Rad4<sup>TopBP1</sup> Associates with Srr2, an Spc1 MAPK-regulated Protein, in Response to Environmental Stress*

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Lorena Taricani and Teresa S. F. Wang

From the Department of Pathology, Stanford University School of Medicine, Stanford, California 94305-5324

Rad4<sup>TopBP1</sup> is a scaffold in a protein complex containing both replication proteins and checkpoint proteins and plays essential roles in both replication and checkpoint responses. We have previously identified four novel fission yeast mutants of rad4<sup>TopBP1</sup> to explore how Rad4<sup>TopBP1</sup>, a single protein, can play multiple roles in genomic integrity maintenance. Among the four novel mutants, rad4<sup>-c17TopBP1</sup> is a thermosensitive mutant. Here, we characterized rad4<sup>-c17TopBP1</sup> and identified a rad4<sup>-c17TopBP1</sup> allele-specific suppressor named srr2<sup>+</sup> (suppressor of Rad4<sup>TopBP1</sup> R2 domain). srr2<sup>+</sup> has previously been identified as an environmental stress-responsive gene (GenBank<sup>TM</sup> accession number AL049644.1, locus sppc191.01). srr2<sup>+</sup> null cells are sensitive to hydroxyurea (HU) at elevated temperatures. Deletion of srr2<sup>+</sup> in rad4<sup>-c17TopBP1</sup> exacerbates the HU sensitivity of the mutant. Overexpression of srr2<sup>+</sup> suppresses the rad4<sup>-c17TopBP1</sup> mutant sensitivity to temperature and HU and restores the compromised ability of rad4<sup>-c17TopBP1</sup> to activating Cds1 kinase in response to HU treatment. Furthermore, stress-activated MAPK, Spc1 (also known as Sty1 or Phh1), induces the expression and phosphorylation of the Srr2 protein. Significantly, environmental stress induces co-precipitation of Srr2 protein with Rad4<sup>TopBP1</sup>, and the co-precipitation is compromised in the rad4<sup>-c17TopBP1</sup> mutant. These results have led us to propose a model; Rad4<sup>TopBP1</sup> exists in a large protein complex to coordinate genomic perturbations with checkpoint responses to maintain genomic integrity. In addition, when cells experience environmental stress, Rad4<sup>TopBP1</sup> associates with Srr2, an Spc1 MAPK-responsive protein, to survive the stress, potentially by providing a link of the Spc1 MAPK response to checkpoint responses.

Fission yeast Rad4<sup>TopBP1</sup> protein contains four BRCT<sup>2</sup> (BRCA1 carboxyl terminus) domains and functions as a scaffold in a protein complex containing both DNA replication proteins and checkpoint proteins (1). Rad4<sup>TopBP1</sup> coordinates replication with checkpoint responses to replication stress and DNA damage for genomic integrity maintenance (1, 2). In response to DNA damage, fission yeast Rad3<sup>ATR</sup> phosphorylates the checkpoint clamp protein Rad9 at Thr<sup>412</sup>/Ser<sup>423</sup>. The phosphorylated form of Rad9 then associates with the two C-terminal BRCT domains of Rad4<sup>TopBP1</sup> to promote activation of the Chk1 damage checkpoint response but not the activation of Cds1 replication checkpoint (3). How Rad4<sup>TopBP1</sup> functions in promoting the activation of Cds1-mediated replication checkpoint response is not yet clear. Rad4<sup>TopBP1</sup> of human cells has been shown to play a critical role in maintaining genomic stability during normal S-phase and following genotoxic stress (4). Furthermore, Rad4<sup>TopBP1</sup> of both Xenopus and human has been shown to play a critical role in activation of ATR for initiating the ATR-dependent checkpoint signaling processes (5).

