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Direct Role for the Replication Protein Treslin (Ticrr) in the ATR Kinase-mediated Checkpoint Response*

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Background: Treslin/Ticrr plays an important role in the initiation of DNA replication.
Results: Treslin/Ticrr stimulates ATR phosphorylation of Chk1 both *in vitro* and *in vivo*.
Conclusion: Treslin/Ticrr is a dual replication/checkpoint protein that directly participates in the ATR-mediated checkpoint.
Significance: Identification of the essential components of the ATR checkpoint response is crucial for understanding this important signal transduction pathway.

TopBP1 (topoisomerase IIβ-binding protein 1) is a dual replication/checkpoint protein. Treslin/Ticrr, an essential replication protein, was discovered as a binding partner for TopBP1 and also in a genetic screen for checkpoint regulators in zebrafish. Treslin is phosphorylated by CDK2/cyclin E in a cell cycle-dependent manner, and its phosphorylation state dictates its interaction with TopBP1. The role of Treslin in the initiation of DNA replication has been partially elucidated; however, its role in the checkpoint response remained elusive. In this study, we show that Treslin stimulates ATR phosphorylation of Chk1 both *in vitro* and *in vivo* in a TopBP1-dependent manner. Moreover, we show that the phosphorylation state of Treslin at Ser-1000 is important for its checkpoint activity. Overall, our results indicate that, like TopBP1, Treslin is a dual replication/checkpoint protein that directly participates in ATR-mediated checkpoint signaling.

In eukaryotic cells, the duplication of the genome involves the orchestrated assembly of proteins onto origins of replication (1). During G_1, the origin recognition complex recognizes origins of replication and binds Cdc6 and Cdt1, which facilitates the loading of the Mcm2-7 helicase complex onto DNA to form the pre-replication complex (2). The loaded helicase is activated by CDK (cyclin-dependent kinase) upon entry into S phase, and additional proteins, including TopBP1 (topoisomerase IIβ-binding protein 1), Cdc45, and the GINS complex, are recruited at this phase (3–5). Once activated, the bidirectional replisomes unwind DNA around the origins of replication to create a template for DNA polymerases to replicate the DNA.

DNA damage checkpoints delay cell cycle progression when damage is detected and thus ensure the fidelity of genome propagation. Several DNA damage checkpoints exist, such as the G_1/S checkpoint, the G_2/M checkpoint, and the replication checkpoint (which includes the intra-S and S/M checkpoints). Although the different checkpoints are distinct, the damage sensor molecules that activate the various checkpoints appear to be shared by all (6). In particular, the G_2/M checkpoint blocks mitotic entry when DNA is damaged by exposure to UV light or ionizing radiation in G_2 phase. The replication checkpoint (S/M) prevents the cell from prematurely entering mitosis before it has fully replicated its genome (7). Abrogation of the S/M pathway allows cells to enter mitosis with incompletely replicated chromosomes (8). ATR (ataxia telangiectasia-mutated and RAD3-related) is required for the UV light-induced G_2/M and S/M checkpoints (6). Once activated, ATR phosphorylates and activates the Chk1 (checkpoint kinase 1) kinase, which in turn inhibits CDK activation. Thus, ATR activation blocks cell cycle progression.

During checkpoint responses, TopBP1 serves as a direct activator of ATR (9). TopBP1 contains multiple BRCT motifs (nine in humans), which mediate protein-protein interactions, and pairs of BRCT domains act as phosphopeptide-binding motifs (10, 11). In addition to its checkpoint function, TopBP1 is also a replication protein (12). Studies with conditional and separation-of-function mutants show that the checkpoint function of TopBP1 can be separated from its replication function (13, 14). The N-terminal part of TopBP1 was shown to be both necessary and sufficient for DNA replication, whereas domains in the C-terminal half of TopBP1 are involved in the DNA damage and replication stress response (13–15). Specifically, a region between BRCT domains 6 and 7 of human TopBP1, which is conserved in *Xenopus* and yeast, has been shown to be important for ATR-dependent phosphorylation of Chk1 and is thus called the ATR activation domain (AAD)[2] (9, 16–21).

A genetic screen for G_2/M checkpoint regulators in zebrafish identified *ticrr* (TopBP1-interacting, checkpoint, and replication regulator) as a novel gene required for DNA replication (11). Ticrr, also called Treslin (TopBP1-interacting replication-stimulating protein), was also discovered as a binding partner for TopBP1 in *Xenopus* egg extracts (15). Treslin acts in collaboration with TopBP1 at an early step in replication to facilitate the loading of Cdc45 onto chromatin (15). Phosphorylation of a

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[2] The abbreviations used are: AAD, ATR activation domain; FL, full-length; FR, fragment.

single conserved site in Treslin (Ser-1000) by CDK/cyclin regulates both its binding to TopBP1 and its ability to promote DNA replication (22, 23).

Data from the Ticrr study in zebrafish indicated that Ticrr-deficient cells enter mitosis with partially replicated DNA and also fail to arrest in mitosis in response to ionizing radiation (11). These data suggested a possible role for Ticrr in checkpoint regulation. However, there was no direct evidence that Ticrr participated in the checkpoint response, and the G/M checkpoint screen that identified Ticrr did not eliminate the possibility that the observed effect was a secondary consequence of the replication function of Ticrr (11).

In this study, we demonstrate that human Treslin/Ticrr plays a direct role in the checkpoint response. We show that Treslin activates ATR-mediated and TopBP1-dependent Chk1 phosphorylation both *in vitro* in a defined system and *in vivo*. Furthermore, we show that the phosphorylation state of Treslin and its binding to TopBP1 are important for this activation.

