, 0.01% NP40, 10% glycerol, and 10 μg/mL BSA) at room temperature for 30 min. The beads were retrieved, washed three times with Binding Buffer, and incubated with the indicated amounts of ATRIP and/or TopBP1 at room temperature for 30 min. The beads were retrieved, washed, and bound proteins were eluted by boiling with SDS-loading buffer. The eluted proteins were separated on SDS-PAGE and analyzed by immunoblotting with the corresponding antibodies.

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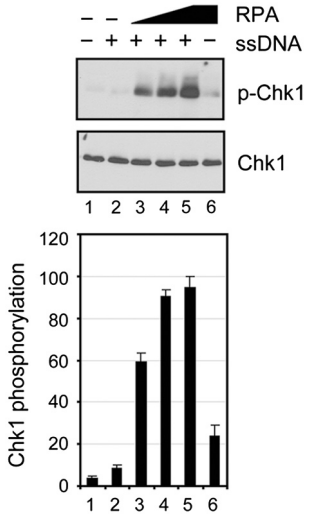

Fig. S1. Titration of RPA. The reactions were carried out without RPA (lane 1, 2) and with 80, 240, or 720 fmol RPA (lanes 3, 4, 5) as indicated. Lane 6 contained RPA (720 fmol) but no DNA.

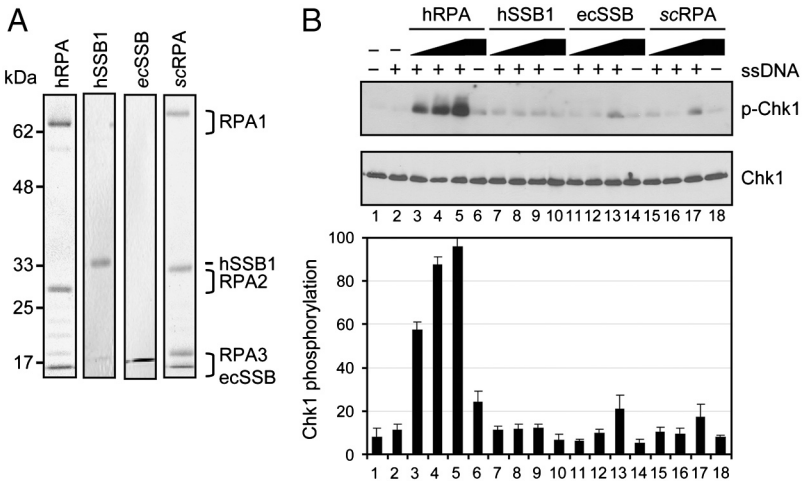

Fig. S2. The stimulatory effect of RPA-ssDNA on ATR is specific for human RPA. (*A*) Analysis of ssDNA-binding proteins. Purified human RPA (hRPA), human SSB1 (hSSB1), *E. coli* SSB (ecSSB), and *S. cerevisiae* RPA (scRPA) were separated on a 10% SDS-PAGE and visualized by Coomassie Blue staining. (*B*) Effects of ssDNA-binding proteins on TopBP1-dependent ATR activation in the presence of ssDNA. The reactions were carried out under standard conditions in the presence of increasing amounts of ssDNA-binding proteins (80, 240, or 720 fmol). The results from three independent experiments were quantified and are presented (bottom).

