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The 14-3-3 Protein Rad24p Modulates Function of the Cdc14p Family Phosphatase Clp1p/Flp1p in Fission Yeast

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Summary

Schizosaccharomyces pombe **cells divide through the use of an actomyosin-based contractile ring. In response to perturbation of the actomyosin ring, *S. pombe* cells delay in a "cytokinesis-competent" state characterized by continuous repair and maintenance of the actomyosin ring [1] and a G2 delay [2, 3]. This checkpoint mechanism requires the function of the Cdc14p-family phosphatase Clp1p/Flp1p [4, 5] and the septation initiation network (SIN) [1–3]. In response to cytokinetic defects, Clp1p, normally nucleolar in interphase, is retained in the cytoplasm until completion of cell division in a SIN-dependent manner [1, 4, 5]. Here, we show that a phosphorylated form of Clp1p binds the 14-3-3 protein Rad24p and is retained in the cytoplasm in a Rad24p-dependent manner in response to cytokinesis defects. This physical interaction depends on the function of the SIN component, Sid2p. In the absence of Rad24p, cells are unable to maintain SIN signaling and lose viability upon mild cytokinetic stress. The requirement of Rad24p in this checkpoint is bypassed by ectopic activation of the SIN. Furthermore, SIN-dependent nuclear exclusion of Clp1p is dependent on Rad24p function. We conclude that Rad24p-mediated cytoplasmic retention of Clp1p/Flp1p is important for cell viability upon stress to the division apparatus.**

Results and Discussion

The Clp1p phosphatase localizes to the SPB and the nucleolus in interphase cells. In contrast, Clp1p is cytoplasmic during mitosis and is also enriched in the actomyosin ring and the spindle midzone [4, 5]. Upon cytokinesis delays, Clp1p is retained in the cytoplasm, even in interphase, until the previous cytokinesis event is completed [1]. Thus, cytoplasmic retention of Clp1p appears to represent a major mechanism of prolonging the duration of SIN signaling in order to allow for the completion of cytokinesis. We therefore sought to identify molecular mechanisms involved in the cytoplasmic retention of Clp1p. To this end, we screened mutants defining molecules known to be involved in nucleocytoplasmic transport. These included nonessential components of the nucleopore apparatus [6] as well as the 14-3-3 molecules, Rad24p and Rad25p [7]. Mutants defining these proteins (Supplemental Data) were screened by exposure to low doses (0.2 μM) of Latrunculin A, an inhibitor of actin polymerization (hereafter referred to as LatA treatment). The dose of LatA used in this study slows down cytokinesis but does not lead to morphogenetic defects or lethality in wild-type cells (Figures 1A and 1B) [1] and does not affect the velocity of actin patch movement [8]. Among the strains tested, mutants defective in *rad24*, which encodes a 14-3-3 protein, were unable to form colonies in conditions in which wild-type cells were viable. Interestingly, cells defective in the related 14-3-3 protein Rad25p showed wild-type sensitivity to LatA (Figure S1). Microscopic examination of *rad24*Δ cells after treatment with LatA revealed that the lethality was due to an inability of cells to complete cytokinesis. Whereas wild-type and *rad25*Δ cells treated with LatA were predominantly binucleate, with their nuclei separated by an improper but complete septum, similarly treated *rad24*Δ cells were multinucleate and only accumulated fragmented septal material (Figures 1B and 1C; Figure S1). Interestingly, *rad24*Δ mutants under these conditions resembled SIN mutants (at the restrictive temperature) [9] as well as LatA-treated *clp1*Δ cells, both of which become multinucleate and are unable to form proper septa. Given that *rad24*Δ mutants are smaller and somewhat spherically (*orb*) shaped [7] as compared to wild-type, we were interested to determine whether the failure of the cytokinesis checkpoint was due to an alteration in cell shape. Significantly, other morphogenetic mutants like *orb6* [10] and *orb3* [11] mutants were able to complete septation and were largely binucleate upon exposure to LatA (Figure 1C and data not shown; Figure S1). Thus, failure of the cytokinesis checkpoint in *rad24*Δ cells appears to be unrelated to its morphogenetic defect. The 14-3-3 mutant, *rad24*Δ, therefore appeared to be similar to *clp1*Δ in that both mutants accumulated unseptated multinucleate cells upon exposure to LatA and lost viability under these conditions.