EXPERIMENTAL PROCEDURES

Materials—The oligonucleotides used in this study were synthesized by Integrated DNA Technologies. Anti-FLAG M2 affinity gel (catalog no. A2220) and FLAG peptide (catalog no. F4799) were purchased from Sigma-Aldrich. Anti-phospho-Chk1 (Ser-345) antibody was purchased from Cell Signaling (rabbit mAb 2348), anti-TopBP1 antibody was from Millipore (catalog no. AB3245), anti-Treslin antibody was from Bethyl Laboratories (catalog no. A303-472A), and anti-Chk1 antibody was purchased from Santa Cruz Biotechnology (catalog no. sc-8408).

Plasmid Construction—Plasmid containing full-length (FL) human Treslin (pcDNA5TO-FL-Treslin) was a gift from Dr. W. Dunphy (15). This plasmid was the template for PCR with the following Treslin fragment (FR) oligonucleotides: forward, 5′-GCGATCCGTGATATCGGATCCGCCACCATGGCTCCT-CAGCAGCCTTCCCAGCCAGTGAAAG-3′; and reverse, 5′-ACGCATGCGGCTAGCTTGTCATCGTCGTCCTTGT-AATCCATGAAGGCTGCTGCTCTCGGAGGTGTTCTG-AGAG-3′. The PCR product was digested with restriction enzymes EcoRV and NheI and cloned into these sites of the backbone vector pcDNA5TO-3Myc-NLS, obtained from the original plasmid (pcDNA5TO-FL-Treslin). As a result, plasmid pcDNA5TO-Treslin-FR-FLAG-3Myc-NLS was obtained, which expresses amino acids 893–1257 of Treslin, which, together with the tags, result in a 428-amino acid fragment with a mass of ~48 kDa. Treslin-FR was also cloned into the NcoI/XhoI sites of the pET28a(+) vector for expression in *Escherichia coli*. Constructs expressing FL-TopBP1 and its derived fragments were reported previously (18). Plasmid expressing LacR-FL-TopBP1 was constructed earlier in our laboratory (24). Constructs for expressing Treslin-FR-S1000A, FL-Treslin-S1000A and LacR-FL-TopBP1-W1145R were produced by PCR using standard molecular biology techniques. All plasmid sequences were verified by sequencing at the Genome Analysis Facility of the University of North Carolina at Chapel Hill.

Purification of Treslin Proteins—HEK293T cells in four 150-mm plates (~90% confluency) were transfected with the Treslin-FR-expressing plasmids using FuGENE HD (Promega). Cells were harvested after 24 h, washed with PBS, and lysed in lysis buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1 mM PMSF, and protease inhibitor mixture). Treslin was purified by immunoaffinity chromatography on FLAG-agarose resin (Sigma) according to the manufacturer's recommendations and using the column format. However, we included a 12-column volume high salt wash step with TBS500 (50 mM Tris buffer (pH 7.4) and 500 mM NaCl) to specifically remove endogenous TopBP1 that might bind to Treslin. Subsequently, the column was equilibrated with TBS150 (50 mM Tris buffer (pH 7.4) and 150 mM NaCl) before competitively eluting with FLAG peptides. DTT (5 mM) and glycerol (10%) were finally added, and aliquots were stored at −80 °C. Treslin-FR was also purified from *E. coli* cells by immunoaffinity chromatography on FLAG-agarose resin according to the manufacturer's recommendations.

Purification of Checkpoint Proteins and Protein Binding Assays—Native ATR-ATRIP (ATR-interacting protein), GST-TopBP1, and His-FLAG-tagged kinase-dead Chk1 were purified as described previously (16, 25, 26). Plasmids expressing FLAG-tagged Treslin-FR were transfected into HEK293T cells and harvested 24 h later. FLAG-tagged proteins were bound to agarose beads, washed with TBS150, washed with TBS500 to remove endogenous TopBP1 bound to Treslin, and then equilibrated back with TBS150. GST-tagged FL-TopBP1 or fragments were then added to the beads and rotated for 2 h at 4 °C. Beads were washed three times with TBS150 and eluted with SDS-PAGE sample buffer.

Kinase Assay—The kinase assay used was essentially the same as described previously (27). Reactions contained 14 mM HEPES (pH 7.9), 30 mM KCl, 1.2 mM $MgCl_2$, 0.4 mM ATP, 0.3 mM DTT, and 1 μM microcystin 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-FLAG-tagged kinase-dead Chk1 and the indicated amounts of Treslin and TopBP1. The reactions were terminated by the addition of SDS-PAGE sample buffer and separated by SDS-PAGE. Chk1 phosphorylation was detected by immunoblotting using anti-phospho-Chk1 (Ser-345) antibodies, and the levels of total Chk1 protein were subsequently detected by immunoblotting the same membrane. Levels of phosphorylation were quantified using ImageQuant 5.0 software (Molecular Dynamics) 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 three independent experiments were graphed.