We have previously identified four novel rad4<sup>TopBP1</sup> mutants to better understand how Rad4<sup>TopBP1</sup> could play multiple genome maintenance tasks in cells (1). Among the four novel mutants, rad4<sup>-c17TopBP1</sup> has a mutation in the second BRCT domain (R2 domain) and is a thermosensitive mutant. Mutant rad4<sup>-c17TopBP1</sup> has a compromised ability to fully activate Cds1 kinase when cells were cultured in rich media and arrested by hydroxyurea (HU) at its restrictive temperature of 36 °C, but it is proficient in Chk1 activation in response to camptothecin (CPT) treatment at its permissive temperature of 30 °C (1). In this study we identified a gene, overexpression of which suppresses the temperature sensitivity and HU sensitivity of rad4<sup>-c17TopBP1</sup> and restores its ability of Cds1 kinase activation in response to HU treatment at its permissive temperature. We named the suppressor srr2<sup>+</sup> (suppressor of Rad4<sup>TopBP1</sup> R2 domain). Analysis of srr2<sup>+</sup> suggests that Rad4<sup>TopBP1</sup> in addition to its essential role in replication and checkpoint, may also provide a link of checkpoint response to the Spc1 MAPK pathway in response to the environmental stress.

**EXPERIMENTAL PROCEDURES**

**Strains and Media—Schizosaccharomyces pombe** (fission yeast) strains (Table 1) were grown in YES or minimum medium (EMM) containing nutritional supplements as necessary. Standard genetic methods, molecular biological techniques, and generation of tagged strains were as described in Moreno et al. (6) and Bahler et al. (7). All strains used in this study are listed in Table 1.

**Screen for the rad4-c17 Mutant Suppressor**—The rad4-c17 mutant strain was transformed with the pREP3X nmt1 overexpression library (8). Transformed cells (>500,000 independent colonies) were plated on selective EMM plus 10 μM thiamine media and incubated for 6 days at 25 °C. Colonies were then replica-plated onto EMM plates with and without thia-
mine for 3–4 days at 36 °C. Colonies that grew in the absence of thiamine were patched out onto EMM plates and incubated at 36 °C for 3–4 days. Plasmids from colonies survived the 36 °C incubation were recovered and further verified by transformation into the rad4-c17 mutant strain to re-test their ability to rescue the growth defect at 36 °C. Four plasmids that suppressed the temperature sensitivity of rad4-c17 at 36 °C were obtained. After sequencing the plasmids, two were rad4 with a deletion of the first N-terminal 58 amino acids, and the other two were an unknown gene spec191.01 (10), which we have named srr2.

Construction of Δsrr2 Strain—The entire srr2 open reading frame was replaced with the ura4+ gene using PCR-mediated gene disruption (7).

Preparation of Cell Extracts, Immunoblotting, and Immunoprecipitation—Proteins extraction and immunoprecipitation of GFP-Rad4TopBP1 and GFP-Rad4-c17TopBP1 proteins were performed as described in Taricani and Wang (1). Preparation of cell extracts and immunoblot of Myc-Srr2 were performed as described in Rhind and Russell (11). Twenty μg of proteins determined by the Bio-Rad protein assay were separated on 10% SDS-PAGE, electroblotted onto polyvinylidene difluoride membranes (Bio-Rad), and detected by mouse anti-Myc (9E10) (1:2000). Immunoreactive bands were revealed with horseradish peroxidase-conjugated secondary goat antimouse IgG antibody (1:10,000) (New England Biolabs) and the luminol-based ECL detection kit (PerkinElmer Life Sciences).

Phosphatase Treatment—Myc-Srr2 was immunoprecipitated from cell extracts with anti-Myc antibodies (9E10) cross-linked to protein G. Immunoprecipitated Myc-Srr2 was treated with λ phosphatase (40 units) (New England Biolabs) at 30 °C for 30 min.

Cds1 Kinase Assay—Immunoprecipitation of Cds1 protein and Cds1 kinase activity were performed as described (12) with the exception of growing the cells in EMM-containing supplements as necessary instead of rich media (YES).

Immunofluorescence—Cells were collected and fixed in 100% methanol at −20 °C for at least 20 min. Cells were then washed 3 times in 1× PEM buffer (100 mM Pipes, pH 6.9, 1 mM EGTA, 1 mM MgCl2) (13), resuspended in 20–50 μl of 1× PEM, and stained with 1 μg/ml propidium iodide. All images were photographed with a Nikon PCM confocal microscope.

Flow Cytometry Analysis—Cells were harvested, washed in water, fixed in ice-cold 70%, and stained with propidium iodide as described previously (14). DNA content was determined using a Coulter fluorescence-activated cell sorter.