Previous studies have shown that *clp1*Δ cells are sensitive to a variety of perturbations to the cell-division apparatus. We found that Rad24p was also essential for the viability of several cytokinesis mutants under semipermissive conditions. The mutants tested included several components of the actomyosin ring and the division septum synthesizing machinery (Figure 1D). Previous studies have demonstrated that in response to cytokinetic failure, mutants such as *cps1*-191, *cdc3*-124, *cdc12*-112, *myo2*-E1, *rng3*-65, and *cdc15*-140, block as binucleate cells (or tetranucleate cells after prolonged shift to the restrictive temperature) in which the nuclei are delayed in G2. Unlike the single mutants, *cps1*-191 *rad24*Δ, *cdc3*-124 *rad24*Δ, and *cdc12*-112 *rad24*Δ accumulated multiple nuclei without significant G2 delay (Figures 1E and 1F and data not shown for *cdc3*-124 *rad24*Δ and *cdc12*-112 *rad24*Δ). Thus, Rad24p behaves like Clp1p in all aspects tested and is required for G2 delay and the completion of cytokinesis in re-

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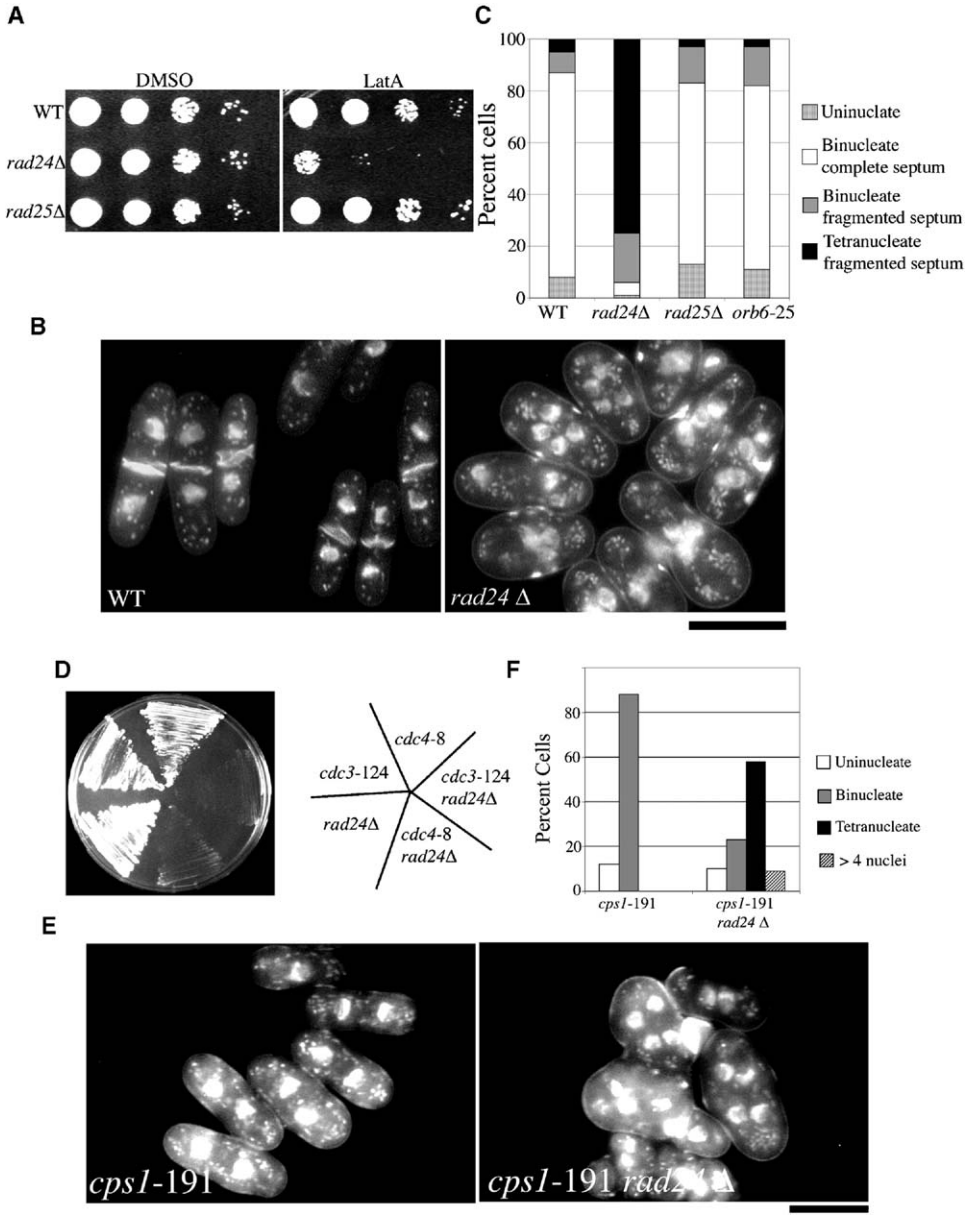

