MOLECULAR AND CELLULAR BIOLOGY, Aug. 2006, p. 6056–6064 Vol. 26, No. 16
0270-7306/06/$08.00+0 doi:10.1128/MCB.00492-06

Claspin Operates Downstream of TopBP1 To Direct ATR Signaling towards Chk1 Activation‡

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Received 21 March 2006/Returned for modification 28 April 2006/Accepted 2 June 2006

TopBP1 and Claspin are adaptor proteins that facilitate phosphorylation of Chk1 by the ATR kinase in response to genotoxic stress. Despite their established requirement for Chk1 activation, the exact way in which TopBP1 and Claspin control Chk1 phosphorylation remains unclear. We show that TopBP1 tightly colocalizes with ATR in distinct nuclear subcompartments generated by DNA damage. Although depletion of TopBP1 by RNA interference (RNAi) strongly impaired phosphorylation of multiple ATR targets, including Chk1, Nbs1, Smc1, and H2AX, it did not interfere with ATR assembly at the sites of DNA damage. These findings challenge the current concept of ATR activation by recruitment to damaged DNA. In contrast, Claspin, like Chk1, remained distributed throughout the nucleus both before and after DNA damage. Consistently, the RNAi-mediated ablation of Claspin selectively abrogated ATR's ability to phosphorylate Chk1 but not other ATR targets. In addition, downregulation of Claspin mimicked Chk1 inactivation by inducing spontaneous DNA damage. Finally, we show that TopBP1 is required for the DNA damage-induced interaction between Claspin and Chk1. Together, these results suggest that while TopBP1 is a general regulator of ATR, Claspin operates downstream of TopBP1 to selectively regulate the Chk1-controlled branch of the genotoxic stress response.

In response to DNA damage or replication stalling, cells activate genome surveillance pathways that cooperate to preserve genomic integrity (50). One such pathway is the ATR signaling cascade. In this pathway, ATR phosphorylates and activates a number of downstream targets that coordinate cell cycle progression with DNA repair. One of the best-studied ATR substrates is Chk1, a kinase that amplifies ATR signaling and directs it to the desired cell cycle and DNA repair effectors. Chk1 is phosphorylated by ATR on serines 317 and 345 in a DNA damage-dependent manner. These phosphorylations are critical for Chk1 activation (49) but may also play other roles, such as promoting Chk1 dissociation form chromatin (34) or restraining Chk1 export from cell nuclei (17). Once activated, Chk1 induces degradation of Cdc25A (8, 18, 29), followed by inhibition of cyclin-dependent kinases (Cdk) and cell cycle delay (32). Such a rapid and reversible cell cycle arrest is believed to be essential to provide time for efficient DNA repair. Besides having an important role in the cellular response to exogenous DNA damage, the ATR-Chk1-Cdc25A-Cdk pathway has a well-established role in the unperturbed cell cycle. Thus, disruption of either the ATR or the Chk1 gene is embryonically lethal in mice (12, 42). On the cellular level, small interfering RNA (siRNA)- or drug-mediated inhibition of Chk1 activity also leads to unscheduled DNA replication resulting in massive DNA damage and phosphorylation of ATR targets (40). Thus, the ATR-Chk1 pathway appears essential to monitor the fidelity of the replication process.

The term "checkpoint mediators" is collectively applied to proteins that support timely and effective signaling by the upstream DNA damage-activated kinases (37). Specifically, it has been proposed that checkpoint mediators assist in promoting interactions between ATR/ATM and their substrates and/or aid the retention of critical factors in close proximity to the actual DNA lesions. As the ATM and ATR pathways differ in a number of genetic and spatiotemporal aspects, they have different checkpoint mediators assigned to them. Thus, the DNA damage checkpoint mediators Mdc1, 53BP1, and BRCA1 seem to be largely linked to the ATM pathway (39), whereas TopBP1 and Claspin, together with the newly identified microcephalin, have been proposed to coregulate the ATR pathway (14, 22, 45).

TopBP1 contains eight BRCA1 C-terminal (BRCT) repeats, the phosphate-binding interaction modules that can be found in many checkpoint mediators. Though TopBP1 was initially identified as a DNA topoisomerase IIβ-binding protein (hence its name) (46), its involvement in the DNA damage response was soon established (30). Thus, TopBP1 was shown to be instrumental for efficient Chk1 phosphorylation by ATR, a role which is conserved in its yeast orthologs (Rad4/Cut5 in fission yeast [*Schizosaccharomyces pombe*] and Dpb11 in budding yeast [*Saccharomyces cerevisiae*], respectively) (14). In mammalian cells, TopBP1 forms ionizing radiation (IR)-induced foci, which have been reported to be dependent on the integrity of its fifth BRCT domain (47). Besides its role in the DNA damage response, other functions of TopBP1 have been proposed. Most notably, TopBP1 has been linked to repression of E2F1-induced apoptosis (24, 25) and to regulation of normal S phase (19).

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‡ Supplemental material for this article may be found at http://mcb.asm.org/.

Claspin was identified as a Chk1-interacting protein in *Xenopus laevis* (20). Although there is a large body of evidence that Claspin is an important regulator of Chk1 phosphorylation in vertebrates (9), there are no apparent structural homologues in yeast. However, Mrc1 has been proposed as a functional equivalent of Claspin in budding and fission yeast through its ability to contribute to activation of Rad53 and Cds1, respectively, in response to replication stress (2, 43). The mechanism by which Claspin assists ATR in Chk1 phosphorylation has been documented best with *Xenopus* extracts (21) and partly confirmed to be conserved in humans (11). Thus, upon DNA damage, Claspin and Chk1 form a complex, dependent on phosphorylation of at least two highly conserved sites (Thr-916 and Ser-945 in human Claspin). Although the motifs surrounding these phosphorylation sites do not resemble the ATR consensus sites, their phosphorylation requires, at least in *Xenopus*, active ATR. Thus, it seems that ATR activates a hitherto-unknown kinase, required to promote Claspin-Chk1 complex formation and Chk1 phosphorylation. Studies of the molecular details underlying the physical association between Claspin and Chk1 revealed that Claspin binds directly to the kinase domain of Chk1 and that the affinity of the two proteins for each other declines following phosphorylation of Chk1 by ATR (16). Given the evidence that human Claspin interacts also with ATR (10), this is consistent with a model in which Claspin recruits Chk1 to ATR, only to disengage once the phosphorylation and activation of Chk1 have taken place.