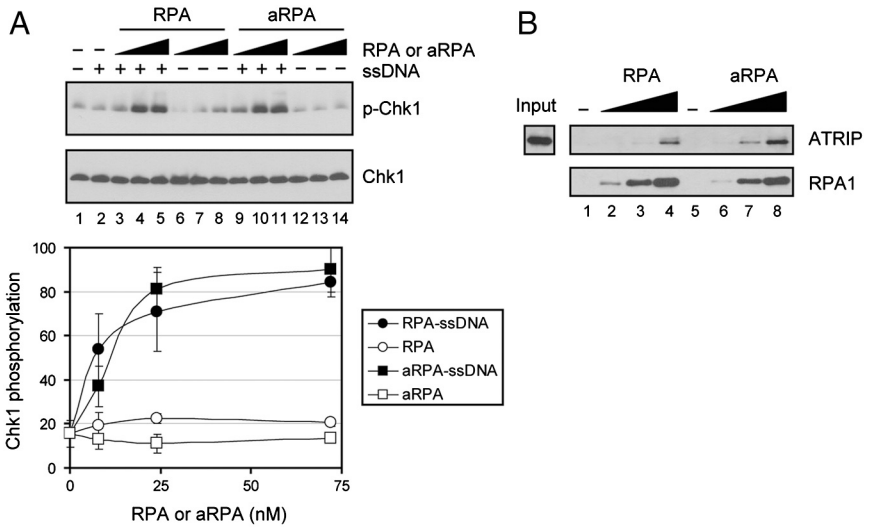

Fig. S3. TopBP1-dependent ATR activation is stimulated by canonical and alternative RPA. (*A*) Stimulation of TopBP1-dependent ATR kinase by RPA or aRPA. Where indicated, the reaction contained 80, 240, or 720 fmol of RPA or aRPA. Bottom shows quantitative analysis of three experiments. (*B*) Recruitment of ATRIP to ssDNA by RPA or aRPA. Biotinylated 80-mer oligonucleotide (1 pmol)-bound streptavidin beads were incubated with RPA or aRPA (0.2, 0.5, or 1 pmol). The beads were retrieved, washed, and incubated with ATRIP (0.5 pmol). The beads were then isolated and washed, and bound proteins were analyzed by immunoblotting with the corresponding antibodies.

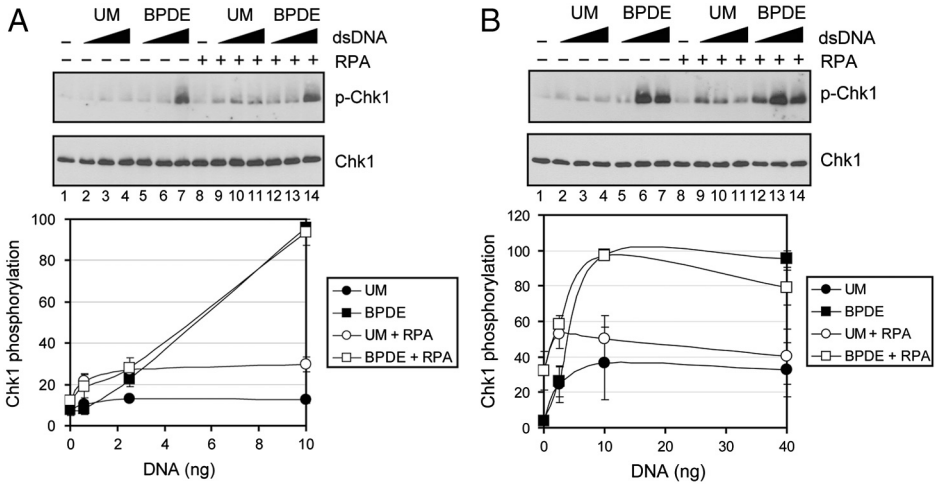

Fig. S4. Damaged DNA-dependent stimulation of ATR by TopBP1 is not significantly stimulated by RPA. (*A*) Effect of RPA on damaged DNA-dependent stimulation of ATR by TopBP1. The reactions were performed with unmodified or BPDE-damaged ϕX174 dsDNA (0.6, 2.5, or 10 ng) and full-length TopBP1 (5 fmol) in the absence or presence of RPA (320 fmol) under high ionic strength conditions (85 mM NaCl). Bottom indicates quantitative analysis of Chk1 phosphorylation from three experiments. (*B*) Broad titration of BPDE-damaged DNA. The reactions were carried out as in *A*, except with 2.5, 10, or 40 ng of unmodified or BPDE-damaged dsDNA.

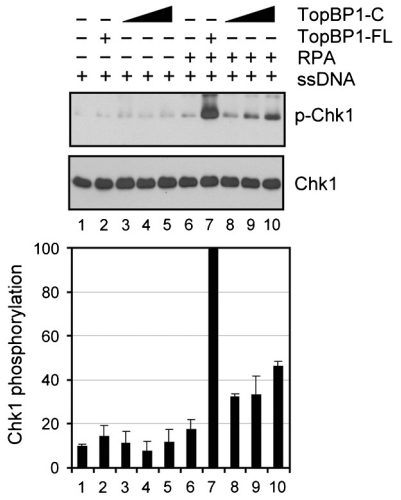

Fig. S5. Defect in RPA-ssDNA-mediated ATR activation by the C terminus of TopBP1. The reactions were carried out with full-length TopBP1 (50 fmol) or TopBP1-C (50, 150, and 350 fmol) in the presence of RPA (320 fmol) and ϕX174 ssDNA (1 ng). Bottom shows averages of three independent experiments.