Figure 1. Cells Lacking Rad24p Are Sensitive to Perturbation of Cell Division Structures

(A) 10-fold serial dilutions of logarithmically growing wild-type, *rad24*Δ, and *rad25*Δ cells were plated on YES media containing 0.2 μM LatA or DMSO (solvent control) and incubated at 30°C for 3 days.

(B) Cells of the indicated genotype were grown to mid-log phase at 30°C and then treated with 0.2 μM LatA for 5 hr before fixing and staining with DAPI (nucleus) and aniline blue (cell wall/septa). Scale bar, 10 μm.

(C) Quantitative data for cells treated as in Figure 1B.

(D) Cells of the indicated genotype were streaked on YES plate and assayed for colony formation after 3 days at 30°C.

(E) *cps1*-191 and *cps1*-191 *rad24*Δ mutants were grown to mid-log phase at 24°C and then shifted to 36°C for 4 hours before fixing and staining with DAPI and aniline blue. Scale bar, 10 μm.

(F) Quantitative data for cells treated as in Figure 1E.

sponse to mild perturbation of the cell-division apparatus.

Having established that Rad24p was important for cytokinesis checkpoint maintenance, we addressed if Rad24p was important for controlling the cytoplasmic localization of Clp1p. To this end, we treated wild-type and *rad24*Δ cells expressing Clp1p-GFP with LatA or DMSO (solvent control). The localization of Clp1p was similar in wild-type and *rad24*Δ cells treated with DMSO in that Clp1p was present in the nucleolus and SPB in interphase and in the cytoplasm, actomyosin ring, and the mid-spindle during mitosis (Figure 2A). Interestingly, whereas Clp1p signal in the cytoplasm was roughly similar to that in the nucleus in LatA-treated wild-type