One important issue that is not completely understood is why both TopBP1 and Claspin are required to mediate Chk1 phosphorylation and how these proteins cooperate in doing so. Additionally, it is not known to which extent these proteins regulate additional ATR substrates. Here, we set out to investigate the overlapping versus unique roles of TopBP1 and Claspin in the DNA damage response and the mechanism of how these two proteins converge on regulating Chk1 activity.

MATERIALS AND METHODS

Plasmids and siRNA. The expression plasmid for Venus-PCNA was generated by inserting PCR-amplified PCNA cDNA in frame with yellow fluorescent protein-Venus in pcDNA3.1 (provided by A. Miyawaki). Wild-type and kinase-dead versions of FLAG-tagged Chk1 in pCI-Neo, as well as a mutant version of this construct with serines 317 and 345 changed to alanine (Chk1-2A), were previously described (35). Inducible short hairpin RNA (shRNA) expression vectors targeting TopBP1 and Claspin were generated by annealing oligonucleotides and ligating the fragments into BglII/HindIII-digested pSUPERIOR (OligoEngine). For the TopBP1 targeting vector, the oligonucleotides corresponded to the target sequence (5′-CCTGAAGAAACCTATTTTG-3′). For the Claspin targeting vector, the oligonucleotides corresponded to the target sequence (5′-GCA ATGAAACTCCGAAGGT-3′).

siRNA was used to transiently downregulate the expression of TopBP1 (5′-AGACCUUAAUGUAUCAGUA-3′) and Claspin (5′-GCACAUACAU GAUAAAGAA-3′). For control, a previously described siRNA against HSP70B was used (7).

Antibodies. Antibodies used in this study included phosphospecific rabbit polyclonal antibodies against Chk1 S317, Chk1 S345, Nbs1 S343, and Smc1 S966 (all from Cell Signaling), total TopBP1 (Abcam), Claspin (Bethyl and Abcam), Chk1 (Santa Cruz), and 53BP1 (Santa Cruz) and goat polyclonal antibodies against Mcm6 (Santa Cruz). In addition, mouse monoclonal antibodies against γ-H2AX (Upstate), FLAG (clone M2; Sigma), Chk1 (DCS-316), Cdk7 (MO-7), Mcm7 (DCS-141), and the p32 subunit of RPA (Neomarkers) and a rat monoclonal antibody against bromodeoxyuridine (BrdU) (OBT0030CX; Immunologicals Direct) were used.

Cell culture and DNA-damaging treatments. Human U-2-OS-derived cell lines were cultivated in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum and penicillin-streptomycin. In some experiments, cells were incubated in the presence of 10 mM caffeine (Sigma). For live-cell imaging, cells were grown in CO_2-independent medium (Invitrogen) in Lab-Tek microscope glass chambers (Nalge Nunc International). Transfection of siRNAs was performed with Oligofectamine (Invitrogen). For stable and transient DNA transfections, we used FuGene 6 (Roche) according to the manufacturer's instructions. For generation of stable cell lines with inducible expression of shRNA, naïve U-2-OS cells were transfected with shRNA vector and a plasmid encoding the tetracycline repressor for 24 h and selected with puromycin (1 μg/ml; Sigma) and blasticidin (5 μg/ml; InvivoGen). Expression of the shRNA was achieved by adding 2 μg/ml of doxycycline (BD Biosciences) to the culture medium. The U-2-OS cell line stably expressing green fluorescent protein (GFP)-ATR was described previously (15).

Cells were exposed to ionizing radiation, as described previously (41), by an X-ray generator (Pantak HF160; 150 kV, 15 mA, dose rate of 2.18 Gy/min). Exposure to UV light was performed with a Stratalinker 1800 (Stratagene). Prior to exposure, the medium was removed, and the cells were washed with phosphate-buffered saline. To induce replication fork stalling, 2 mM hydroxyurea (HU) (Sigma) was added to the culture medium for 1 h. Laser microirradiation was performed essentially as described previously (26, 27). Briefly, cells were grown in the presence of 10 μM BrdU for 24 h to sensitize the cells to the double-stranded break (DSB)-generating insult. After shifting the cells to CO_2-independent medium (see above), approximately 200 cells were microirradiated with a 337-nm UV-A laser.

Immunochemical techniques. Cells were lysed in a buffer containing 20 mM Tris, 150 mM NaCl, 1 mM EDTA, and 0.5% NP-40 supplemented with inhibitors. For immunoprecipitation, lysates containing 1 to 1.5 mg protein were first precleared with 40 μl of a 50% protein G-Sepharose slurry for 30 min. Second, the supernatants were incubated with 2 μg of monoclonal FLAG antibody for 1 h, followed by protein G-Sepharose for another hour. Subsequently, the beads were isolated and washed four times. Immunoprecipitates as well as lysates were subjected to immunoblotting as described previously (13). For detection of immunoprecipitated FLAG-Chk1, horseradish peroxidase-coupled protein A (Amersham) was used as a secondary reagent to circumvent cross-reaction between the Chk1 signal and the FLAG antibody heavy chain. For immunofluorescence, cells were grown on glass coverslips (Menzel), fixed in 4% paraformaldehyde (15 min at room temperature), and permeabilized in 0.2% Triton X-100. The cells were immunostained with the indicated primary antibodies and secondary antibodies coupled to Alexa dyes with excitation wavelengths of 488, 568, and 647 nm (Molecular Probes). Where indicated, cells were counterstained with the DNA intercalating dye ToPro3 (Molecular Probes) before being mounted on glass slides (Menzel). Images were acquired through a PLAN-Neofluar 40×/1.3 oil immersion objective (Carl Zeiss) by use of an LSM510 confocal laser scanning microscope (Carl Zeiss).

Flow cytometry. For flow cytometric analysis of cellular DNA content, cells were harvested by trypsinization and fixed in 70% ethanol. Upon permeabilization in 0.25% Triton X-100, the DNA was stained by incubation with propidium iodide (0.1 mg/ml in phosphate-buffered saline) containing RNase for 30 min at 37°C. The samples were analyzed with a FACSCalibur flow cytometer (BD Biosciences) using CellQuest software.

