Tethering DNA Damage Checkpoint Mediator Proteins
Topoisomerase IIβ-binding Protein 1 (TopBP1) and Claspin
to DNA Activates Ataxia-Telangiectasia Mutated and
RAD3-related (ATR) Phosphorylation of Checkpoint
Kinase 1 (Chk1)*

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The ataxia-telangiectasia mutated and RAD3-related (ATR) kinase initiates DNA damage signaling pathways in human cells after DNA damage such as that induced upon exposure to ultraviolet light by phosphorylating many effector proteins including the checkpoint kinase Chk1. The conventional view of ATR activation involves a universal signal consisting of genomic regions of replication protein A-covered single-stranded DNA. However, there are some indications that the ATR-mediated checkpoint can be activated by other mechanisms. Here, using the well defined Escherichia coli lac repressor/operator system, we have found that directly tethering the ATR activator topoisomerase IIβ-binding protein 1 (TopBP1) to DNA is sufficient to activate ATR phosphorylation of Chk1 in an in vitro system as well as in vivo in mammalian cells. In addition, we find synergistic activation of ATR phosphorylation of Chk1 when the mediator protein Claspin is also tethered to the DNA with TopBP1. Together, these findings indicate that crowding of checkpoint mediator proteins on DNA is sufficient to activate the ATR kinase.

DNA damage checkpoints delay cell cycle progression in response to DNA damage to maintain genomic integrity. In mammalian cells, checkpoint response signaling pathways are initiated primarily by two large phosphoinositide 3-kinase-related serine-threonine kinases, ataxia-telangiectasia mutated (ATM) and ATM and RAD3-related (ATR). ATM is activated mainly, but not exclusively, by double-stranded breaks, and ATR is activated by ultraviolet (UV) and UV-mimetic chemical agents as well as other conditions that result in replication fork stalling. Upon activation, ATR activates the key signal-transducing kinase Chk1 by phosphorylating it on Ser317 and Ser345 (1, 2), and the mediator protein Claspin is required for the efficient phosphorylation of Chk1 (3–5). ATR activation also requires topoisomerase IIβ-binding protein 1 (TopBP1), which has been shown to activate ATR directly in defined systems in vitro (6–11) as well as in vivo (6, 12).

The current model for ATR activation is as follows. Single-stranded DNA (ssDNA) generated at sites of DNA damage during repair, transcription, or replication is bound by replication protein A (RPA), which then recruits ATR through a physical interaction between RPA and the ATR-binding partner, ATR-interacting protein (ATRIP). Independently, Rad17-RFC loads the 9-1-1 (Rad9-Rad1-Hus1) checkpoint complex at primer/template junctions, where it recruits TopBP1 in the proximity of ATR. We have previously described an in vitro system that recapitulates ATR phosphorylation of Chk1 dependent on RPA-coated ssDNA and TopBP1 (8). However, RPA is not required for maximal ATR kinase activity in this system when the ssDNA is replaced with DNA containing bulky DNA base adducts (9, 10). In fact, we found that TopBP1 binds directly to damaged DNA and that the DNA binding activity of TopBP1 is required to confer damaged DNA-dependent activation of ATR. Thus, we hypothesized that TopBP1 may directly recognize damaged DNA in the cell to activate ATR. It is difficult to assess the direct contribution of DNA damage in the activation of ATR because of the large number of cellular processes that generate ssDNA when DNA damage is encountered (i.e. replication, transcription, and repair). Therefore, to address the question of whether direct binding of TopBP1 to DNA can activate ATR, we have tethered TopBP1 to DNA by fusing TopBP1 to the lac repressor (LacR), which has high binding affinity for DNA containing the lac operator (LacO) sequence. We find that in the presence of LacO DNA, LacR-TopBP1 activates ATR phosphorylation of Chk1 both in vitro and in vivo. In addition, when LacR-Claspin is also tethered to the DNA it functions synergistically with LacR-TopBP1. These findings indicate that concentrating TopBP1 and Claspin on DNA is sufficient for Chk1 phosphorylation by ATR.