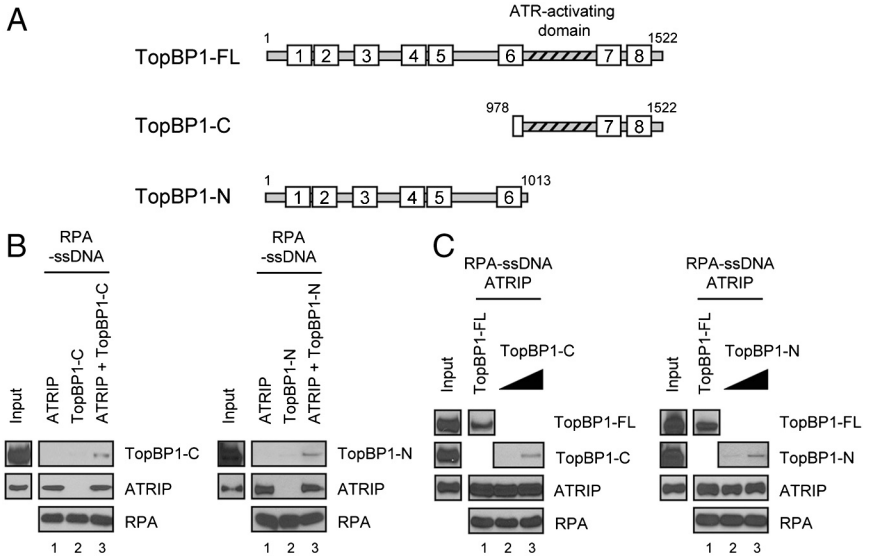

Fig. S6. Efficient recruitment of TopBP1 by ATRIP to RPA-ssDNA requires both the C- and N-terminal domains of TopBP1. (*A*) Schematic of full-length, the C terminus, and the N terminus of TopBP1. (*B*) ATRIP-dependent recruitment of TopBP1 fragments to RPA-ssDNA. DNA pull-down assays were carried out with biotinylated 80-mer oligonucleotide (1 pmol)-bound streptavidin beads, 3 pmol of TopBP1-C (left, lane 2 and 3) or TopBP1-N (right, lane 2 and 3), and 0 (lane 2) or 1 pmol (lane 1 and 3) of ATRIP. (*C*) Recruitment of full-length TopBP1 vs. TopBP1 fragments to RPA-ssDNA. DNA pull-down assays were performed as in (*B*), except with the C or N terminus of TopBP1 (1 or 3 pmol) compared with full-length TopBP1 (1 pmol).

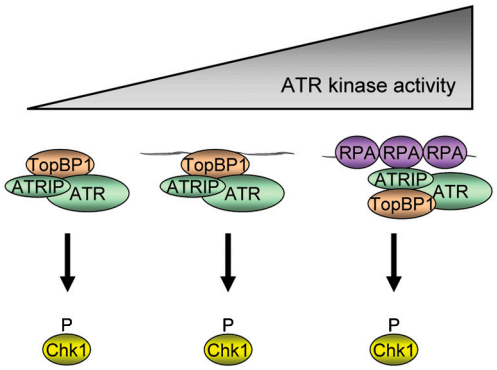

Fig. S7. TopBP1-dependent ATR activation under different reaction conditions. TopBP1 directly activates ATR kinase (left). Under more stringent conditions, TopBP1-dependent ATR kinase activity becomes dependent on DNA (middle), and RPA-ssDNA confers the most efficient stimulation of ATR by TopBP1 (right).

Table S1. Purification of human ATR-ATRIP from HeLa cells.

Fractions	Volume	Total protein	ATR*		Purification factor†	Yield	ATR Kinase activity‡
	mL	mg	%	μg		%	
1. WCE	66	528	0.05	264	1	100	1
2. 35% SAS	34	221	0.08	183	1.7	69.3	2.5
3. Phosphocellulose	15	15.7	0.23	35.4	4.5	13.4	3.8
4. Mono Q	4	0.4	1.89	7.5	37.8	2.8	41.4

*The percent ATR in whole cell extract (WCF) was taken from previous work (7). The percent in subsequent fractions is relative to that value and the total protein in the particular fraction.

†Purification factor was determined by the ratio of ATR (semiquantitative western) to the total amount of protein in the fraction.

‡The TopBP1-dependent Chk1 S345 phosphorylation (arbitrary number) divided by total amount of protein in the fraction.