RESULTS

Generation of RNA interference-based knockdown models. To study the roles of TopBP1 and Claspin in the DNA damage response, we designed shRNA constructs to conditionally reduce the levels of the respective proteins in living mammalian cells in a doxycycline-dependent manner (see Materials and Methods). This approach has the advantage of ensuring near-identical genetic backgrounds between different knockdown and control cell lines. To validate these models, we first examined the kinetics and efficiency of the shRNA-mediated downregulation of TopBP1 and Claspin by inducing the respective shRNAs for up to 4 days. Western blotting of lysates prepared from the indicated time points revealed a rapid and quantitative Claspin downregulation, while TopBP1 depletion occurred with somewhat slower kinetics (see Fig. S1A in the supplemental material), reaching a nadir around 72 h after shRNA induction. This analysis also revealed that the two proteins did

not regulate each other in terms of expression levels or nuclear localization (see Fig. S1A in the supplemental material; also data not shown). To address the immediate effects of downregulation of TopBP1 and Claspin on the cell cycle, we monitored the cell cycle distributions by flow cytometry during 4 days after shRNA induction. The shTopBP1 cell line showed no detectable changes in cell cycle distribution over the course of 4 days of shRNA induction. However, downregulation of Claspin was accompanied by some degree of cell death and accumulation in S phase at later time points (3 days after shRNA induction) (see Fig. S2A in the supplemental material). Despite these long-term adverse effects of Claspin removal, the earlier time points (when the downregulation was already maximal) were not associated with any detectable effects in cell cycle distribution. We also addressed whether TopBP1-deficient cells showed any sign of replication stress by a BrdU incorporation assay. We consistently observed replication patterns reminiscent of cells in early, middle, and late S phase (36) in both TopBP1-proficient and -deficient cells, and the relative proportions of BrdU-positive cells were indistinguishable between the two cell populations (see Fig. S2B in the supplemental material). Consequently, 72 h of doxycycline induction for the shTopBP1 cell line and 24 h for the shClaspin cell line were used for most experiments in this study.

Impact of TopBP1 and Claspin on DNA damage-induced, ATR-mediated phosphorylations. To further characterize our experimental models, we determined the impact of TopBP1 and Claspin downregulation on Chk1 phosphorylation. First, we induced the respective shRNAs in the cell lines and assayed the dynamics of Chk1 phosphorylation on serine 317 in a time course after exposure of the cells to a low dose of UV-C light (λ = 254 nm, 10 J/m^2), the known inducer of the ATR-Chk1 pathway. In both cell lines, we observed a strong attenuation of serine 317 phosphorylation following shRNA induction (see Fig. S1B and S1C in the supplemental material). Throughout the study, we consistently observed a stronger effect of TopBP1 downregulation, despite the fact that the degree of its downregulation was similar or even slightly less efficient than that of Claspin.

Next, we addressed whether other ATR substrates are regulated by TopBP1 and Claspin by assaying the phosphorylation statuses of several proteins known to function downstream of ATR. We exposed the knockdown cell lines to UV (10 J/m^2) and the replication inhibitor HU (2 mM) and assayed the phosphorylation statuses of Chk1, Nbs1, and Smc1 by Western blotting with phosphospecific antibodies 1 h after these treatments. Consistently, we observed a strong attenuation of Chk1 phosphorylations (on both serine 317 and serine 345) in both cell lines, accompanied by an almost-complete abrogation of the shift in Chk1 mobility (Fig. 1A). Strikingly, while phosphorylations of Nbs1 on S343 and Smc1 on S966 were clearly attenuated in the TopBP1-depleted cells, downregulation of Claspin had no effect (Fig. 1A). Thus, these data suggest that while the ATR-mediated phosphorylations of Chk1 and other substrates, such as Nbs1 and Smc1, requires TopBP1, Claspin selectively regulates the ATR-Chk1 cross talk.

ATM and ATR share many substrates, but the potential of these kinases to become activated relies on the type of DNA damage (1, 33). ATR is the main kinase to be activated as a result of replication stress. ATM is the main kinase responsible for phosphorylation of most downstream substrates after IR, which creates DNA double-stranded breaks. However, ATR also becomes activated by DSB-generating insults, albeit with a delay, caused by the time required for resection of DSBs and generation of stretches of single-stranded DNA (ssDNA) (15, 51). We therefore tested whether and how TopBP1 and Claspin contribute to Chk1 phosphorylation after IR. When we exposed the knockdown cell lines to a low dose of IR (2 Gy), we observed equally robust phosphorylations of Nbs1 and Smc1 in both Claspin- and TopBP1-depleted cells (Fig. 1A). Thus, the ability of ATM to phosphorylate these substrates is not dependent on TopBP1. However, phosphorylation of Chk1 (on both serine 317 and serine 345) clearly was dependent on both TopBP1 and Claspin (Fig. 1A). This is consistent with the emerging view that ATR is the only kinase that can phosphorylate Chk1 on these residues, regardless of the type of DNA-damaging insults (15), and suggests that both TopBP1 and Claspin are involved in this process.

The three major phosphatidylinositol 3-kinase-like kinases ATM, ATR, and DNA-dependent protein kinase have the ability to phosphorylate the C-terminal tail of the histone variant H2AX on serine 139. Whereas this phosphorylation is carried out exclusively by ATM or DNA-dependent protein kinase after ionizing irradiation (38), ATR is responsible for the reaction in response to replication stress (44). Replication stress occurs when replication forks stall, for example, due to their collision with DNA lesions (after UV). Indeed, in UV-irradiated cells stably expressing PCNA fused with the yellow fluorescent protein variant Venus, phosphorylation of H2AX was evident only in replicating cells. High-resolution confocal imaging revealed that the UV-generated γ-H2AX signal tightly colocalized with the PCNA foci (see Fig. S3A in the supplemental material). Thus, the replication stress-induced signaling represented here by γ-H2AX is generated by ATR at the sites of stalled replication forks. To address whether H2AX phosphorylation by ATR also needs the mediator functions of TopBP1, we exposed our knockdown cell lines to UV irradiation and assayed the ability of the cells to phosphorylate H2AX by Western blotting. Indeed, we found that TopBP1-depleted cells were severely impaired in H2AX phosphorylation compared to control cells. Importantly, although Claspin downregulation by itself induced some γ-H2AX (see also the following sections), it did not interfere with the ability of ATR to phosphorylate this target in response to UV-induced DNA damage (Fig. 1B). This analysis adds H2AX to the list of ATR substrates whose phosphorylation requires TopBP1 but is independent of Claspin.