Cell Lines—HEK293T cells were from our laboratory collection. NIH2/4 (NIH3T3 cells containing LacO arrays) were described previously (28). NIH2/4 cells were cultured in DMEM with 10% FBS, penicillin/streptomycin, and 100 μg/ml hygromycin. For transient expression of LacR fusion proteins, 3×10^5 cells in 6-well plates were transfected with the indicated amount of plasmid using 6.5 μl of Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The cells were harvested 18 h after transfection, rinsed with PBS, and lysed with SDS-PAGE sample buffer. After brief sonication and

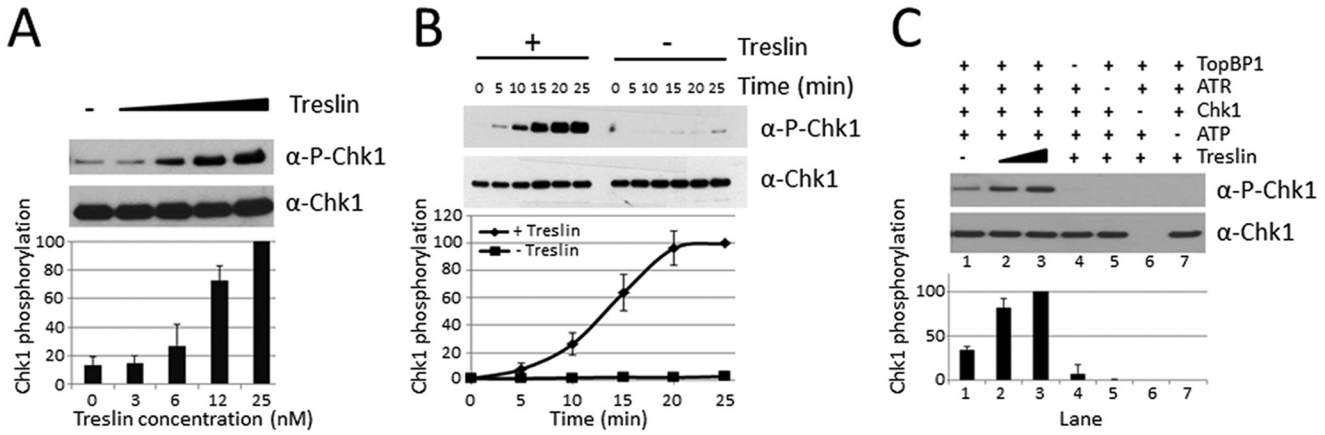

FIGURE 1. **Treslin stimulates Chk1 phosphorylation by ATR in a defined system.** *A*, Treslin-dependent phosphorylation of human Chk1 *in vitro*. Purified Treslin-FR (3, 6, 12, and 25 nM) was added to kinase reactions containing TopBP1, Chk1, and ATR as described under "Experimental Procedures." Blots for anti-phospho-Chk1 (Ser-345) antibody (*α-P-Chk1*; *upper panel*) and anti-Chk1 antibody (*lower panel*) are displayed. Phospho-Chk1 bands were quantified using ImageQuant 5.0 software and are plotted relative to the highest intensity band. *B*, kinetics of Chk1 phosphorylation in the presence and absence of Treslin-FR. Samples were taken from a master mixture every 5 min, mixed immediately with SDS-PAGE loading buffer, and boiled. Phospho-Chk1 and Chk1 were blotted and quantified as described for *A*. *C*, requirement for both TopBP1 and Treslin-FR for Chk1 phosphorylation by ATR. At its highest concentration in *lane 3*, the Treslin concentration was maintained for the remaining lanes at 25 nM (*lanes 4–7*). *Error bars* indicate S.D.

boiling, samples were loaded onto SDS-polyacrylamide gel for immunoblotting as described above.

RESULTS

Chk1 Phosphorylation by ATR Is Stimulated by Treslin in a Defined ATR Checkpoint System—Although an unbiased genetic screen identified Treslin as a novel replication/checkpoint regulator (11), and biochemical studies have discovered an interaction of Treslin with TopBP1 (15, 22), the essential role of Treslin and TopBP1 in DNA replication initiation has prevented the analysis of Treslin function in checkpoint signaling. To further investigate this potential role, we decided to test Treslin in a defined *in vitro* ATR kinase assay developed in our laboratory. We chose a Treslin fragment (amino acids 893–1257) previously reported to interact strongly with TopBP1 (23). We first expressed and purified Treslin from HEK293T cells and then added it to an ATR kinase assay containing human Chk1 as a substrate. The result is displayed in Fig. 1*A* and shows stimulation of Chk1 phosphorylation by Treslin. The increase in Chk1 phosphorylation was dose-dependent and resulted in up to 6-fold stimulation. A time course experiment shown in Fig. 1*B* reveals significant stimulation in the rate of Chk1 phosphorylation when Treslin was added to the kinase reaction.

Stimulation of ATR-mediated Chk1 Phosphorylation by Treslin Is TopBP1-dependent—To test the contribution of each of the reaction components in our defined Chk1 phosphorylation assay, we carried out the experiment in Fig. 1*C*, in which individual components were successively eliminated while maintaining a high Treslin concentration. Our results show that omitting TopBP1 or ATR from the kinase reaction abolished Chk1 phosphorylation under our reaction conditions (compare *lanes 4* and *5* with *lane 3*). These results indicate that stimulation of Chk1 phosphorylation by Treslin is both TopBP1- and ATR-dependent.

Fig. 2 (*A* and *B*) shows the purification of WT-Treslin and its S1000A mutant, which was previously reported to have reduced binding to TopBP1 (23). The high salt wash step (labeled *W2*) removed the endogenous TopBP1 bound to Treslin (*lane 4*). This, in conjunction with the results from Fig. 1*C*, suggests that the stimulation of Chk1 phosphorylation can indeed be attributed to Treslin and not additional TopBP1. It is also noticeable that ATR was detected on the agarose beads of WT-Treslin (Fig. 2*A*, *lane 12*), but not those of the Treslin mutant, which bound less TopBP1 (Fig. 2*B*, *lane 12*), suggesting that Treslin and ATR function in the same pathway and that the interaction of Treslin with ATR is mediated through TopBP1.