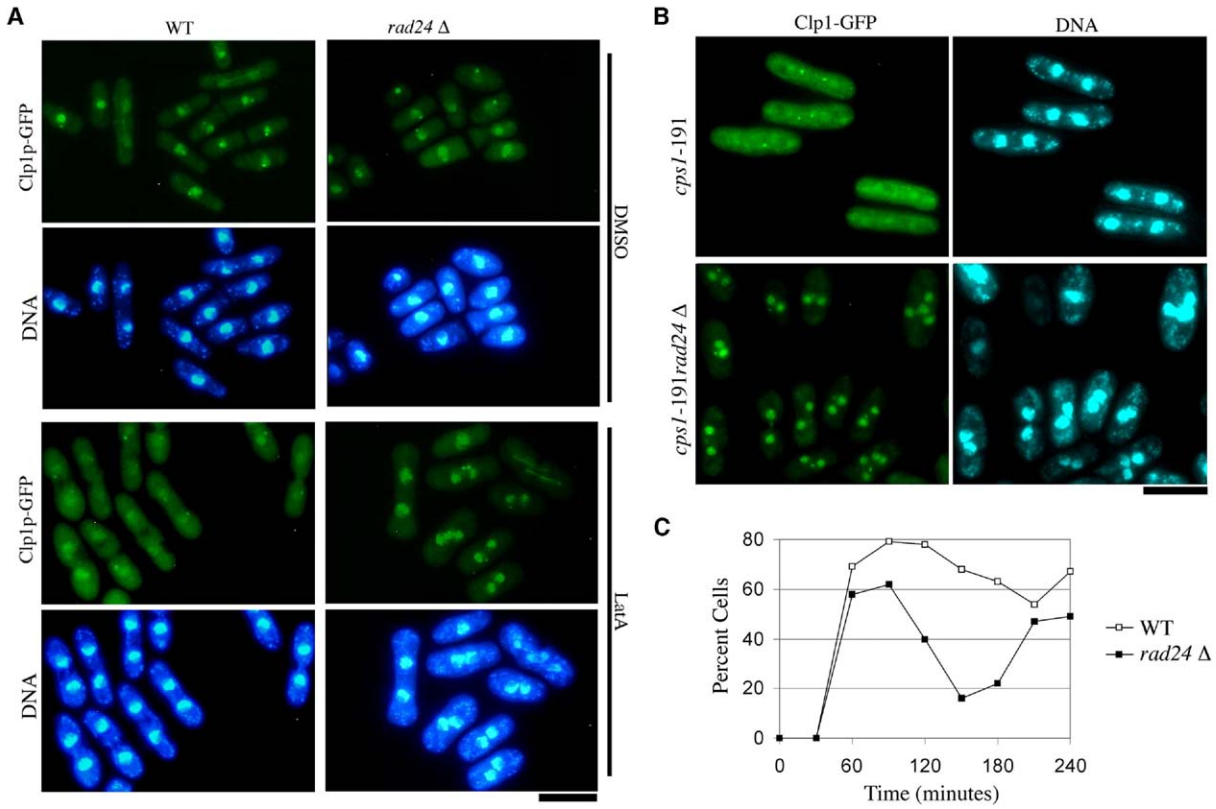

Figure 2. Rad24p Is Required for Cytoplasmic Retention of Clp1p and for Prolonged SIN Signaling upon Cytokinetic Perturbation

(A) Wild-type and *rad24*Δ cells carrying an integrated copy of Clp1p-GFP were grown to were grown to mid-log phase at 30°C and then treated with 0.2 μM LatA for 3.5 hr before fixing and staining with DAPI and aniline blue. Clp1p-GFP localization was monitored by using GFP autofluorescence. Scale bar, 10 μm.

(B) *cps1*-191and *cps1*-191*rad24*Δ cells carrying an integrated copy of Clp1p-GFP were grown to mid-log phase at 25°C and then shifted to 36°C 4 hr before fixing and staining with DAPI and aniline blue. Clp1p-GFP localization was monitored by using GFP fluorescence. Scale bar, 10 μm.

(C) Wild-type and *rad24*Δ cells carrying integrated copies of Cdc7p-GFP were cultured to mid-log phase at 30°C, synchronized in early G2 by centrifugal elutriation, and then treated with 0.2 μM LatA or DMSO (solvent control) and cultured at 30°C. Cells were fixed at 30 min intervals, stained with antibodies against GFP, and scored for localization of Cdc7p-GFP to the spindle pole body.

cells (nucleus/cytoplasmic intensity ratio of 1.30 ± 0.22 out of 60 cells), Clp1p was largely nuclear in *rad24*Δ cells treated with LatA (nucleus/cytoplasmic intensity ratio of 1.91 ± 0.43 out of 60 cells). These studies suggested that continued cytoplasmic localization of Clp1p upon LatA treatment depended on Rad24p function. Similarly, Clp1p-GFP localization to the cytoplasm of heat-arrested *cps1*-191 cells (which activates a cytokinesis delay by a different means) also depended on Rad24p function. Whereas, Clp1p-GFP signal (Figure 2B) in the nucleus was comparable to that in the cytoplasm in heat-arrested *cps1*-191 cells (nucleus/cytoplasmic intensity ratio of 1.06 ± 0.14 out of 60 cells), Clp1p-GFP signal was enriched in the nucleus of similarly treated *cps1*-191 *rad24*Δ cells (nucleus/cytoplasmic intensity ratio of 2.19 ± 0.51 out of 60 cells). Thus, under a variety of conditions that perturb the cell-division apparatus, Rad24p appears to be involved in the cytoplasmic retention of Clp1p.