Since both TopBP1 and Claspin impact Chk1 phosphorylation, we decided to compare their relative contributions to this process. We used siRNA to deplete the two proteins either individually or in combination and assayed the phosphorylation status of Chk1 1 h after exposure of the cells to 10 J/m^2 of UV (Fig. 1C; see also Fig. S4 in the supplemental material). By this approach, we again found the TopBP1-depleted cells to display the most severe defect in Chk1 phosphorylation. In addition, we consistently observed no further effect of Claspin downregulation on Chk1 phosphorylation in cells already depleted of TopBP1. Thus, it appears that Claspin operates downstream of TopBP1 on a common pathway designed to target Chk1 for ATR-mediated phosphorylation.

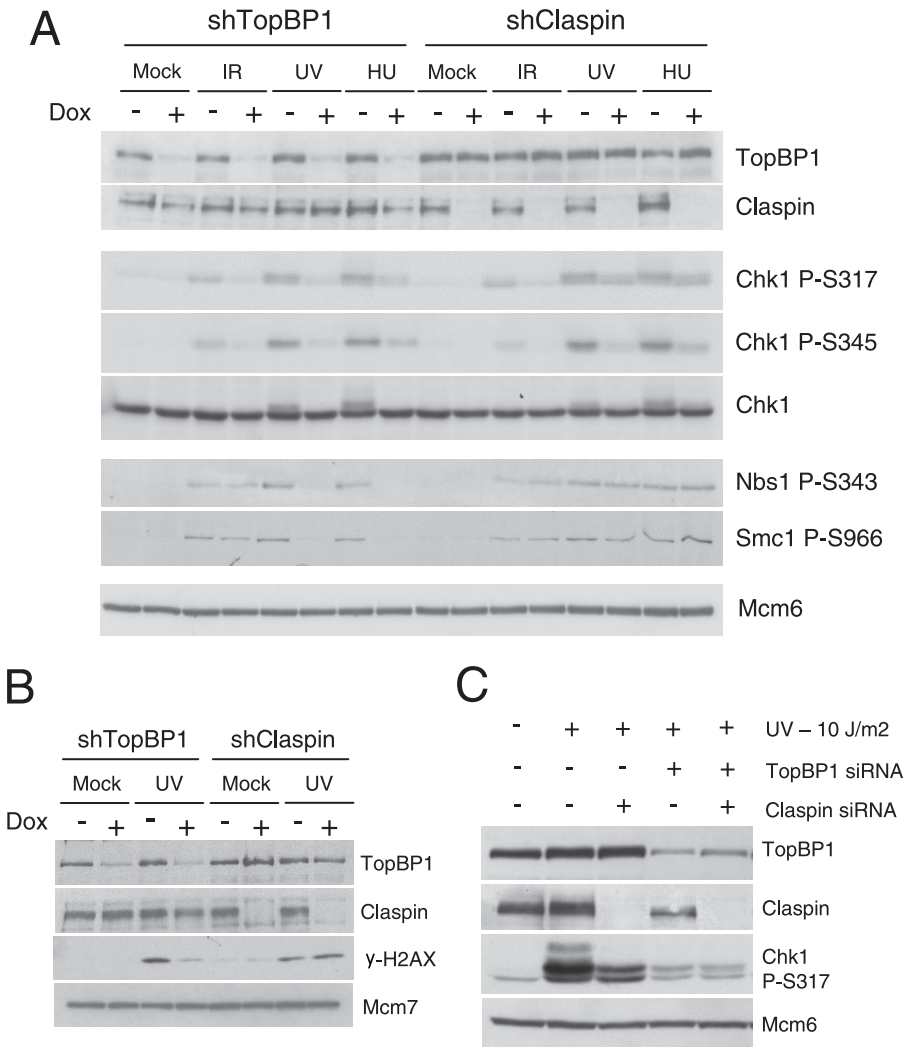

FIG. 1. Impact of TopBP1 and Claspin on ATR- and ATM-mediated phosphorylations. (A) Cells conditionally expressing shRNA to TopBP1 or Claspin were incubated (+) or not incubated (−) with doxycycline (Dox) and treated or not treated with 2 Gy IR, 10 J/m^2 of UV, or 2 mM HU. One hour after these treatments, cells were harvested and lysed. Total and phosphorylated (P) proteins were analyzed by immunoblotting with the indicated antibodies. (B) shTopBP1 and shClaspin cells were induced as described for panel A and treated or not treated with 10 J/m^2 of UV. Lysates were prepared 1 h later, and the efficiency of knockdown as well as the extent of H2AX phosphorylation was assayed by immunoblotting. (C) U-2-OS cells were transfected for 48 h with siRNAs targeting TopBP1 and Claspin, either individually or in combination, as indicated. The remaining dishes were transfected with control siRNA. One hour after exposure to 10 J/m^2 of UV, cells were lysed and the efficiency of knockdown and the extent of Chk1 phosphorylation were assayed by immunoblotting.

The spatial properties of TopBP1 and Claspin recapitulate those of ATR and Chk1, respectively. We employed local laser microirradiation (26, 27) combined with immunostaining to dissect the patterns of TopBP1 and Claspin localization after DNA damage. We recently used a similar approach to describe the existence of several distinct spatial compartments generated by laser as well as IR (6). One of these compartments is represented by the relatively large chromatin regions marked by phosphorylated H2AX, to which ATM and its associated mediators (e.g., Mdc1, 53BP1, and BRCA1) bind. The other, and significantly smaller, compartment is delineated by single-stranded DNA, the resection to which is allowed only during S and G_2 phases of the cell cycle (6, 15). The latter microcompartment harbors proteins involved in homologous recombination repair, ATR, and proteins involved in ATR-mediated signaling (e.g., Rad17 and Rad9). Finally, some checkpoint proteins interact with DNA lesions only transiently (without cytologically discernible accumulation) and remain homogenously distributed in the nucleus throughout the duration of the checkpoint response. Importantly, the latter group also includes Chk1 (6). When we stained microirradiated cells with Claspin antibodies, no enrichment of Claspin could be detected along the laser tracks, despite clear H2AX phosphorylation within these regions (Fig. 2A). Similar results were obtained after exposing the cells to IR (data not shown). Thus, Chk1 and Claspin cluster to the same spatial category, consistent with Claspin being a key mediator specifically linked to Chk1. In a parallel experiment, we microirradiated cells stably expressing GFP-ATR (15) and stained for endogenous TopBP1 and phosphorylated H2AX. This approach revealed TopBP1 to tightly colocalize with