We also cloned the Treslin fragment into the pET28a vector and purified it from *E. coli*. To our surprise, Treslin purified from *E. coli* failed to stimulate Chk1 phosphorylation. In Fig. 2*C*, we added increasing concentrations of TopBP1 to kinase reactions while maintaining a constant concentration of Treslin. Treslin purified from HEK293T cells stimulated Chk1 phosphorylation ~5-fold, whereas Treslin purified from *E. coli* had no effect. We reasoned that Treslin produced in *E. coli* lacked a proper post-translational modification, such as phosphorylation, important for stimulation. In Fig. 2*D*, we compared the mobility of the Treslin proteins treated with phosphatase on SDS-polyacrylamide gel. The Treslin made in HEK293T cells had slower mobility on SDS-polyacrylamide gel relative to the Treslin made in *E. coli*, and upon phosphatase treatment, it exhibited the same mobility as the protein made in *E. coli*, indicating that Treslin purified from HEK293T cells is phosphorylated. Phosphatase treatment of the Treslin produced in *E. coli* (*lane 2*) did not alter its mobility compared with the untreated protein (*lane 1*).

The N Terminus of TopBP1 Is Important for Activation—The *in vitro* checkpoint assays discussed so far included only FL-TopBP1 protein. Because Treslin specifically interacts with the N terminus of TopBP1 (23), we asked whether the N terminus of TopBP1 is required for stimulation of Chk1 phosphorylation by ATR. We have previously demonstrated that fragments of TopBP1 containing the AAD (depicted in Fig. 3*A*) are functional in our *in vitro* checkpoint assay (18). We tested the ability of Treslin to stimulate ATR in reactions containing these