In fission yeast, actomyosin ring maintenance during late stages of cytokinesis and division septum assembly depends on the SIN. Previous studies have shown that the duration of SIN signaling is prolonged in wild-type cells treated with LatA but that SIN signaling declines rapidly in *clp1*Δ cells treated with LatA. Because Clp1p is known to prolong SIN signaling [1, 4], we asked if the loss of Rad24p also led to a rapid decrease of SIN signaling. To this end, we synchronized wild-type and *rad24*Δ cells expressing Cdc7p-GFP by centrifugal elutriation, and small G2 cells were treated with LatA. The SIN component Cdc7p localizes to one of the two SPBs late in mitosis, and remains there until the completion of cytokinesis, and has been correlated with an active SIN configuration (reviewed in [9]). Interestingly, whereas Cdc7p was retained at one SPB for more than 2 hr in wild-type cells treated with LatA, Cdc7p was rapidly lost from the SPB when *rad24*Δ cells were treated similarly (Figure 2C). These studies established that Rad24p, similarly to Clp1p, was required for increasing the duration of SIN signaling in response to stressors of the cell-division apparatus.

Given the conclusion that Rad24p was important for

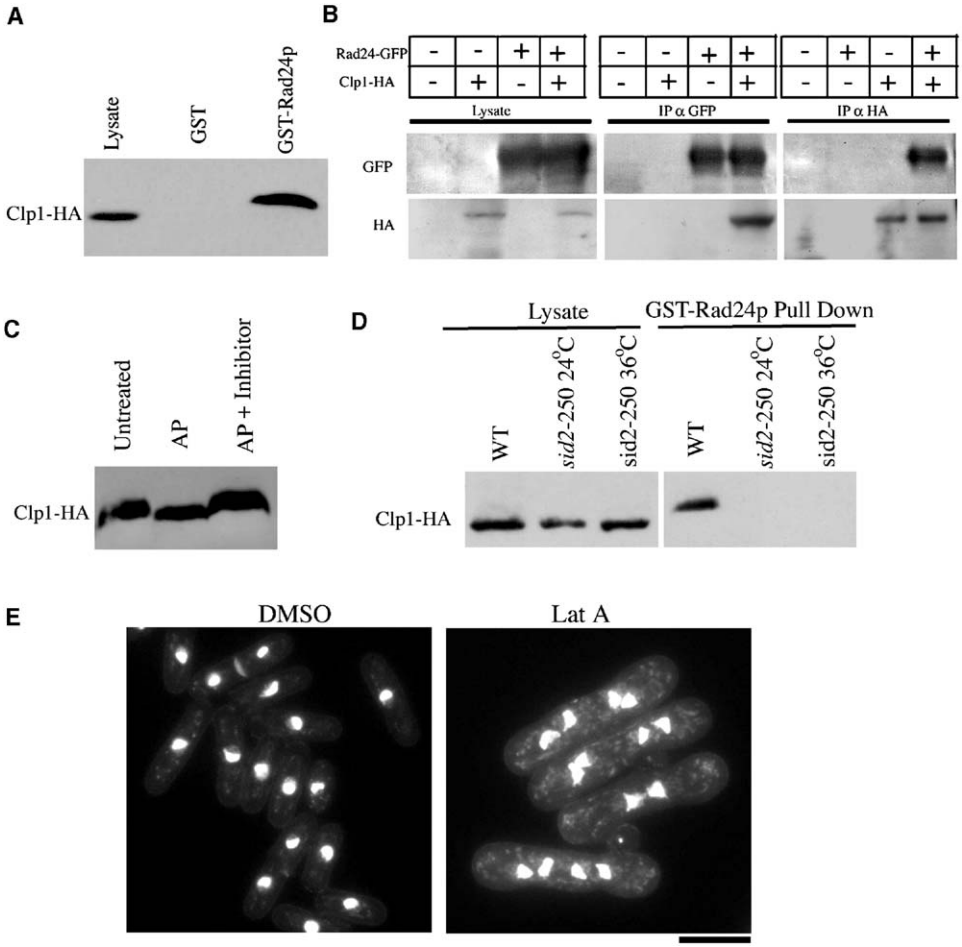

Figure 3. Rad24p Physically Associates with Phosphorylated Clp1p In Vitro

(A) GST and Rad24-GST were produced in bacteria and mixed with protein extracts prepared from *clp1*-HA cells. The complexes precipitated with glutathione-agarose were Western blotted and probed with mAB 12-CA5 (anti-HA).