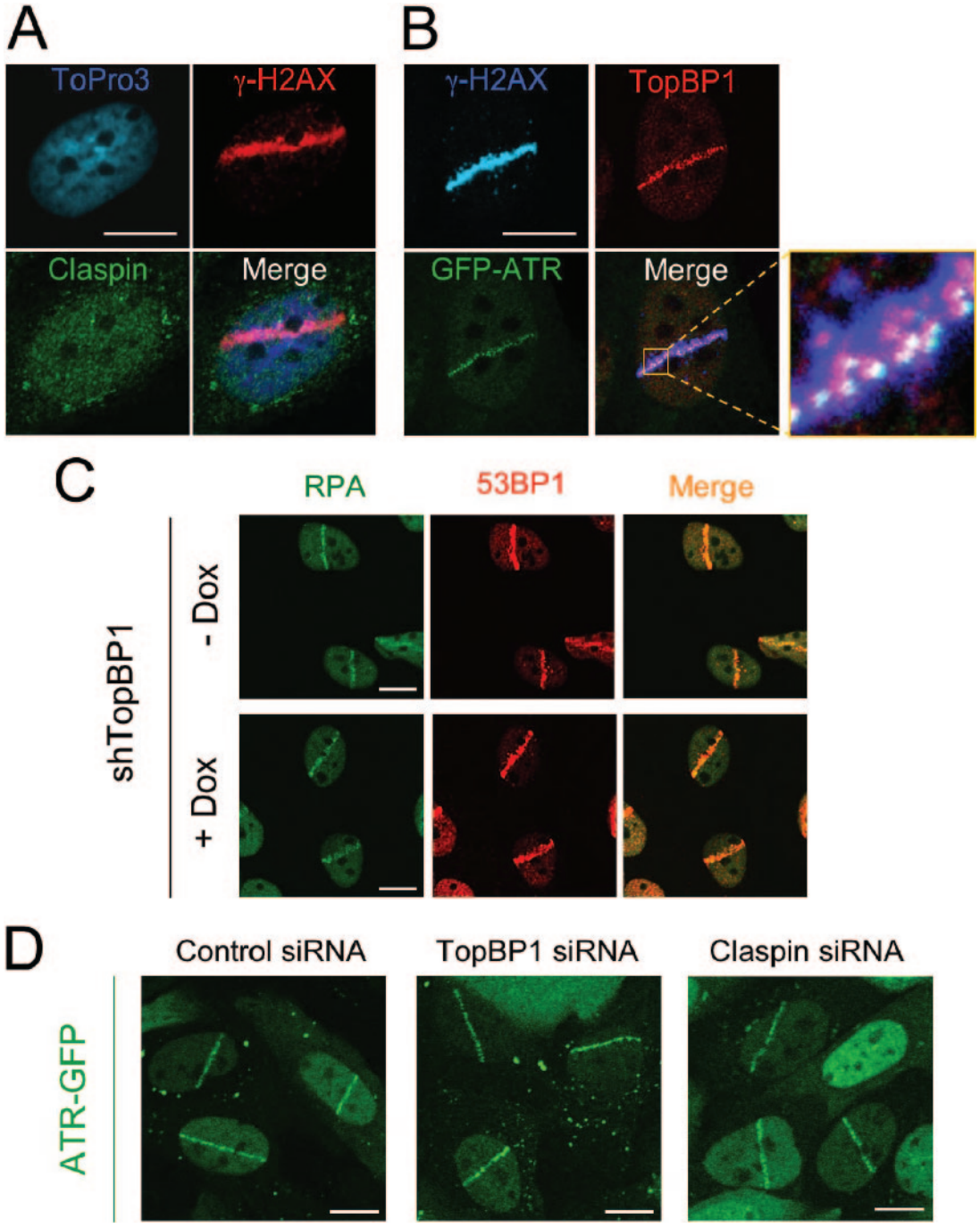

FIG. 2. Redistribution of ATR, TopBP1, and Claspin in response to laser-generated DNA damage. (A) U-2-OS cells were grown on glass coverslips and incubated in the presence of 10 μM BrdU for 24 h. One hour after exposure to UV-A laser (see Materials and Methods), cells were fixed and immunostained for endogenous Claspin and γ-H2AX. In addition, the nuclear DNA was counterstained with ToPro3. (B) U-2-OS cells stably expressing GFP-ATR were treated as described for panel A and immunostained for endogenous TopBP1 and γ-H2AX. (C) shTopBP1 cells were incubated (+) or not incubated (−) for 72 h with doxycycline (Dox), treated as described for panel A, and immunostained with antibodies towards RPA and 53BP1 (the latter protein was used as a marker of DNA damage). (D) U-2-OS cells stably expressing GFP-ATR were transfected with control siRNA for 72 h, siRNA targeting TopBP1 for 72 h, or siRNA targeting Claspin for 24 h. BrdU was added to the cultures for the last 24 h. A representative field of cells from each culture was microirradiated as described for panel A, and the ability of GFP-ATR to accumulate at sites of DNA damage was assayed by confocal microscopy. Bar = 10 μm.

GFP-ATR in discrete microfoci, within a larger area of γ-H2AX-modified chromatin (Fig. 2B). Thus, TopBP1 shows the same spatial behavior as ATR, consistent with a tight functional link between these two proteins.