**RESULTS**

Characterization of rad4-c17TopBP1—rad4-c17TopBP1 is a temperature-sensitive mutant among the four novel rad4TopBP1 mutants that we identified (1). Our previous studies have shown that rad4-c47TopBP1 mutant exhibits reduced Cds1 kinase activity in response to HU treatment at the restrictive temperature of 36 °C when cultured in rich media but is fully proficient in activation of the Chk1 damage checkpoint at its permissive temperature of 30 °C in response to CPT treatment (1). This novel temperature-sensitive mutant of rad4TopBP1 contains a S171N substitution within the second BRCT domain (R2 domain) (Fig. 1, A and B). Notably, the mutation site resides in a conserved Cys/Ser in the third α-helix of the secondary structure of Rad4TopBP1 found in all BRCT family domains including BRCA1 (S1841N) (15). Similar to the previously well-characterized thermosensitive mutant rad4-c11TopBP1, rad4-c17TopBP1 exhibits a cut (cells ultimately torn) phenotype at the restrictive temperature of 36 °C. To test whether mutation in rad4-c17TopBP1 causes a defect in the cell cycle progression, rad4-c17TopBP1 with rad4-116TopBP1 as a comparison was arrested at the restrictive temperature of 36 °C and analyzed for their DNA contents by FACScan. cdc10-m17 and cdc17-K42 mutants at their restrictive temperature of 36 °C were used as 1C and 2C control, respectively. rad4-c11TopBP1 and rad4-c17TopBP1 accumulate the majority of the cells with 1C DNA with a minor population in less than 1C DNA content as described previously (16), whereas rad4-c11TopBP1 and rad4-c17TopBP1 cells accumulate only in 1C DNA content (Fig. 1C). Results of the cut phenotype and the 1C FACScan profile of rad4-c17TopBP1 suggest that rad4-c17TopBP1 at the restrictive temperature of 36 °C, similar to rad4-116TopBP1, is defective in both checkpoint response and DNA replication.
Rad4TopBP1 Links Checkpoint Response to MAPK Pathway

Rad4TopBP1 is a protein that links checkpoint response to MAPK pathway.

**Figure 1.** Characterization of rad4-c17TopBP1. A, a schematic location of the novel rad4-c17TopBP1 mutation allele. Rad4TopBP1 consists of four BRCT domains (R1, R2, R3, and R4) and two hydrophilic (acidic and basic) domains. Locations of rad4-c17TopBP1 and rad4-116TopBP1 in R1 and R2 BRCT domain are marked. B, rad4-c17TopBP1 mutant is temperature-sensitive. Cells were cultured to log phase, and then 10-fold serial dilutions of 1 x 10^7 cells were spotted onto YES plates and incubated at 30 and 36 °C for 3 days. WT, wild type. C, fluorescence-activated cell sorter (FACS) analysis of rad4-c17TopBP1 mutant at 36 °C. The DNA contents of the rad4-c17TopBP1 mutant cells at 36 °C for 4 h were analyzed by Coulter FACS. cdc10-k17 and cdc17-k42 mutants incubated at 36 °C for 4 h were used as control for 1C and 2C DNA contents, respectively.

**Figure 2.** Characterization of srr2. B, srr2 is an allele-specific suppressor for the temperature sensitivity of rad4-c17TopBP1. A, overexpression of srr2 suppresses the temperature sensitivity of rad4-c17TopBP1. rad4-c17TopBP1 strain was independently transformed with plasmids pREP3x (empty vector control), prad4TopBP1 (positive control), pREP3x + ΔN58aa rad4TopBP1, and pREP3x + srr2. rad4-c17TopBP1 strain was independently transformed with plasmids pREP3x + ΔN58aa rad4TopBP1 and pREP3x + srr2. Transforms (1 x 10^7) were serial diluted (1:10), spotted on media without thiamine to activate the gene expression, and incubated at 30 and 36 °C for 3 days. B, Srr2 protein sequence. The four potential MAPK phosphorylation sites (Ser-Pro/Thr-Pro) are marked in gray, and the potential Thr (T) or Ser (S) phosphorylation sites are underlined.