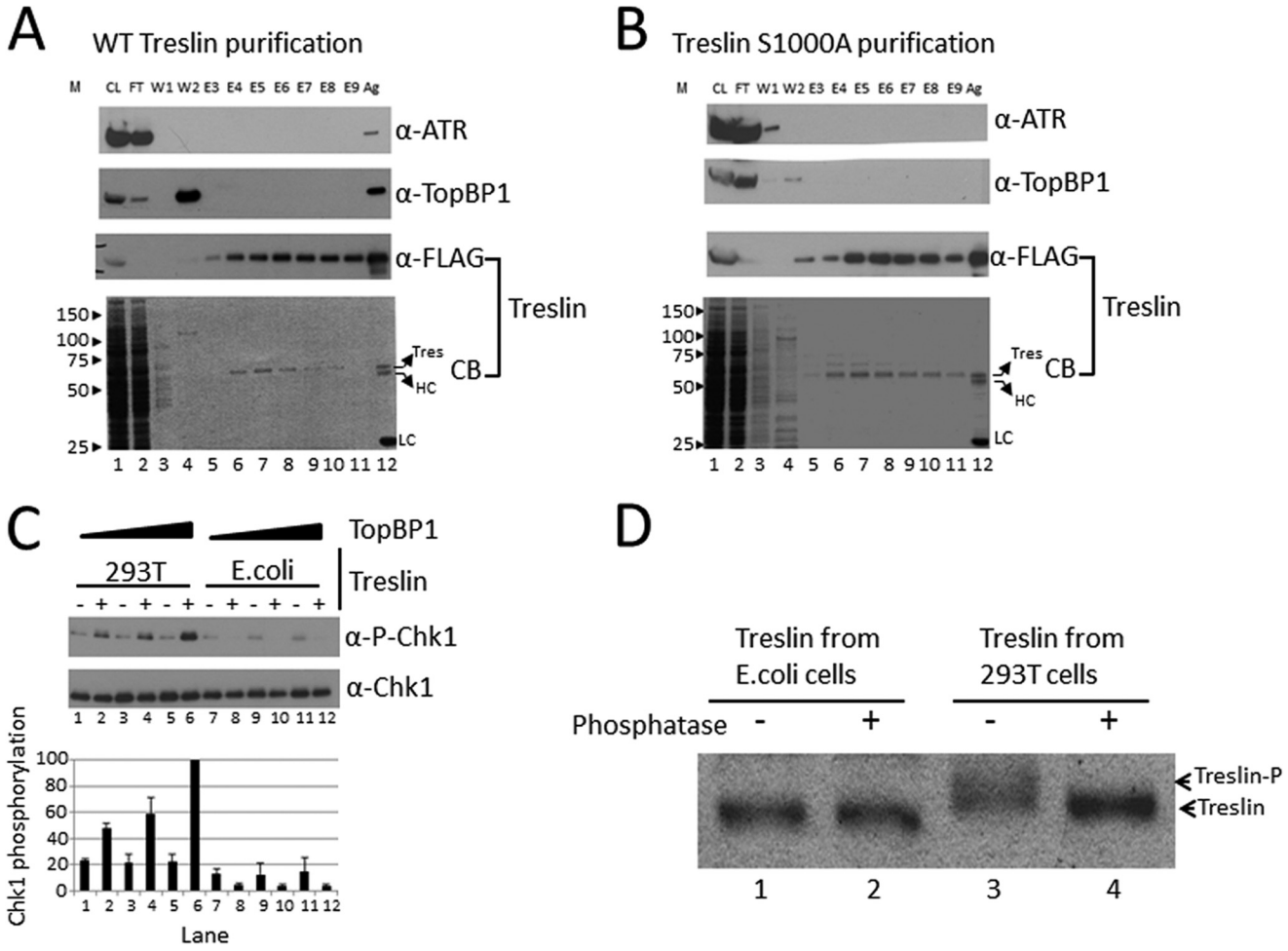

FIGURE 2. **Purified Treslin-FR from HEK293T cells is active in checkpoint stimulation.** *A*, purification of WT-Treslin-FR from HEK293T cells. *M*, molecular mass markers; *CL*, cleared lysate; *FT*, flow-through; *W1*, low salt wash at 150 mM NaCl; *W2*; high salt wash at 500 mM NaCl; *E3–9*, elutions; *Ag*, agarose beads; *CB*, Coomassie Blue-stained SDS-polyacrylamide gel; *Tres*, Treslin; *HC*, heavy chain; *LC*, light chain. *B*, purification of Treslin-FR-S1000A from HEK293T cells. *C*, kinase reactions with Treslin-FR purified from HEK293T or *E. coli* cells. *Lanes 2*, *4*, *6*, *8*, *10*, and *12* contain the same amount of Treslin. *Error bars* indicate S.D. *D*, Antarctic phosphatase treatment of Treslin-FR proteins purified from *E. coli* or HEK293T cells. The two constructs used for expression code for the same polypeptide. Note the change in mobility of Treslin from HEK293T cells upon treatment with phosphatase.

TopBP1 fragments, and the results are shown in Fig. 3*B*. As expected, Treslin stimulated Chk1 phosphorylation by ATR in the presence of FL-TopBP1 (*lanes 2–4* and *9–11*). However, neither of the TopBP1 fragments lacking the N terminus could support Treslin-dependent stimulation (*lanes 5–7* and *12–14*). These results indicate that the N terminus of TopBP1 plays an important role in Treslin-mediated ATR activation. The C-terminal fragments activated ATR similar to FL-TopBP1, indicating that the AADs are folded properly (compare *lanes 5* and *12* with *lanes 2* and *9* relative to *lane 1* without TopBP1). Next, we examined the interaction of Treslin with the TopBP1 fragments used in these assays. The results from binding studies are displayed in Fig. 3*C* and show that Treslin bound to the TopBP1 fragments lacking the N terminus only 5–10% as well as it bound to FL-TopBP1. Therefore, the N terminus of TopBP1 is required for efficient Treslin binding and activation of ATR kinase activity.

S1000A Mutant Treslin Is Deficient in ATR Activation—Because it appeared that checkpoint stimulation by Treslin was dependent on its interaction with TopBP1, we wished to use a Treslin mutant that could not interact with TopBP1 in our kinase assay to further confirm this conclusion. It has been reported that Treslin interacts with TopBP1 upon phosphorylation by CDKs at a specific amino acid residue (Ser-1000 in human Treslin) (23) and that mutation of this amino acid disrupts the interaction. We first analyzed the interaction of wild-type and S1000A mutant Treslin with TopBP1 in extracts from HEK293T cells transfected with FLAG-tagged wild-type or S1000A mutant Treslin plasmid. The results from pulldown assays are displayed in Fig. 4*A* and show a 90% reduction in the amount of TopBP1 bound to mutant Treslin (compare *lanes 5* and *6*). Remarkably, when added to the kinase assay, the S1000A mutant failed to stimulate Chk1 phosphorylation (Fig. 4*B*) in contrast to WT-Treslin. Together, these results indicate that phosphorylation of Treslin is essential for its interaction with TopBP1 and a subsequent ability to stimulate ATR kinase.

Treslin Activates ATR in Vivo—Because we observed a stimulating effect of Treslin on checkpoint activation in our *in vitro* system in the absence of DNA damage signaling, we wished to determine whether this stimulation could be observed *in vivo*. We have previously shown that LacR-TopBP1 induces ATR phosphorylation of Chk1 in mammalian cells containing an