EXPERIMENTAL PROCEDURES

Antibodies, Reagents, and DNA—Chk1 Ser(P)345 antibodies were purchased from Cell Signaling Technology (2348), TopBP1 antibodies were from Millipore (AB3245), RPA2 anti-

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2 The abbreviations used are: ATM, ataxia-telangiectasia mutated; ATR, ATM and RAD3-related; ATRIP, ATR-interacting protein; C, C-terminal; Chk1, checkpoint kinase 1; -FL, full-length; IPTG, isopropyl β-D-thiogalactopyranoside; kd, kinase-dead; LacO, lac operator; LacR, lac repressor; RPA, replication protein A; ssDNA, single-stranded DNA; TopBP1, topoisomerase IIβ-binding protein 1.

3 Ataxia-telangiectasia (ATX), breast cancer 1 susceptibility (BRCA1), breast cancer 2 susceptibility (BRCA2), checkpoint kinase 1; -FL, full-length; IPTG, isopropyl β-D-thiogalactopyranoside; kd, kinase-dead; LacO, lac operator; LacR, lac repressor; RPA, replication protein A; ssDNA, single-stranded DNA; TopBP1, topoisomerase IIβ-binding protein 1.

4 The abbreviations used are: ATM, ataxia-telangiectasia mutated; ATR, ATM and RAD3-related; ATRIP, ATR-interacting protein; C, C-terminal; Chk1, checkpoint kinase 1; -FL, full-length; IPTG, isopropyl β-D-thiogalactopyranoside; kd, kinase-dead; LacO, lac operator; LacR, lac repressor; RPA, replication protein A; ssDNA, single-stranded DNA; TopBP1, topoisomerase IIβ-binding protein 1.
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bodies were from Calbiochem (NA18), and Claspin (sc-48771) and Chk1 (sc-8408) antibodies were purchased from Santa Cruz Biotechnology. Isopropyl β-D-thiogalactopyranoside (IPTG) was purchased from Promega (V3955) and was dissolved in water. LacO plasmid is a ~15-kb plasmid that contains 256 Lac operator repeats cloned into pHBluescript (Addgene plasmid 17655, named Lac-I-SceI-Tet) (13). The control plasmid was selected because of its similar size of 14.15 kb and was generated by cloning the ATR gene into the pcDNA5-FRT/TO-LacI plasmid.

**Plasmid Construction**—The lac repressor sequence from pKM208 (14) (Addgene plasmid 13077) was the template for PCR with LacI forward oligonucleotide (5'-GGCCGAGATCT-ACACCTATGCGGATTATACGAATATGCG-3') and LacI reverse oligonucleotide (5'-GGCCGTCGACGGCGGGCGTGATCGAGCTCCACCTTCCTCTTCTTCTGGGTGCGGAAACCTGTGCTGGCCG-3') which was digested with restriction enzymes HindIII and XhoI and cloned into these sites of pcDNA5-FRT/TO-LacI plasmid.

**Kinase Assays**—The procedure was essentially as described previously (11). Briefly, kinase assay reactions contained 14 mM Hepes, pH 7.9, 30 mM KCl, 1.2 mM MgCl₂, 0.4 mM ATP, 0.3 mM DTT, 1% glycerol, and 1 μM microcin in a 10-μl final volume. Purified ATR-ATRIP (0.25 nM) was incubated in the reaction buffer for 20 min at 30 °C with 12 nM His-Chk1-kd and the indicated amounts of DNA and recombinant TopBP1 and Claspin. The reactions were terminated by the addition of SDS-PAGE loading buffer and separated by 10% SDS-PAGE. Chk1 phosphorylation was detected by immunoblotting using Ser(P)⁴⁴⁵ antibodies, and the levels of total Chk1 protein were subsequently detected by immunoblotting the same membrane. Levels of phosphorylation were quantified using ImageQuant 5.2 software after scanning the immunoblots. The highest level of Chk1 phosphorylation in each experiment was set equal to 100, and the levels of phosphorylated Chk1 in the other lanes were determined relative to this value. The averages from at least three independent experiments were graphed, and the error bars indicate the S.D.

**DNA Binding Assay**—LacO oligonucleotide 5'-TGTGGAA-TTGTTAGGCCTACAATTCCACA-3' or control oligonucleotide 5'-ACACCTAAACACTCGGAGTGGTTAAGGTGT-3' was 5' end-labeled with γ-³²P by polynucleotide kinase according to the manufacturer's instructions (New England Biolabs), self-annealed, and used in electrophoretic mobility shift assays (EMSA). The indicated amounts of LacR-TopBP1 or LacR-Claspin-C were preincubated for 90 min on ice with or without 150 μM IPTG (concentration in final reactions) and then further incubated for 30 min at room temperature with 10 nM labeled oligonucleotides in 0.5× TBE (25 mm Tris, 25 mm boric acid, 0.6 mm EDTA), 3% glycerol, and 1 μM DTT. The reactions were loaded directly onto a 5% w/v polyacrylamide (30:1 acrylamide: bisacrylamide) gel, cast, and run in 0.5× TBE. Electrophoresis was carried out for 60 min at 25 mA. After drying, the gel was analyzed by phosphorimaging.