(B) Wild-type, *clp1*-HA, *rad24*-GFP, and *clp1*-HA *rad24*-GFP cells were grown to mid-log phase and lysed under native conditions. Part of the lysate were subjected to anti-HA and anti-GFP immunoprecipitations. Both total lysates and immunoprecipitates were resolved by SDS page and immunoblotted by either mAB 12-CA5 or anti-GFP polyclonal antibodies.

(C) The Rad24p-GST pull-down complex was treated with calf-intestinal alkaline phosphatase in the presence or absence of phosphatase inhibitor and was analyzed by Western blotting with 12CA5 antibodies.

(D) Clarified protein extracts prepared from *clp1*-HA or *clp1*-HA *sid2*-250 grown either at permissive temperature of 25°C or 4 hr temperature shift to the restrictive temperature of 36°C were mixed with Rad24-GST. The complexes precipitated with glutathione-agarose were Western blotted and probed with mAB 12-CA5 (anti-HA).

(E) *sid2*-250 cells were grown to mid-log phase at 25°C and then treated with 0.2 μM LatA for 8 hr at 25°C before fixing and staining with DAPI (nucleus) and aniline blue (cell wall/septa). Scale bar, 10 μm.

cytoplasmic retention of Clp1p and the checkpoint response, we addressed if Rad24p physically bound to Clp1p. To address this question, we utilized two approaches. In the first approach, we assayed the ability of GST-Rad24p expressed in *E. coli* to bind HA-tagged Clp1p isolated from *S. pombe* cell lysates. Interestingly, whereas Clp1p-HA did not bind to the control GST beads, Clp1p-HA was efficiently isolated on GST-Rad24p beads, suggesting physical interaction between Rad24p and Clp1p (Figure 3A). The GST-Rad24p bound Clp1p-HA migrated slower than that isolated in total lysates from asynchronous cells, suggesting that a potentially phosphorylated form of Clp1p might bind Rad24p. To independently confirm this physical interaction between Rad24p and Clp1p, we utilized a coimmunoprecipitation assay. To achieve this, we constructed a strain in which the endogenous Rad24p was tagged with GFP and the endogenous Clp1p was tagged with the HA-epitope. Immune complexes generated with the mAB 12-CA5 (anti HA-antibodies) also contained Rad24p-GFP, and immune complexes generated with GFP-antibodies also contained Clp1p-HA (Figure 3B). Rad24p has also been isolated by McCollum and colleagues (accompanying study by Trautmann and McCollum in this issue of *Current Biology* [12]) as a Clp1p binding partner. Thus, Rad24p physically interacts with Clp1p.

Clp1p-HA was isolated on GST-Rad24p beads and incubated with alkaline phosphatase or with alkaline

phosphatase and its inhibitors to test if Clp1p bound to GST-Rad24p was indeed phosphorylated. We found that Clp1p-HA treated with AP was faster migrating compared with GST-Rad24p bound Clp1p-HA and Clp1p-HA treated with AP and inhibitors (Figure 3C). These experiments suggested that a phosphorylated form of Clp1p-HA bound to Rad24p. Given that Clp1p and the SIN participate in the cytokinesis-checkpoint response and function in a positive-feedback loop, it was possible that phosphorylation of Clp1p was mediated by elements of the SIN. We therefore assayed the ability of Clp1p-HA isolated from *sid2*-250 cells grown at 24°C and 36°C. Surprisingly, we found that Clp1p-HA isolated from *sid2*-250 grown at the permissive and restrictive temperatures was unable to bind GST-Rad24p beads (Figure 3D), although Clp1p-HA isolated from wild-type cells under identical conditions bound Rad24p. Given that Clp1p-HA isolated from permissive-temperature-grown *sid2*-250 did not bind Rad24p, we predicted that *sid2*-250 might be cytokinesis-checkpoint defective at 24°C. Consistently, we found that *sid2*-250 cells accumulated multiple nuclei upon exposure to LatA but not DMSO (Figure 3E). We conclude that a phosphorylated form of Clp1p binds Rad24p and that this binding requires function of Sid2p and possibly other members of the SIN.