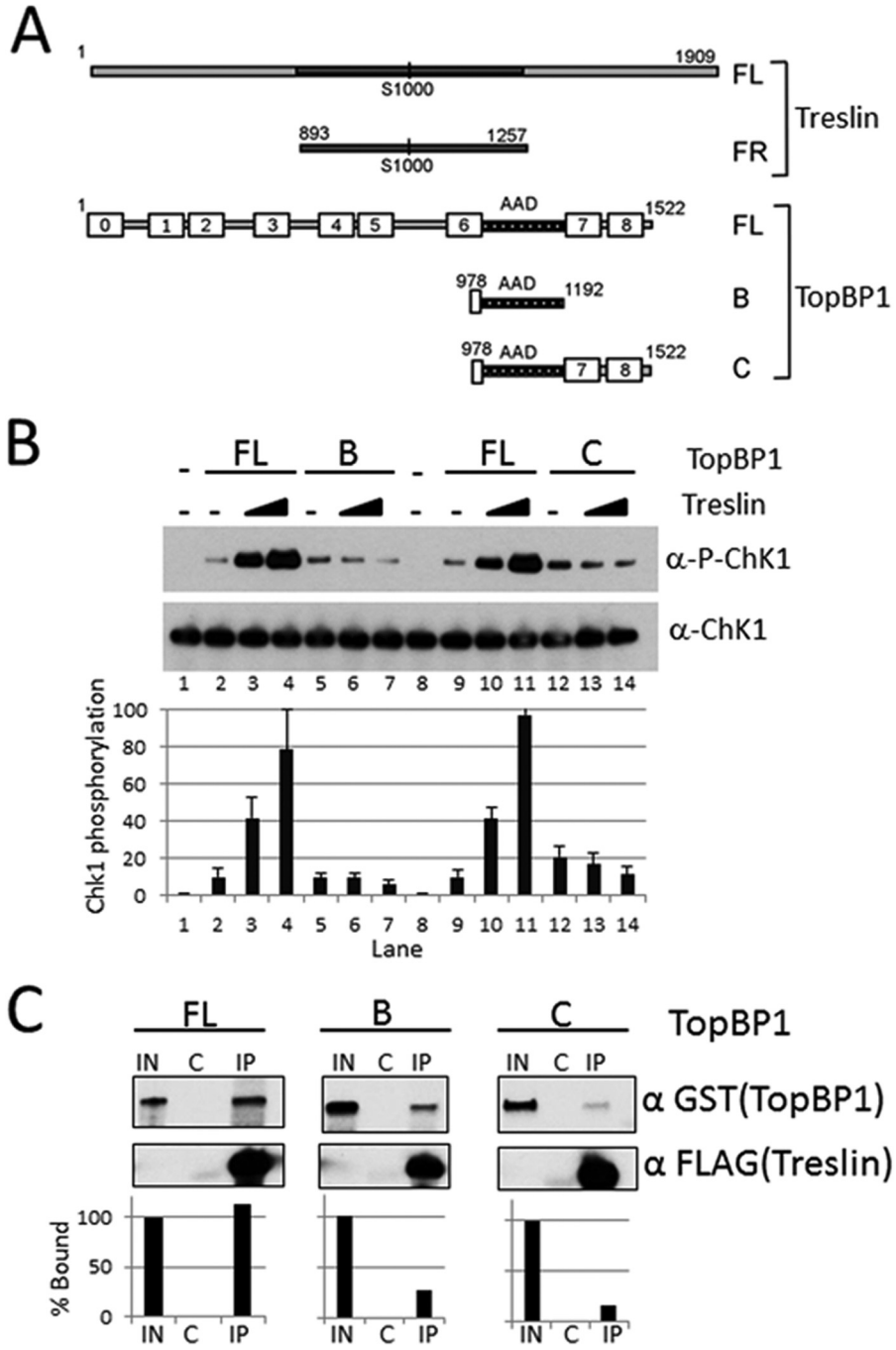

FIGURE 3. **The N terminus of TopBP1 is important for Treslin-dependent checkpoint activation.** *A*, *upper*, schematic of the Treslin protein. FL-Treslin comprises amino acids 1–1909, and Treslin-FR comprises amino acids 893–1257 and contains the phosphorylated Ser-1000 residue. *Lower*, schematic of the TopBP1 fragments used. FL-TopBP1 comprises amino acids 1–1522; fragment B comprises amino acids 978–1192 and contains essentially the AAD; and fragment C comprises amino acids 978–1522 and contains BRCT domains 7 and 8 in addition to the AAD. *B*, kinase reactions with different TopBP1 fragments. Lanes marked with a *triangle* contain increasing concentrations of Treslin (12 and 25 nM) while maintaining a constant concentration of the indicated TopBP1 protein or its fragments. FL-TopBP1 and fragments B and C were used at 1, 16, and 0.1 nM, respectively. *Error bars* indicate S.D. *C*, binding of Treslin to different TopBP1 fragments. FLAG-tagged Treslin-FR was bound to agarose beads, washed with TBS150, washed with TBS500, and then equilibrated with TBS150. Different TopBP1 fragments purified from *E. coli* were added to the beads and rotated with mixing for 2 h at 4 °C. Beads were washed five times with TBS150 and eluted with SDS-PAGE loading buffer. For the input (*IN*) lanes, 1% of the total reaction was loaded on the gel. *C*, control; *IP*, immunoprecipitate.

array of the LacO sequence (24). Therefore, we used this system to determine whether Treslin affects checkpoint activity. We transiently expressed LacR-TopBP1 and FL-Treslin in the NIH2/4 cell line containing a LacO array stably integrated into its genome (28), and the results are shown in Fig. 5*A*. Because transfecting NIH2/4 cells with high concentrations of LacR-TopBP1 or FL-Treslin plasmid activated ATR on its own, we transfected limiting amounts of these plasmids so that the Chk1 phosphorylation signal was just above the background with either plasmid alone (*lanes 2* and *3*). However, coexpression of LacR-TopBP1 and FL-Treslin resulted in an ~3-fold increase in Chk1 phosphorylation in comparison with cells expressing only