We next asked whether TopBP1 or Claspin was required for ATR accumulation into nuclear foci. To this end, we first microirradiated uninduced and induced shTopBP1 cells and assayed, by immunostaining, the ability of the RPA subunit p32

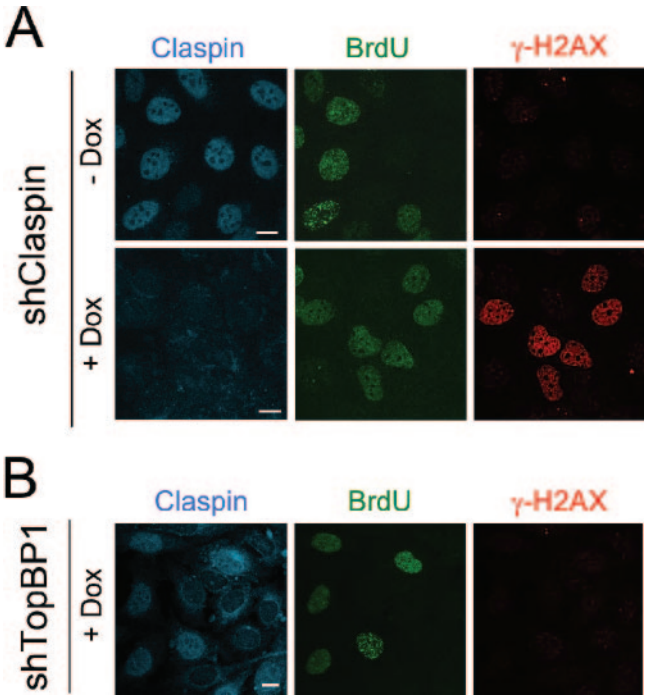

FIG. 3. Downregulation of Claspin, but not that of TopBP1, triggers H2AX phosphorylation in replicating cells. (A) Cells conditionally expressing Claspin-targeting shRNA were grown on glass coverslips in the presence (+) or absence (−) of doxycycline (Dox) for 48 h. Subsequently, BrdU (25 μM) was added to the medium for 1 h to label S-phase cells. After fixation, the coverslips were treated with DNase to expose the incorporated BrdU and stained with antibodies towards Claspin, BrdU, and γ-H2AX. (B) shTopBP1 cells were treated as described for panel A, except that they were treated with doxycycline for 72 h. Bar = 10 μm.

to decorate single-stranded DNA in DSB-containing laser tracks (Fig. 2C). We consistently observed that the TopBP1-depleted cells were proficient in generating RPA-coated ssDNA compartments, a key step required for the recruitment of ATR to the sites of DNA damage. Second, we depleted TopBP1 or Claspin from GFP-ATR-expressing cells by siRNA and assayed the ability of GFP-ATR to assemble into microirradiated tracks (Fig. 2D). Depletion of either protein did not prevent GFP-ATR from accumulating at the sites of DNA damage, despite the fact that these treatments impaired the ability of ATR to phosphorylate Chk1 (Fig. 1C; see also Fig. S4 in the supplemental material) (Fig. 1C also shows the effectiveness of TopBP1 downregulation by siRNA). Thus, although mammalian TopBP1 is required for multiple ATR-dependent phosphorylations, it does not appear to regulate ATR on the level of its recruitment to ssDNA.

Removal of Claspin, but not TopBP1, confers a phenotype reminiscent of that achieved by Chk1 inhibition. We noted that a subset of Claspin-deficient cells showed elevated phosphorylation of H2AX, even though these cells were not exposed to any external DNA damage (Fig. 3A). This was reminiscent of the consequences of chemical inhibition of Chk1, which leads to unscheduled replication and activation of ATR (40). Although the intensity of the γ-H2AX response in Claspin-depleted cells was weaker than after Chk1 inhibition or exposure to exogenous DNA damage, it was significant, reproducible, and clearly elevated over the background level detected in Claspin-proficient cells. By coimmunostaining with BrdU, we found that the γ-H2AX signal was invariably restricted to S-phase cells (Fig. 3A). Besides γ-H2AX, we noticed moderately increased phosphorylation of other DNA damage-regulated substrates, such as p53, Smc1, and Nbs1 (data not shown). Importantly, we did not observe any sign of elevated ATR activity in S-phase cells depleted of TopBP1 (Fig. 3B), despite the previous evidence that TopBP1 is required for Chk1 function. Only after a substantial increase of the detection sensitivity on our microscopes were we able to detect a slightly elevated incidence of γ-H2AX foci in the TopBP1-deficient cells (see Fig. S3B in the supplemental material). However, this γ-H2AX response was much weaker than that generated by Claspin depletion (Fig. 3A) and it likely reflects increased incidence of double-stranded breaks followed by ATM activation, as recently described (19). Thus, despite the fact that TopBP1-deficient cells likely suffered replication stress and/or DNA damage, the lack of the ATR response indicated that TopBP1 regulates a general step in ATR activation and/or that it mediates ATR interactions with a broader range of substrates. In contrast, Claspin emerged from these experiments as a specific mediator of Chk1 activation.

TopBP1 regulates Claspin's ability to interact with Chk1. Activation of Claspin includes its phosphorylation by ATR and at least one additional kinase activated by DNA damage. This activation is required for interaction between Claspin and Chk1 and subsequent activation of Chk1 (10, 21). To further dissect the roles of TopBP1 in Chk1 activation, we also assayed whether TopBP1 regulates the ability of Claspin to physically interact with Chk1. First, by immunoprecipitating transiently transfected FLAG-tagged Chk1 from U-2-OS lysates, we confirmed that Claspin and Chk1 interact specifically after DNA damage (in this case, UV) and that this could be abrogated by caffeine, an inhibitor of ATR (Fig. 4A). This result supports previously published data from both human cells and *Xenopus* extracts (10, 21). In addition, we found that the two major ATR phosphorylation sites in Chk1 (S317 and S345 [49]) were dispensable for the DNA damage-induced association between Claspin and Chk1 (Fig. 4B). In several experiments, we observed a slight decrease in the amount of coimmunoprecipitated Claspin upon transfection of the kinase-dead version of Chk1 (Fig. 4B) or after treatment with UCN-01 (not shown). This is probably due to the fact that the region of Chk1 recognized by activated Claspin locates to its kinase domain (16). To test whether TopBP1 also contributes to the formation of productive Chk1-Claspin complexes, we induced the TopBP1 shRNA, transfected the cells with wild-type FLAG-Chk1 for another 24 h, and exposed the cultures to UV (25 J/m^2). Western blotting of the FLAG immunoprecipitates revealed a strong decrease in the Chk1-Claspin association in the TopBP1-depleted cells (Fig. 4C). This result indicated that TopBP1 indeed impacts not only the direct phosphorylation of Chk1 on S317 and S345 but also the ability to form productive Claspin-Chk1 complexes. These data are consistent with the previous finding that ATR promotes Claspin-Chk1 interaction (21), further corroborate these results by showing that TopBP1 is directly involved in this process, and place Claspin downstream of TopBP1 in the DNA damage response.