**Figure 3.** Characterization of srr2. —The Srr2 protein is a small protein that consists of 178 amino acids. The Srr2 protein has a poly-Glu motif at the C terminus and four potential consensus MAPKs (Ser-Pro/Thr-Pro) phosphorylation sites in the C-terminal region, namely Thr-109 (TSTP), Thr-139 (MPTP), Ser-159 (PPSP), and Ser-158 (PESP) (Fig. 2B). Cells with a disruption of srr2 gene by replacing of the entire srr2 coding region with ura4+ were viable, indicating that srr2+ is not essential for cell viability. Cells harboring the srr2::ura4+ are not sensitive to HU at 30 °C (Fig. 3, A and C). In contrast, rad4-c17TopBP1 is sensitive to HU treatment at the permissive temperature of 30 °C. Notably, deletion of srr2+ in rad4-c17TopBP1 exacerbates the sensitivity of the cells to HU treatment (Fig. 3). We further examined the HU sensitivity of srr2::ura4+ mutant at 32 °C (Fig. 3B). srr2::ura4+ was not sensitive to HU treatment at 32 °C. However, disruption of srr2+ in rad4-c17TopBP1 overtly exacerbated the sensitivity of the double mutant to HU at 32 °C, indicating that Srr2 is required for maintaining the viability of rad4-c17TopBP1 when cells experience HU treatment at the semipermissive temperature (Fig. 3B, upper panel). Importantly, srr2::ura4+ cells are sensitive to HU treatment at 36 °C.
Both myc:srr2::ura4+ 107 cells were spotted onto rich media YES plates with or without 5 mM HU and incubated at 30 °C for 3 days.

Characterization of srr2+. A, sensitivities of the srr2::ura4+ mutant to HU. Cells were cultured to log phase, and then 10-fold serial dilutions of 1 × 10⁷ cells were spotted onto rich media YES plates with or without 5 mM HU and incubated at 30 °C for 3 days. WT, wild type; B, sensitivities of the srr2::ura4+ mutant to HU and temperature. Cells were cultured to log phase, and then 10-fold serial dilutions of 1 × 10⁷ cells were spotted onto YES plates with or without 2.5 and 5 mM HU and incubated at 32 and 36 °C for 3 days. C, UV sensitivity of srr2::ura4+ mutant. Wild-type, srr2::ura4+, rad4-c17TopBP1, and srr2::ura4+ rad4-c17TopBP1 strains were grown in YES to early log phase and plated in triplicate on YES plates. Cells were irradiated with the indicated doses of UV and incubated at 30 °C for 5 days. Data shown represent the average results of three independent experiments. D, the cellular localization of GFP-Srr2 protein. Top left panel shows the cytoplasmic localization of GFP-Srr2 in the absence of stress, and the bottom left panel shows the nuclear localization of GFP-Srr2 after 4 h of 12 mM HU treatment. Nuclei were stained by propidium iodine (PI).

Overexpression of srr2+ suppresses the hydroxyurea sensitivity. WT, wild type.

srr2+ was overexpressed via pREP3X vector in rad4-c17TopBP1 at the permissive temperature of 30 °C, with empty vector pREP3X and prad4TopBP1+ as the negative and positive control, respectively. As shown in Fig. 4A, overexpression of srr2+ suppresses the HU sensitivity of rad4-c17TopBP1.

We have previously shown that rad4-c17TopBP1 exhibits a reduced ability to activate Cds1 kinase activity when cultured in rich media at 36 °C (1). Given that overexpression of srr2+ suppresses the HU sensitivity of rad4-c17TopBP1, we tested whether overexpression of srr2+ could have an effect on the ability of rad4-c17TopBP1 to activate Cds1 kinase in response to HU treatment. Mutant rad4-c17TopBP1 was cultured in minimal media to express srr2+ via pREP3X vector and was treated with or without HU at 30 and 36 °C. Wild-type cells harboring an empty vector were used as a control. Cds1 proteins immunoprecipitated from the wild-type cells and from rad4-c17TopBP1: pREP3X+srr2+ mutant were used to assay for Cds1 kinase activities (12). rad4-c17TopBP1 in minimal media at 30 °C exhibited reduced ability to activate Cds1 kinase activity upon HU treatment, similar to our previous finding of rad4-c17TopBP1 cultured in rich media at 36 °C (1). Interestingly, overexpression of srr2+ in rad4-c17TopBP1 restored the ability of rad4-
cells cultured in rich media at 36 °C (1), resulting in compromising the Cds1 kinase activation. The ability of restoring the Cds1 kinase activation in rad4-c17TopBP1 in minimal media at 30 °C suggests a role of Srr2 in stress response. This result also suggests that suppression of rad4-c17TopBP1 temperature sensitivity at 36 °C by overexpression of srr2+ is not entirely due to restoration of the ability of the mutant to activate Cds1 kinase.