**Cell Lines**—NIH2/4 (LacO array-containing NIH3T3 cells) (13) and NIH3T3 cells were cultured in DMEM-H supplemented with 10% fetal calf serum, penicillin/streptomycin, and L-glutamine. The NIH2/4 cells were also cultured with 100 μg/ml hygromycin. For transient expression of LacR fusion proteins, 1×10⁵ cells in 24-well plates were transfected with the indicated amount of plasmid using 1.25 μl of Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The cells were harvested 18 h after transfection by rinsing twice with 1× PBS and lysing with 50 μl of SDS sample buffer (62.5 mm Tris-HCl, pH 6.8, 2% w/v SDS, 10% glycerol, 50 mm DTT, 0.01% bromphenol blue). After brief sonication and boiling, 15 μl of the sample was loaded onto 10% SDS-PAGE for immunoblotting as described above.

**RESULTS**

Chk1 Phosphorylation by ATR Is Stimulated When TopBP1 Is Tethered to DNA in a Defined System—Because we have previously shown that DNA stimulates ATR kinase activity in a manner dependent on TopBP1 binding to DNA (9), we reasoned that when fused to LacR, TopBP1 should stimulate ATR kinase activity specifically when in the presence of LacO DNA even in the absence of DNA damage. To test this directly, we purified LacR-TopBP1 (Fig. 1A) and added it to kinase reactions where we can analyze the site-specific phosphorylation of Chk1 by ATR. Fig. 1B shows the results from adding increasing concentrations of LacR-TopBP1 to ATR kinase reactions without DNA (lanes 1–4), with LacO DNA (lanes 5–8), or with control DNA (lanes 9–12). LacR-TopBP1 stimulates ATR phosphorylation of Chk1 in reactions containing the LacO DNA up to 10-fold more than in reactions lacking DNA (compare lanes 8 and 4). The activity is specific for DNA containing the LacO sequence because an equal concentration of DNA lacking the LacO repeats does not stimulate ATR kinase activity (lanes 9–12). We observe the highest LacO sequence dependence at low concentrations of DNA, such as used in this experiment, where there is a negligible effect of adding DNA lacking LacO (see Fig. 3B for higher DNA concentrations). Because we have previously observed an effect of DNA length on the activation of ATR (10), it is important to note that the LacO-containing and control DNAs used in this study are of similar size (Fig. 1C). These data, along with our previous finding that recruitment of ATR to DNA by TopBP1 bound to bulky base adducts was sufficient for its activation, lead us to conclude that recruitment of ATR to DNA by whatever means is sufficient to activate it.
DNA-tethered TopBP1 and Claspin Synergistically Stimulate ATR Phosphorylation of Chk1 in a Defined System—

Consistent with data in vivo, we have previously reported that the phosphorylation of Chk1 by ATR is highly dependent on Claspin in vitro (11). Furthermore, a C-terminal fragment of Claspin is sufficient for its activity in vitro. We, therefore, purified the C-terminal fragment of Claspin fused to the LacR (Fig. 1A) to determine whether tethering Claspin to DNA would affect ATR phosphorylation of Chk1. Fig. 2A shows the results from adding LacR-Claspin to reactions with or without LacR-TopBP1 in the presence or absence of LacO-containing DNA. Under these more limiting reaction conditions we only observe significant ATR kinase activity in reactions containing LacR-TopBP1, LacR-Claspin, and LacO-containing DNA (lane 8). The limiting concentration of LacR-TopBP1 used in these reactions is insufficient to activate ATR even in the presence of LacO DNA (lane 5). Reactions lacking TopBP1 do not have significant Chk1 phosphorylation even in the presence of LacR-Claspin and LacO DNA (lane 11), suggesting that the dimerization of LacR is not simply bringing multiple DNAs together to activate ATR. These results indicate that LacR-Claspin works synergistically with LacR-TopBP1 in the ATR-Chk1 kinase reaction by recruiting the ATR substrate Chk1 in the presence of LacO-containing DNA and further reinforce the concept that ATR recruitment to DNA by any means is sufficient to activate its kinase function.