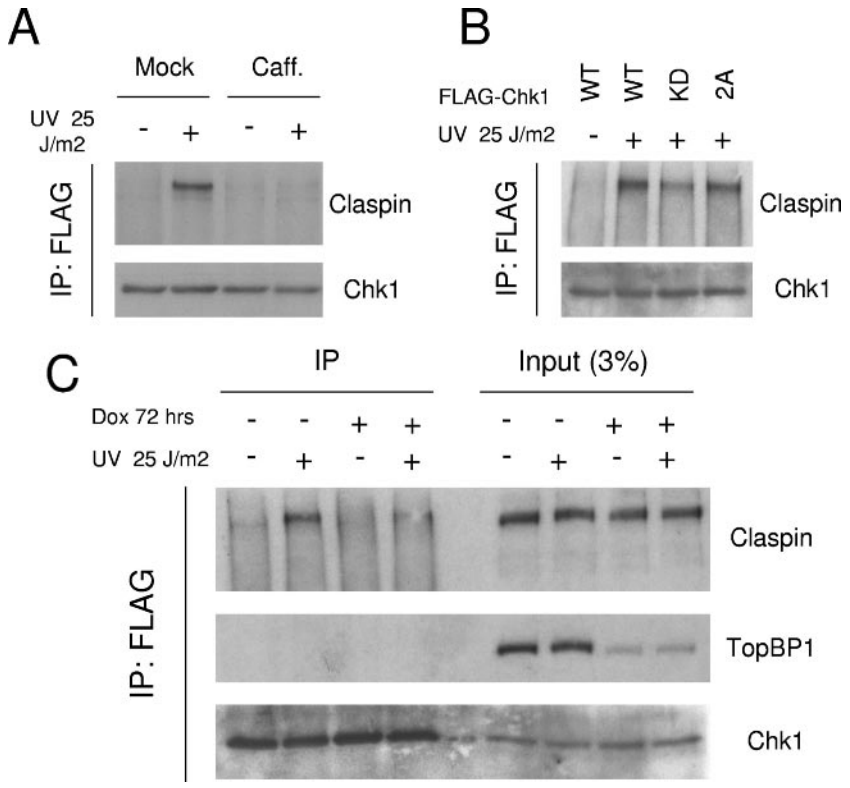

FIG. 4. DNA damage-induced interaction between Claspin and Chk1 depends upon TopBP1. (A) U-2-OS cells were transiently transfected with FLAG-Chk1 expression plasmid, incubated in the presence or absence of 10 mM caffeine (Caff.) for 1 h, and exposed (+) or not exposed (−) to 25 J/m^2 of UV. Cells were lysed after 1 h and immunoprecipitated (IP) with monoclonal FLAG antibody. The levels of Chk1 and Claspin in the immunoprecipitates were assayed by immunoblotting. (B) U-2-OS cells were transiently transfected with wild-type (WT), kinase-dead (KD), or S317A/S345A (2A) versions of FLAG-tagged Chk1, as indicated, and exposed to 25 J/m^2 of UV. FLAG immunoprecipitates were immunoblotted for Claspin and Chk1. (C) shTopBP1 cells were incubated or not incubated with doxycycline (Dox) for 72 h and transfected with wild-type FLAG-Chk1 construct. FLAG immunoprecipitates and lysates used for immunoprecipitation were immunoblotted for the indicated proteins.

DISCUSSION

Given its central importance in preserving genome integrity, the ATR-Chk1 pathway is subject to several layers of regulation. Tight control of Chk1 activity is critical to impose timely delay in cell cycle progression after DNA damage (5) and to support the physiological pace of the unperturbed cell cycle (42). Here, we elucidated how TopBP1 and Claspin cooperate in regulating the ability of ATR to phosphorylate Chk1. We found that TopBP1 tightly colocalizes with ATR on RPA-coated ssDNA generated after resection of chromosome breaks. In addition, ATR-mediated phosphorylation of Chk1, Smc1, Nbs1, and H2AX required TopBP1. Conversely, Claspin possessed the same spatial properties as Chk1 (pan-nuclear distribution after local DNA damage), a finding that likely reflects the unique capacity of Claspin to selectively regulate ATR-mediated Chk1 phosphorylation. Additionally, we found that Claspin is required for Chk1 activity also during the unperturbed cell cycle by showing that Claspin-deficient cells suffer replication problems reminiscent of those generated by Chk1 inhibitors (Fig. 3A) (40). Notably, we did not observe a similar response in TopBP1-downregulated cells, despite the fact that depletion of TopBP1 causes replication errors and inhibition of basal Chk1 activity to a similar extent as Claspin downregulation (reference 19 and the present study).

When combined with the published results, our results led us to propose a model for the respective functions of TopBP1 and Claspin in the ATR-controlled genome surveillance pathway (Fig. 5). In this model, TopBP1 regulates most (if not all) ATR phosphorylations after DNA damage or replication stalling. Claspin, on the other hand, is assigned exclusively to link ATR with Chk1. Although we were limited to investigate a larger array of substrates by the availability of suitable reagents (phosphospecific antibodies), we suspect that the list of ATR-mediated phosphorylations that require TopBP1 is in fact much longer. Such a scenario is also supported by a recent publication describing the requirement of TopBP1 for ATR-mediated phosphorylation of Rad1 and Hus1 (28). Consistent with the notion that TopBP1 contributes to ATR activation, our results provide evidence that TopBP1 operates upstream of Claspin in a common pathway leading to Chk1 activation (Fig. 5). This is supported by our findings that Chk1 phosphorylation was impaired more severely in TopBP1-depleted cells than in Claspin-depleted cells. In addition, Chk1 inhibition in TopBP1-deficient cells was already maximal and was not further downregulated by concomitant Claspin ablation. Furthermore, we show that TopBP1 is required for DNA damage-induced Claspin-Chk1 complex formation. Collectively, our present data are consistent with TopBP1 being an essential