Spc1 MAPK Up-regulates Srr2 Expression in Response to Environmental Stress—A previous analysis of global transcription response to environmental stress in fission yeast has identified the up-regulation of srr2+ transcript to a wide range of environmental stresses (10). Finding that deletion of srr2+ exacerbates the rad4-c17TopBP1 sensitivity to HU (Fig. 3B) and overexpression of srr2+ suppresses the rad4-c17TopBP1 HU sensitivity and restores the ability of rad4-c17TopBP1 to activate Cds1 in response to HU treatment at 30 °C (Fig. 4, A and B) led us to further explore the response of Srr2 protein expression to various environmental stresses. We found that Srr2 protein levels were elevated in response to the treatment of hydrogen peroxide (H₂O₂). A comparable extent of increase of Srr2 protein levels was also observed in response to treatments of methyl methanesulfonate, CPT, HU, and heat shock (HS) (Fig. 5A). Furthermore, Srr2 protein fractionated as a slower mobility protein in gel in response to various environmental stresses. Phosphatase treatment abolishes the slow mobility of Srr2 protein, indicating that the Srr2 protein is phosphorylated in response to stress (Fig. 5, A, C, and D).

Examination of the promoter sequence of srr2+ identified a CESR (core environmental stress response) motif (ttacgt) and an Spc1-dependent motif (tctttactt) (Fig. 5B). To test whether these motifs regulate Srr2, we investigated the Srr2 protein expression in spc1Δ and pyp1Δ mutant backgrounds because deletion of spc1+ inactivates the MAPK pathway (17), whereas deletion of pyp1+, an inhibitor of Spc1, activates the pathway (18). Wild-type cells express a low basal

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**FIGURE 4. Effect of overexpression of srr2+ on rad4-c17TopBP1 HU sensitivity and Cds1 activation.** A, expression of srr2+ suppresses the HU sensitivity of rad4-c17TopBP1 at 30 °C (yellow). rad4-c17TopBP1 strain was independently transformed with plasmids pREP3X (empty vector control), prad4-1TopBP1 (positive control), and pREP3X+srr2+. Wild-type (WT; leu1–32) strain was independently transformed with pREP3X (empty vector, as the positive control). Transformants (1 x 10⁷) were serial-diluted (1:10), spotted on media without thiamine to activate the gene expression, and incubated at 30 °C in the absence or presence of 5 mM HU. B, expression of srr2+ in rad4-c17TopBP1 strain restores the Cds1 kinase activity in rad4-c17TopBP1 at 30 °C (yellow). rad4-c17TopBP1 strain was independently transformed with plasmids pREP3X (empty vector control), prad4-1TopBP1 (positive control), and pREP3X+srr2+. The wild-type strain was independently transformed with pREP3X (empty vector, positive control). Strains were grown in minimum EM medium containing nutritional supplements in the absence of thiamine for 24 h. Cells extracts were performed as described under “Experimental Procedures.”

**FIGURE 5. Srr2 protein expression is up-regulated in response to genotoxic stress.** A, myc:srr2+ cells were grown in YES media and treated with 0.5 mM H₂O₂ for 1 h, 0.02% methyl methanesulfonate for 1 h, 30 μM CPT for 2 h, and 12 μM HU for 4 h at 30 °C and heat-shocked (HS) at 39 °C for 15 min. B, schematic illustration of the regulatory motifs in srr2+ promoter. The CESR is located at −360 to −355 bp, and the Spc1-dependent motif is located at −158 to −151 bp. C, cells harboring Δspc1 myc:srr2+ and Δpyp1 myc:srr2+ were grown in YES media and treated with 0.5 mM H₂O₂ for 1 h, 0.02% methyl methanesulfonate (MMS) for 1 h, 30 μM CPT for 2 h, and 12 μM HU for 4 h at 30 °C and heat-shocked at 39 °C for 15 min. Srr2 protein was detected by immunoblotting with mouse anti-Myc (9E10). Cdc2 (PSTAIRE) was used as the loading control. Asyn, asynchromous. D, Myc:Srr2 was immunoprecipitated from extracts of cells cultured in the presence or absence of HU as described under “Experimental Procedures.” The immunoprecipitates were treated without (−) or with (+) with phosphatase (PPhos) in the absence of phosphatase inhibitor.