To measure the specific contribution of tethering Claspin to DNA to activation of ATR, we added LacR-Claspin to reactions containing TopBP1 fused to either GST or LacR (Fig. 2B). We observe 10-fold more Chk1 phosphorylation in reactions containing LacR-Claspin together with LacR-TopBP1 in the presence of LacO-containing DNA than in the presence of control DNA (compare lanes 4 and 11). However, in reactions containing LacR-Claspin together with GST-TopBP1 there is no significant difference in the kinase activity with or without DNA containing the LacO sequence (compare lanes 5–7 and 12–14). Note that a higher concentration of GST-TopBP1 was used in these reactions so that the signal would be above background. These results indicate that GST-TopBP1 does not function synergistically with LacR-Claspin and LacO DNA, suggesting that both checkpoint mediators must be co-localized on the DNA to stimulate ATR phosphorylation of Chk1 effectively.

Mutations in the Chk1 binding domain of Claspin abolish the ability of Claspin to mediate ATR phosphorylation of Chk1 in vivo (17) and in vitro (11, 18). We purified mutated LacR-Claspin (3A) (Fig. 1A) to test whether mutations in the Chk1 binding domain of LacR-Claspin (Thr916, Ser945, Ser982 to alanine) would disrupt its function in our defined system. Fig. 2C shows the results from adding increasing amounts of wild-type (WT) or mutant (3A) LacR-Claspin to reactions containing LacR-TopBP1 and either LacO or control DNA. We find that there is up to 10-fold more Chk1 phosphorylation in reactions contain-
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A, ATR kinase reactions contained no mediator proteins (lanes 1–3), 0.125 nM LacR-TopBP1 (lanes 4–6), 6 nM LacR-Claspin-C (lanes 10–12), or both (lanes 7–9). Reactions 1, 4, 7, and 10 contain no DNA (−), reactions 2, 5, 8, and 11 contain 3 pm LacO DNA (L), and reactions 3, 6, 9, and 12 contain 3 pm control DNA (C). B, GST-TopBP1 is not synergistic with LacR-Claspin, indicating that the mediators must co-localize to stimulate ATR effectively. ATR kinase reactions contained 6 nM LacR-Claspin and either 3 pm LacO DNA (lanes 1–7) or 3 pm control DNA (lanes 8–14), and either no TopBP1 (lanes 1 and 8), 0.0417, 0.125, or 0.375 nM LacR-TopBP1 (lanes 2–4 and 9–11), or 0.417, 1.25, or 3.75 nM GST-TopBP1 (lanes 5–7 and 12–14). C, mutations in the Chk1 binding domain of LacR-Claspin (Thr916, Ser945, Ser982 changed to alanine) abolish its ability to synergize with LacR-TopBP1 to activate ATR phosphorylation of Chk1. ATR kinase reactions contained 0.125 nM LacR-TopBP1, 3 pm LacO DNA (lanes 1–7), or 3 pm control DNA (lanes 8–14), and either no LacR-Claspin (lanes 1 and 8), 0.7, 2, or 6 nM LacR-Claspin-C WT (lanes 2–4 and 9–11), or mutant (3A, lanes 5–7 and 12–14).