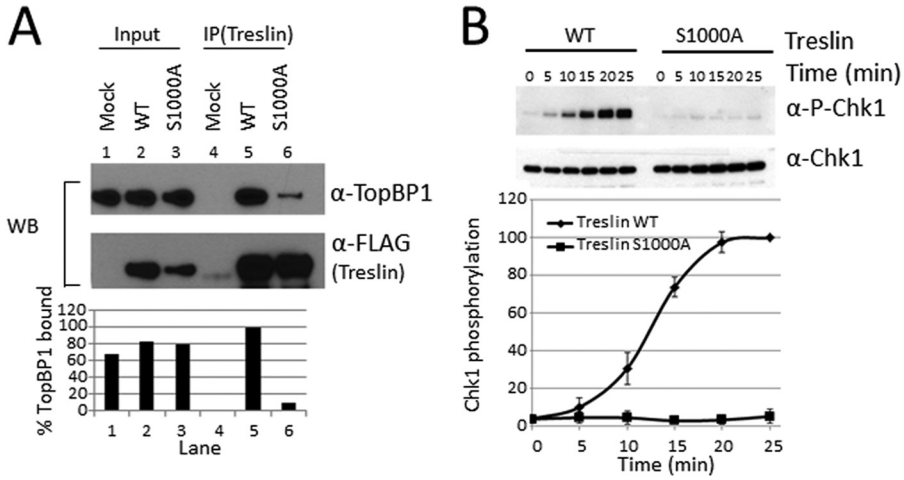

FIGURE 4. **S1000A mutation in Treslin abrogates its ATR kinase stimulatory activity.** *A*, interaction of WT-Treslin and the S1000A mutant with TopBP1. HEK293T cells were transfected with FLAG-tagged WT-Treslin or Treslin-S1000A plasmids, Treslin was immunoprecipitated (*IP*) with anti-FLAG-agarose beads, and Western blots were probed for TopBP1. *B*, kinetics of Chk1 phosphorylation in the presence of wild-type or mutant Treslin-FR purified from HEK293T cells. Reactions contained the same concentration of Treslin (25 nM). Samples were removed from a master mixture every 5 min and mixed immediately with SDS-PAGE loading buffer and boiled. *Error bars* indicate S.D.

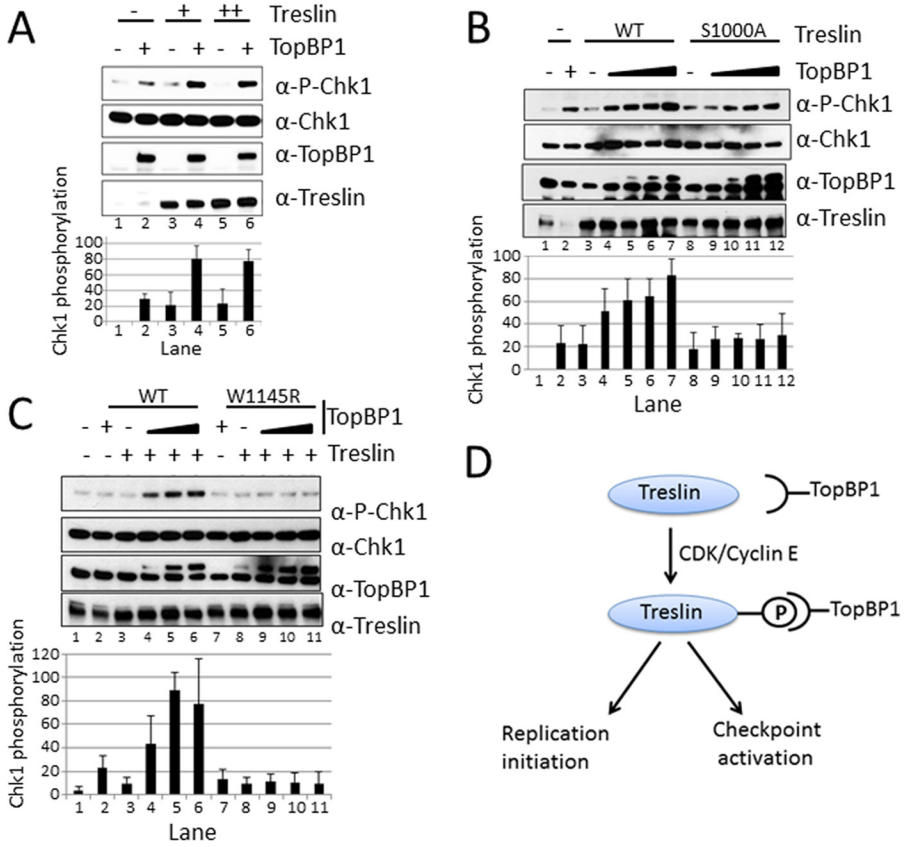

FIGURE 5. **Coexpression of Treslin and LacR-TopBP1 activates Chk1 phosphorylation *in vivo*.** *A*, coexpression of LacR-TopBP1 and FL-Treslin stimulates Chk1 phosphorylation. NIH2/4 cells were transfected with pcDNA3-LacR-TopBP1 and pcDNA5TO-FL-Treslin plasmids and empty pcDNA3 plasmid such that all of the transfections had equal amounts of DNA. The LacR-TopBP1 plasmid was used at 250 ng, and FL-Treslin was used at 62 and 124 ng/well. *B*, titration of wild-type or S1000A mutant FL-Treslin with TopBP1 *in vivo*. The concentration of wild-type or mutant FL-Treslin was kept constant at 125 ng/well. TopBP1 was used at 31, 62, 125, and 250 ng/well while keeping the total amount of transfected DNA constant with pcDNA3. Background signal values from *lane 1* of each experiment were subtracted from the rest of the lanes. *C*, titration of FL-Treslin with wild-type or W1145R mutant TopBP1 *in vivo*. The concentration of FL-Treslin was kept constant at 125 ng/well. TopBP1 was used at 62, 125, and 250 ng/well while keeping the total amount of transfected DNA constant by pcDNA3. *Error bars* indicate S.D. *D*, model for the role of Treslin in the initiation of DNA replication and checkpoint activation. Treslin is phosphorylated in a CDK2/cyclin E-dependent manner, which increases its affinity for binding to TopBP1 and contributes to its replication and checkpoint functions.

the individual proteins (compare *lane 4* with *lanes 2* and *3*). Therefore, we conclude that, as was observed *in vitro*, induced proximity of Treslin and TopBP1 activates ATR phosphorylation of Chk1 *in vivo*, indicating that spatial organization of ATR and its activators is a primary mechanism for initiating checkpoint signaling.