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Rad4TopBP1 Links Checkpoint Response to MAPK Pathway

**FIGURE 5.** A, expression of srr2+ suppresses the HU sensitivity of rad4-c17TopBP1 at 30 °C (yellow). rad4-c17TopBP1 strain was independently transformed with plasmids pREP3X (empty vector control), prad4-1TopBP1 (positive control), and pREP3X+srr2+. Wild-type (WT; leu1–32) strain was independently transformed with pREP3X (empty vector, as the positive control). Transformants (1 x 10⁷) were serial-diluted (1:10), spotted on media without thiamine to activate the gene expression, and incubated at 30 °C in the absence or presence of 5 mM HU. B, expression of srr2+ in rad4-c17TopBP1 strain restores the Cds1 kinase activity in rad4-c17TopBP1 at 30 °C (yellow). rad4-c17TopBP1 strain was independently transformed with plasmids pREP3X (empty vector control), prad4-1TopBP1 (positive control), and pREP3X+srr2+. The wild-type strain was independently transformed with pREP3X (empty vector, positive control). Strains were grown in minimum EM medium containing nutritional supplements in the absence of thiamine for 24 h. Cells extracts were performed as described under “Experimental Procedures.”
level of Srr2 protein (see Fig. 5C, myc:Srr2 lanes). In the Δspc1 mutant cells with or without exposure to environmental stress, Srr2 protein is expressed at nominal levels lower than the basal level (Fig. 5C). In marked contrast, significant elevation of Srr2 protein levels and phosphorylation of Srr2 protein was observed in response to H₂O₂, methyl methanesulfonate, CPT, HU, and heat shock treatment in the Δpyp1 mutant strain (Fig. 5C). Notably, the Srr2 protein level is also elevated in Δpyp1 cells without any environmental stress, and the extent of phosphorylation of Srr2 protein is increased (see Fig. 5C, Δpyp1 myc:srr2+ lane). Taken together, these results suggest that Spc1 and Pyp1 cooperatively maintain the basal levels of Srr2 protein in cells. The absence of the Spc1 reduces the Srr2 basal protein levels in cells, whereas the absence of Pyp1, the Spc1 inhibitor, enhances the Srr2 protein levels in cells. Importantly, MAPK regulates both the expression and phosphorylation of Srr2 protein when cells experience environmental stresses.

**Rad4TopBP1 Associates with Srr2 Protein in Response to Environmental Stress, and the Association Is Compromised by the Mutation in rad4-c17TopBP1.**—Studies have shown that nucleotide depletion induced by HU treatment or heat shock is sensed by the Spc1 MAPK pathway (19). Given the facts that overexpression of Srr2 suppresses the temperature sensitivity and HU sensitivity of the rad4-c17TopBP1 mutant and the expression of Srr2 protein is up-regulated in response to various environmental stresses, we investigated whether Srr2 has a direct physical role in linking Rad4TopBP1 to stress responses. We constructed two strains of myc:srr2+ in the GFP-tagged wild-type rad44TopBP1 background and in the rad44-c17TopBP1 mutant background. Co-precipitation of Myc:Srr2 with GFP-Rad4TopBP1 under environmentally stress conditions such as growth at 36 °C or HU treatment was analyzed. Myc:Srr2 protein was expressed in both GFP:rad44TopBP1 cells and GFP:rad44-c17TopBP1 mutant cells to similar levels upon stress inductions (Fig. 6, lower, Input panel). Myc:Srr2 protein was readily detected in the anti-GFP immunoprecipitates from the myc:srr2+:GFP:rad44TopBP1 wild-type cell extracts upon HU treatment by heat shock at 36 °C (Fig. 6). Notably, the co-precipitation was reduced but not completely abolished from the extracts of myc:srr2+:GFP:rad44-c17TopBP1 cells after HU treatment or growing at the restrictive temperature (Fig. 6). These results suggest that the change of Ser171 to Asn in the Rad4TopBP1 R2 domain somewhat compromises the environmental stress-responsive association of Rad4TopBP1 with Srr2.