FIGURE 2. TopBP1 and Claspin function synergistically to stimulate ATR phosphorylation of Chk1 when tethered to DNA in a defined system. A, ATR kinase reactions contained no mediator proteins (lanes 1–3), 0.125 nM LacR-TopBP1 (lanes 4–6), 6 nM LacR-Claspin-C (lanes 10–12), or both (lanes 7–9). Reactions 1, 4, 7, and 10 contain no DNA (−), reactions 2, 5, 8, and 11 contain 3 pm LacO DNA (L), and reactions 3, 6, 9, and 12 contain 3 pm control DNA (C). B, GST-TopBP1 is not synergistic with LacR-Claspin, indicating that the mediators must co-localize to stimulate ATR effectively. ATR kinase reactions contained 6 nM LacR-Claspin-C and either 3 pm LacO DNA (lanes 1–7) or 3 pm control DNA (lanes 8–14), and either no TopBP1 (lanes 1 and 8), 0.0417, 0.125, or 0.375 nM LacR-TopBP1 (lanes 2–4 and 9–11), or 0.417, 1.25, or 3.75 nM GST-TopBP1 (lanes 5–7 and 12–14). C, mutations in the Chk1 binding domain of LacR-Claspin (Thr916, Ser945, Ser982 changed to alanine) abolish its ability to synergize with LacR-TopBP1 to activate ATR phosphorylation of Chk1. ATR kinase reactions contained 0.125 nM LacR-TopBP1, 3 pm LacO DNA (lanes 1–7), or 3 pm control DNA (lanes 8–14), and either no LacR-Claspin (lanes 1 and 8), 0.7, 2, or 6 nM LacR-Claspin-C WT (lanes 2–4 and 9–11), or mutant (3A, lanes 5–7 and 12–14).
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Tethering Both TopBP1 and Claspin to DNA Results in Synergistic Induction of Chk1 Phosphorylation in Mammalian Cells—Because we observed synergistic effects of tethering both TopBP1 and Claspin to DNA in our in vitro system, we wanted to determine whether this synergy could be recapitulated in vivo. Fig. 4B shows the results from transfecting LacR-Claspin with or without LacR-TopBP1 in cells containing the LacO array or in cells without the array. Because transfecting LacO array-containing cells with sufficiently high concentrations of LacR-TopBP1-expressing plasmids activates ATR (Fig. 4A), we transfected a limited amount of LacR-TopBP1 so that there was no significant Chk1 phosphorylation in cells expressing LacR-TopBP1 alone (lane 3). However, co-expressing LacR-Claspin together with LacR-TopBP1 resulted in 5-fold more Chk1 phosphorylation than either alone (compare lane 4 with lanes 2 and 3). Importantly, this effect is only observed in cells containing the LacO array (lane 4) because we do not detect significant Chk1 phosphorylation when LacR-TopBP1 and LacR-Claspin are co-expressed in NIH3T3 control cells that do not have the LacO array (lane 9). Therefore, as was observed in vitro, crowding ATR on DNA with two different tethers that are also known to be an activator (TopBP1) and a mediator (Claspin) is sufficient to activate ATR phosphorylation of Chk1 in vivo.

When Tethered Together on DNA with TopBP1, the C Terminus of Claspin Is Sufficient for Synergistic Stimulation of Chk1 Phosphorylation, and Mutations Abrogate the Function—We have observed that a C-terminal fragment of Claspin (amino acids 679–1332) is sufficient for its function in our in vitro system and that mutations of three amino acids in the Chk1 binding domain of this fragment (Thr916, Ser945, and Ser982) abrogate its ability to mediate ATR phosphorylation of Chk1. We wished to test whether this fragment was also sufficient for the synergistic effect we observe in vitro and whether the mutated Claspin was functional. Fig. 4C shows the results from transfecting NIH2/4 LacO array-containing cells with full-length LacR-Claspin (-FL) or with the C-terminal fragment (-C), which is WT or mutated (3A), with or without LacR-TopBP1. We observe similar levels of Chk1 phosphorylation when either -FL or -C LacR-Claspin is co-transfected with LacR-TopBP1 (lanes 7 and 9). Importantly, the Chk1 phosphorylation is 3-fold more than with LacR-TopBP1 alone (lane 6) or without LacR-TopBP1 (lanes 2 and 4). However, the 3A mutations in the LacR-Claspin-FL (lane 8) or LacR-Claspin-C fragment (lane 10) diminishes the ability of Claspin to function synergistically with TopBP1 to induce Chk1 phosphorylation. Therefore, as is the case in vitro, the C terminus of Claspin is sufficient in vivo for synergistic stimulation of Chk1 phosphorylation when tethered to DNA with TopBP1, and mutations in the Chk1 binding domain of Claspin abrogate this function.

DISCUSSION

In Fig. 5 we present a summary model for the activation of Chk1 phosphorylation by ATR upon tethering an activator and mediator protein to DNA. Here, we report that tethering the checkpoint activator TopBP1 to DNA is sufficient to activate ATR phosphorylation of Chk1 both in vitro and in vivo. We have shown previously that the higher binding affinity of TopBP1 for damaged DNA results in specific activation of ATR

transfected LacR-TopBP1 (where the levels of LacR-TopBP1 are similar to endogenous levels of TopBP1), there is 5-fold more Chk1 phosphorylation than the control condition in which cells were transfected with empty vector (compare lanes 5 and 1). Therefore, DNA-tethered TopBP1 activates ATR phosphorylation of Chk1 in vivo, similar to what we have observed in vitro.
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A