Because we observed that the S1000A mutation abrogated the ability of Treslin to bind TopBP1 and mediate ATR phosphorylation of Chk1 *in vitro*, we decided to test this mutation *in vivo*. Fig. 5*B* shows the results from transfecting NIH2/4 cells with wild-type or S1000A mutant FL-Treslin at a constant concentration while increasing the concentration of Lac-TopBP1. The increase in Chk1 phosphorylation was attenuated with Treslin-S1000A compared with WT-Treslin, especially at the highest concentration of TopBP1 (compare *lanes 7* and *12*). Together, these results agree with the *in vitro* results that disruption of the ability of Treslin to interact with TopBP1 abrogates its effect on checkpoint activation.

One potential complication with the experimental approach described above is that the expression of different replication proteins could alter replication, which would result in the activation of ATR. To address this issue, we introduced a point mutation (W1145R) into the AAD of TopBP1. Mutation of this tryptophan residue, analogous to Trp-1138 of *Xenopus* TopBP1, abrogates TopBP1 activation of ATR (9) and is unlikely to affect replication because the whole AAD in not required for replication (15). Fig. 5*C* shows the results of transfecting NIH2/4 cells with FL-WT-Treslin at a constant concentration while titrating wild-type or W1145R mutant TopBP1. Coexpression of W1145R mutant TopBP1 with FL-Treslin displayed Chk1 phosphorylation levels close to the background, in contrast to coexpression with WT-TopBP1 (compare *lanes 4–6* with *lanes 9–11*). These results indicate that the observed stimulation of checkpoint activation is due in fact to the direct function of Treslin and TopBP1 in ATR activation.

DISCUSSION

Treslin was identified through a G_2/M checkpoint screen in zebrafish exposed to ionizing radiation (11). Treslin-deficient cells failed to arrest before mitosis after DNA damage and thus had a deficient G_2/M checkpoint response. In addition, in the absence of DNA damage, Treslin-deficient cells demonstrated an S/M checkpoint defect by entering mitosis with partially replicated DNA. However, because Treslin is required for proper replication initiation at origins of replication, it is not clear whether these observed phenotypes are due to a role in checkpoint signaling or a reflection of the replication function of Treslin.

In this study, we report that Treslin activates ATR phosphorylation of Chk1 both *in vitro* and *in vivo*. Our results indicate that, in addition to its role in replication initiation, Treslin possesses an additional role in checkpoint signaling. There is precedence for proteins with dual replication/checkpoint functions. The best known example is TopBP1. In addition, it was recently reported that the essential lagging strand maturation factor Dna2 is a component of the replication checkpoint machinery in yeast (29). Thus, there is growing evidence that replication and checkpoint signaling pathways are more intimately associated than previously thought.

Our *in vitro* results show that checkpoint stimulation is dependent on TopBP1. Preventing the interaction of Treslin with TopBP1 (through deletion of the N terminus of TopBP1 or mutation of the corresponding interacting domain in Treslin) abrogates Chk1 phosphorylation by ATR. This highlights the importance of the Treslin-TopBP1 interaction interface in checkpoint stimulation. The precise molecular mechanism for this stimulation of ATR activation is still unclear. When we purified Treslin by immunoaffinity chromatography, the protein preparations contained both TopBP1 and ATR in addition to Treslin (Fig. 2*A*). This supports the presence of a Treslin-TopBP1-ATR ternary complex in mammalian cells. The conditions that lead to the formation of this complex remain to be determined.

Our *in vivo* results validate a model of direct checkpoint activation by Treslin. We used our previously reported system in which TopBP1 tethered to DNA via the LacR/LacO array activates ATR phosphorylation of Chk1 in mammalian cells in the absence of DNA damage (24). This system essentially concentrates checkpoint proteins onto chromatin and thus constitutes a good system to study the role of Treslin outside of replication. Treslin binds to TopBP1 on chromatin when it is phosphorylated at Ser-1000 (23), and consequently, the S1000A mutant displayed a reduced stimulation of Chk1 phosphorylation (Fig. 5*B*). The fact that there was residual Chk1 phosphorylation in reactions with S1000A mutant Treslin can be attributed to the presence of a second TopBP1-interacting domain in the N-terminal region of Treslin (residues 1–463). This fragment is reported to retain significant binding to TopBP1, although weaker compared with fragment 893–1257 (23). In contrast, mutating TopBP1 in its AAD (W1145R) completely eliminated Chk1 phosphorylation and stopped the signal transduction at the TopBP1-ATR interface (Fig. 5*C*).

Although our data clearly demonstrate a role for Treslin in the activation of ATR, the physiological role of Treslin in the ATR-mediated checkpoints remains to be further investigated. ATR has been reported to regulate the timing of DNA replication origin firing in the absence of overt DNA damage (30). Our results provide the conceptual framework for examining activities of Treslin and TopBP1 in ATR signaling at origins of DNA replication. In addition, Treslin could play an important role after DNA damage or replication fork stalling in blocking late origin firing. The yeast homolog of Treslin, Sld3, is phosphorylated by Rad53 (the functional homolog of Chk1), and this phosphorylation has been shown to block late origin firing (31). Moreover, recent data have demonstrated that the interaction of Treslin with TopBP1 is inhibited upon replication fork stalling (22), and this abrogated interaction is Chk1-dependent. Thus, Treslin and TopBP1 may function not only upstream of ATR, being required for ATR-Chk1 pathway activation, but also downstream, as an ultimate effector of Chk1 signaling. Although much work remains to be done to elucidate the role of Treslin in DNA replication and checkpoint signaling, our results demonstrate that Treslin stimulates ATR activity in a TopBP1-dependent manner both *in vitro* and *in vivo*, and thus, Treslin is directly involved in checkpoint activation (model displayed in Fig. 5*D*).

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