**DISCUSSION**

Studies of Rad4TopBP1 in yeast and in vertebrate cells have established that Rad4TopBP1 is an essential protein involved in both replication and checkpoint responses (2). We isolated four novel mutants of rad44TopBP1 to explore how Rad4TopBP1, a single protein, could have multiple functions in cells (1). Here, we characterize a novel thermosensitive mutant rad44-c17TopBP1 and its suppressor Srr2. From our results we propose a model; Rad4TopBP1 is a scaffold in a large protein complex containing both replication proteins and checkpoint proteins. Rad4TopBP1 coordinates replication stress and DNA damage to enforce different checkpoint responses during cell proliferation to maintain genomic integrity. Additionally, when cells experience environmental stress, Rad4TopBP1 can also associate with Srr2, an Spc1 MAPK-responsive protein, to link the Spc1 MAPK response pathway to the checkpoint responses for cell survival. Below, we discuss our results that support the proposed model.

**The Expression and Phosphorylation of Srr2 Protein Are Regulated by the Stress-activated Spc1 MAPK Pathway.**—An analysis of global transcriptional responses of genes in fission yeast to environmental stress has identified srr2+ as a stress-responsive gene (10). Environmental stresses such as osmotic stress, oxidative stress, heat stress, UV radiation, nutrient limitation, nucleotide depletion, and various other genotoxic agents activate MAPK Spc1 in fission yeast (17, 20–25). The srr2+ promoter region has a CESR consensus sequence (TTACGT) at −355 to −360 and an Spc1-dependent, not Atf1p regulatory motif (TCTTAATT), at −151 to −158 (Fig. 5B). The presence of a CESR consensus sequence and Spc1 regulatory motif in the up-stream sequence of srr2+ confirms the stress responsive-inducible transcription of srr2+ (10). In this study we show the up-regulation of Srr2 protein expression and phosphorylation of Srr2 protein when cells are exposed to various stress-inducing agents (Fig. 5). Thus, environmental stresses up-regulate the srr2+ expression at both transcriptional and the protein levels.

We show that inactivation of the Spc1 MAPK pathway by deletion of spc1+ gene results in nominally detectable expression of Srr2 protein under various types of stress inductions. In contrast, deletion of the Spc1 inhibitor gene, pyp1+, enhances the activation of Spc1 MAPK and increases the expression and phosphorylation of Srr2 protein in stressed cells. Moreover, Spc1 and Pyp1 cooperatively regulate the basal levels of Srr2 in...
cells without stress. Taken together our findings demonstrate that the stress-induced activation of Spc1 MAPK pathway regulates the Srr2 protein expression and phosphorylation of Srr2.

Cells with the deletion of srr2+ are not particularly sensitive to HU at 30 and 32 °C (Fig. 5, A and B). The findings suggest that there are additional cellular factors besides Srr2 that play a redundant role in response to environmental stress to ensure cell survival of moderate stress. Srr2, however, is required for the redundant role in response to environmental stress to ensure there are additional cellular factors besides Srr2 that play a suppressor role in response to environmental stress.

Why Is the Temperature-induced Endogenous Srr2 Unable to Suppress the rad4-c17TopBP1 Phenotype?—Environmental stresses such as heat and HU treatment do induce the expression of the endogenous Srr2 protein in rad4-c17TopBP1 mutant (Fig. 6, bottom Input panel). The rad4-c17TopBP1 mutant, however, is thermosensitive. Why is the induced endogenous Srr2 unable to suppress the thermosensitive phenotype of rad4-c17TopBP1?—Environmental stresses such as heat and HU treatment do induce the expression of the endogenous Srr2 protein in rad4-c17TopBP1 mutant (Fig. 6). As shown in Fig. 6, the mutant Rad4TopBP1 protein has a reduced ability to associate with the endogenous Srr2 protein induced by HU treatment and 36 °C. The level of endogenous Srr2 protein induced by 36 °C may not be sufficient to compensate weak and compromised association of Srr2 with the mutant Rad4TopBP1 for stabilizing the Rad4TopBP1 scaffold or initiating the signaling processes, resulting in rad4-c17TopBP1 being thermosensitive. Thus, despite the fact that the srr2+ promoter has the CESR consensus sequence and the Spc1 regulatory motif and Srr2 protein is up-regulated by temperature-activated Spc1 MAPK in the mutant cells, the rad4-c17TopBP1 still has the thermosensitive phenotype.