Cells with LacO Array

LacR-TopBP1
Endo-TopBP1

α-TopBP1
α-P-Chk1
α-Chk1

B

Cells with LacO Array

LacR-TopBP1
LacR-Claspin-FL
HU

α-TopBP1
α-P-Chk1
α-Chk1

C

LacR-TopBP1
LacR-Claspin-FL
LacR-Claspin-C

α-Claspin
α-P-Chk1
α-Chk1
in the presence of damaged DNA (9). In the current report, we have bypassed the requirement for damaged DNA both in vitro and in vivo by fusing TopBP1 to LacR and using DNA containing LacR binding sites. The results from our in vivo studies are somewhat unexpected. It has been reported previously that overexpressing a fragment of TopBP1 containing the ATR-activating domain causes ATR activation in mammalian cells in the absence of DNA damage (6, 12). However, simply tethering the ATR activating domain of TopBP1 to chromatin by fusing it to histone H2B was not sufficient to induce Chk1 phosphorylation in DT40 chicken cells in the absence of DNA damage (20). In our study, LacR-TopBP1 is not overexpressed relative to endogenous TopBP1 (Fig. 4, A and B); however, it is highly concentrated on the chromatin because of the repeated LacO sequence. Thus, there may be a TopBP1 concentration threshold required for ATR activation. In fact, we do observe LacR-TopBP1 concentration-dependent activation of Chk1 both in vitro (Fig. 1B) and in vivo (Fig. 4A). We have found that even though tethering the mediator Claspin to DNA is insufficient to activate ATR phosphorylation of Chk1 either in vitro or in vivo, tethering Claspin to the DNA together with TopBP1 functions synergistically to activate ATR phosphorylation of Chk1 both in vitro and in vivo. Claspin increases the affinity of ATR for Chk1 (11). Mutations in the Chk1 binding domain of Claspin abrogate the phosphorylation of Chk1 by ATR (17, 18, 21, 22). Here, we report that the same mutations also abrogate the ability of Claspin to function when tethered to DNA both in vitro and in vivo. Interestingly, we find that the C-terminal half of Claspin is sufficient for the synergistic activation of ATR phosphorylation of Chk1 both in vitro and in vivo. It is known that the N-terminal half of Claspin has DNA binding activity (23) and is involved in protein-protein interactions required for chromatin association (24); yet, these requirements are bypassed when Claspin is tethered to the DNA.

The lac repressor/operator system has previously been used to study ATM activation in mammalian cells (25) and activation of the ATR homolog, Mec1, in yeast cells (26). Tethering individual mediators in the ATM pathway such as members of the MRN complex (Mre11-Rad50-Nbs1) or Mdc1 was sufficient to activate ATM in the absence of DNA damage (25). In yeast, however, checkpoint activation was observed when a member of the 9-1-1 complex was tethered together with the Mec1-binding partner Ddc2, but not when either was tethered alone (26). Here, we show that in mammalian cells, simply tethering the activator of ATR is sufficient to induce the checkpoint response and that the effect is amplified when the mediator Claspin is also tethered. Taken together, these studies illustrate the generality that activation of the cellular DNA damage response pathways does not require DNA damage but can be
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by stable association of checkpoint proteins with chromatin. For the ATR pathway, there are many potential mechanisms for achieving stable chromatin association of the checkpoint proteins, such as by RPA-coated ssDNA, which, through interactions with ATRIP (27) and the checkpoint mediator protein Tipin (28), bring ATR and Claspin into close proximity to one another to facilitate Chk1 phosphorylation. Similarly, interactions between ATR and DNA repair factors (29–32) as well as direct binding of checkpoint proteins to DNA structures such as branched DNA (23) or DNA-containing bulky adducts (9, 33), may also facilitate activation of the ATR pathway.

In conclusion, we have found that tethering TopBP1 to DNA is sufficient to activate ATR phosphorylation of Chk1 both in vitro and in vivo and that when Claspin is also tethered to the DNA there is a synergistic effect on ATR activation. Our findings taken together with the in vivo studies with recruiting ATM by tethering in human cells and recruiting Mecl by tethering in Saccharomyces cerevisiae, bring into focus that recruitment of phosphoinositide 3-kinase-related serine-threonine kinases members to chromatin by whatever means is sufficient to institute checkpoint signaling. We believe this realization will help define more generally the mechanism by which checkpoint signaling is activated.

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