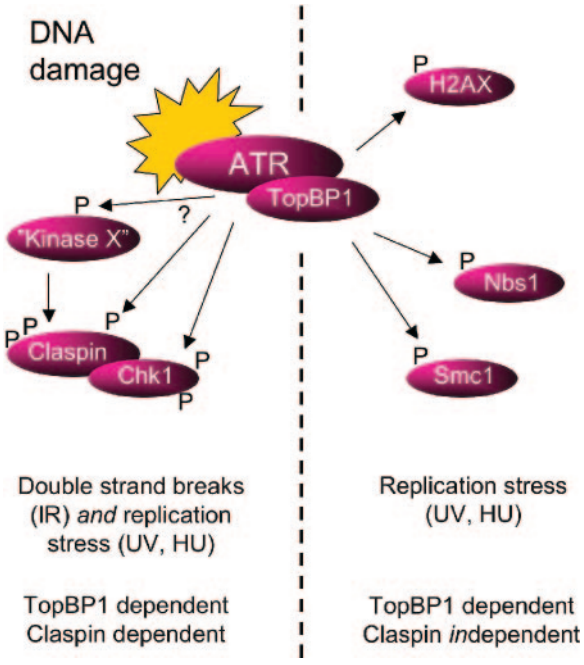

FIG. 5. Roles of TopBP1 and Claspin in the ATR-mediated DNA damage response. TopBP1 directly assists ATR in the phosphorylation of numerous downstream targets at sites of DNA damage, including Chk1, Nbs1, Smc1, and H2AX. Claspin, on the other hand, adds another layer of control in channeling ATR signaling to Chk1. Additionally, TopBP1 regulates Chk1 not only by directly stimulating its phosphorylation by ATR but also by facilitating activation of Claspin and potentiating its ability to bind Chk1.

component of the ATR activation machinery and place TopBP1 upstream of Claspin in the DNA damage response. After completion of this study, Kumagai and coworkers reported that TopBP1 directly activates ATR both in vitro and in vivo (23). Our data support and further corroborate this concept. Most notably, we show that Chk1 stands out among other ATR targets in its requirement for both TopBP1 and Claspin for its activation. Such a dual requirement could be important to separate Chk1 activation from other ATR substrates in certain biological settings and to provide more flexibility for a dynamic adjustment of Chk1 activity.

In this and earlier papers, we studied the intranuclear redistribution of checkpoint and repair proteins in response to DNA damage (6, 7, 27). During these studies, the Chk1 and Chk2 kinases emerged as unique proteins capable of transmitting the DNA damage signal from the site of DNA damage to the rest of the nucleus. Clearly, their very high mobility and a lack of physical retention at the sites of DNA damage are important features required for the efficient signaling to pannuclear checkpoint and/or repair effectors. An important question is how these kinases manage to recognize the sites of damage. Our results suggest that Claspin could be an important determinant to mark damaged DNA or stalled replication forks for a transient immobilization and activation of Chk1. Claspin has been reported to interact with ATR, both in the presence and in the absence of DNA damage (10). Such an interaction is very dynamic (based on our preliminary data that Claspin is a highly mobile protein both before and after DNA damage); yet, it might be sufficient to transiently immobilize Chk1 at the site of DNA damage and thereby physically link Chk1 with the catalytic machinery (containing ATR, ATRIP, and TopBP1) required for its phosphorylation and activation.

Another important question is how TopBP1 exerts its tight control over ATR phosphorylation events. Until now, the prevailing model was that ATR might be constitutively active and that the key regulatory step in firing the ATR response is its physical recruitment to the sites of DNA damage or replication forks where (in the context of adaptor proteins and other auxiliary factors) it can physically bind its substrates (1). However, our finding that TopBP1 deficiency strongly impairs ATR's ability to phosphorylate its substrates without impairing its relocalization to the sites of DNA damage (Fig. 2C and D) challenges this view. In fact, our results indicate that ATR is subject to several layers of regulation and that local accumulation of ATR at the sites of DNA damage is not sufficient for or synonymous with its activation. Interestingly, two recent studies on the ATR-ATRIP complex support this view. First, Ball and colleagues reported an ATRIP mutant deficient in binding RPA, which precluded sustained retention of ATR in nuclear foci. Cells containing this ATRIP mutant were still competent to support Chk1 activation (4). The same group constructed a hybrid of the ATRIP protein by exchanging its native coiled-coil domain with a heterologous dimerization domain from the transcription factor GCN4. When expressed in an ATRIP-deficient background, this mutant supported ATR localization to sites of DNA damage but not Chk1 phosphorylation (3). In the *Xenopus* system, TopBP1 has been reported to be required for binding of ATR, Rad1, and DNA polymerase α to genotoxin-damaged chromatin (31). We cannot exclude that human TopBP1 may also cooperate with other factors to stabilize ATR retention in the vicinity of DNA lesions. However, our in vivo data (Fig. 2D) suggest that, contrary to the *Xenopus* system, this may not be an essential function of TopBP1 in mammals. Indeed, results presented in Fig. 1C and 2D and Fig. S4 in the supplemental material show that it is possible to reduce the level of TopBP1 to an extent that abrogates ATR signaling to the downstream effectors but allows its productive assembly at the sites of DNA damage. Thus, while ATR retention might well be the rate-limiting step for ATR activity, it does not seem to be sufficient to trigger efficient signaling to the downstream effectors.

We propose that the essential trigger to activate ATR is provided by TopBP1. TopBP1 could act on the level of ATR activation, a notion supported by a recent report by Kumagai and colleagues showing that a conserved domain of TopBP1 (distinct from the BRCT repeats) binds ATR and strongly stimulates its kinase activity (23). In addition, TopBP1 can further promote ATR signaling by acting as an assembly factor for substrates at sites of DNA damage and replication stress. This view is supported by another recent report describing the requirement for *Xenopus* TopBP1 in mediating ATR phosphorylation of Chk1 bound to already-activated Claspin (48). With its numerous BRCT domains, TopBP1 is well suited for such a role. In conclusion, the present report identifies TopBP1 as a general regulator of ATR and provides evidence that Claspin generates an additional regulatory layer operating downstream of TopBP1 and connecting ATR signaling specifically with the Chk1-controlled branch of the genotoxic stress response.

ACKNOWLEDGMENTS

We thank A. Miyawaki and Claus Storgaard Sorensen for reagents.

This work was supported by grants from the Danish Cancer Society (DP 03 035), the Danish National Research Foundation, the Danish Medical Research Council, the China Scholarship Council, the European Science Foundation (EuroDYNA 21-04-280), the European Union (DNA repair), and the John and Birthe Meyer Foundation.

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