Why Is the Ectopically Expressed Srr2 Able to Suppress the Phenotype of rad4-c17TopBP1?—Srr2 protein relocates into the nucleus (Fig. 3D) and associates with Rad4TopBP1 in response to environmental stress (Fig. 6). The stress-induced association of Rad4TopBP1 with Srr2 might either play a role in stabilizing the Rad4TopBP1 scaffold or helping/enhancing the initiation of the signaling processes for cells to tolerate the stress at 36 °C. The Rad4-c17TopBP1 mutant protein contains a Ser71 to Asn (S171N) substitution within the second BCRT (R2) domain. Srr2 endogenous protein is up-regulated in the rad4-c17TopBP1 at 36 °C (Fig. 6, see Input panel). The Rad4-c17TopBP1 mutant protein, however, has a reduced ability to associate with the endogenous Srr2 protein induced by the elevated temperature (Fig. 6). Srr2 ectopically overexpressed from pREP3X is in much higher levels; the high levels of Srr2 protein might be able to compensate the weak association of Srr2 with the Rad4-c17TopBP1 mutant protein and, thus, be able to rescue the thermosensitivity of rad4-c17TopBP1 at 36 °C.

Overexpression of Srr2 protein can also suppress the HU sensitivity and restore the HU-induced Cds1 kinase activation at 30 °C (Fig. 4, A and B). Although the ectopically overexpressed Srr2 can suppress the thermosensitivity of rad4-c17TopBP1, it cannot restore the HU-induced Cds1 kinase activity in rad4-c17TopBP1 at 36 °C (Fig. 4B). These findings suggest that the role of the high levels of overexpressed Srr2 to suppress rad4-c17TopBP1 thermosensitivity at 36 °C is not due to its role involved in activating Cds1 kinase in rad4-c17TopBP1.

What Might Be the Physiological Significance of the Stress-induced Association of Rad4TopBP1 with Srr2?—We have previously shown that Rad4TopBP1 co-exists with replication and checkpoint proteins in a large protein complex and serves as a scaffold to coordinate the responses of replication stress and DNA damage by enforcing different checkpoint responses (1). Thus far, Rad4TopBP1 has not been shown to be involved in stress response. Finding that an allele-specific suppressor of rad4TopBP1 mutant, srr2+ is, an environmental stress-responsive gene regulated by the Spc1 MAPK pathway demonstrates for the first time a link of Rad4TopBP1 with stress response. When cells experience environmental stress, the activated Spc1 MAPK induces the expression and phosphorylation of Srr2 protein. The stress-induced Srr2 re-localizes into nucleus to associate with Rad4TopBP1. The association possibly along with other stress response factors might either help to stabilize the Rad4TopBP1 scaffold or to enhance the initiation of a cascade of signaling process for cells to survive stress to maintain genomic stability. The Spc1 MAPK-regulated Srr2 may also function as a conduit by associating with Rad4TopBP1 for Spc1 MAPK to phosphorylate either Rad4TopBP1 itself or Rad4TopBP1-associated factors for cell to survive the moderate stress.

We show here that deletion of srr2+ in rad4-c17TopBP1 exacerbates the HU sensitivity of the mutant (Fig. 3B), whereas ectopic overexpression of srr2+ suppresses the mutant HU sensitivity (Fig. 4A) and restores the ability of the mutant to activate Cds1 kinase (Fig. 4B). These results suggest that Srr2 is also involved in the mutant checkpoint defect. Findings that the association of Rad4TopBP1 and Srr2 is induced by HU treatment (Fig. 6) and Rad4TopBP1 plays a key role in checkpoint response to replication stress (2) support the premise that the association of Rad4TopBP1 and Srr2 links the checkpoint response to the Spc1 MAPK pathway in response to environmental stress. Spc1 MAPK pathway is activated in response to all sorts of environmental stresses. The molecular mechanisms of how the activated Spc1 MAPK response eventually contribute to genomic integrity maintenance is not yet clear. Nonetheless, results of our study reveal a novel role for Rad4TopBP1 via Srr2 in linking MAPK response to stress with checkpoint responses to maintain genomic stability.

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