Previous studies have shown that the function of Clp1p in maintaining the "cytokinesis-competent" state can be largely bypassed by ectopic activation of the SIN [1]. We therefore assayed if the function of Rad24p in the cytokinesis checkpoint could also be bypassed by these means. SIN represents a GTPase-controlled signaling cascade that can be maintained in the active state by overproduction of the Spg1p-GTPase or by mutation of the components of the GAP for Spg1p, Cdc16p, and Byr4p [9]. We therefore constructed a strain of the genotype *cdc16*-116 *rad24*Δ and compared the ability of this strain and the two parental single mutants to complete cytokinesis in response to LatA treatment. We also studied the localization of Clp1p-GFP upon LatA treatment in these strains. As shown previously, *rad24*Δ cells were incapable of completing cytokinesis (Figure 4; Figure S2). In addition, consistent with previous studies [1], *cdc16*-116 cells were able to complete cytokinesis and division-septum assembly upon LatA treatment (Figure 4; Figure S2). Interestingly, mutation of *cdc16* bypassed the requirement for Rad24p in G2 delay and completion of cytokinesis in response to cytokinetic defects. As shown in Figure 4, *cdc16*-116 *rad24*Δ cells, unlike *rad24*Δ single mutants, were largely binucleate and contained a complete septum that bisected the cell. As expected, Clp1p was nucleolar in LatA-treated *rad24*Δ cells (nucleus/cytoplasmic intensity ratio of 3.0 ± 0.8 out of 50 cells) and was not enriched in the nucleolus in LatA-treated *cdc16*-116 cells (nucleus/cytoplasmic intensity ratio of 1.28 ± 0.34 out of 60 cells). Surprisingly, in LatA-treated *cdc16*-116 *rad24*Δ cells, Clp1p was localized in the nucleolus (nucleus/cytoplasmic intensity ratio of 2.47 ± 0.69 out of 50 cells). These experiments suggested that the primary role of Rad24p, similar to Clp1p, was to prolong the duration of SIN signaling in order to allow completion of cytokinesis. These experiments also demonstrated that Clp1p was not required to be present in the cytoplasm in order to allow completion of cytokinesis, if SIN pathway is maintained in an active state. These experiments also revealed that nuclear exclusion of Clp1p under SIN-active conditions depends on Rad24p.

Given the role of Rad24p in prolonging SIN signaling and in its role in the completion of cytokinesis under conditions of stress to the cell division apparatus, we proceeded to study the intracellular localization of Rad24p. To this end, we constructed a strain expressing chromosomally tagged Rad24p-GFP. This fusion protein is fully functional because the cells expressing this fusion resemble wild-type cells in morphology (Figures 5A and 5B) and are able to form colonies on LatA-containing plates and remain largely binucleate, unlike the *rad24*Δ mutant, which dies under similar conditions with multiple nuclei (Figure S3). Rad24p displayed a striking cell-cycle-dependent localization pattern. Rad24p was detected in the cytoplasm at all stages of the cell cycle. Interestingly, Rad24p was also detected at the SPBs and the actomyosin ring in mitotic and postmitotic cells undergoing cytokinesis (Figure 5A). The SPB localization was further confirmed by its colocalization with DsSad1p, a known component of the SPB (Figure 5B). Consistent with a role in maintaining a "cytokinesis-competent" state, Rad24p was detected in the SPB and the actomyosin ring in cells in which cytokinesis was delayed either by mutation of *cps1*-191 (Figure 5C) or upon treatment of cells with Latrunculin A (data not shown).

Because Rad24p plays a key role in modulating the function of Clp1p and the SIN, we were interested to determine whether Clp1p and the SIN affected the localization of Rad24p. Rad24p localization was largely unaffected in *clp1*Δ cells. For analysis of the role of SIN in localization of Rad24p, wild-type, *cdc11*-123, *sid4*-A1, and *sid2*-250 cells expressing Rad24p-GFP were synchronized in early G2 by centrifugal elutriation and shifted to restrictive temperature of 36°C, and the localization of Rad24p followed every 30 min. Rad24p localized to the SPB and the actomyosin ring in mitotic wild-type cells and also in cells lacking the downstream SIN component Sid2p but failed to localize to the SPB in cells lacking Cdc11p and Sid4p (Figures 5D and 5E and data not shown for *sid4*-A1), two known scaffold components of the SIN that are important for the loading of numerous regulators of cytokinesis to the SPB [13, 14]. Approximately 60%–80% of mitotic wild-type and *sid2*-250 cells contained SPB-localized Rad24p, whereas less than 5% of mitotic *cdc11*-123 cells displayed SPB localization of Rad24p at the restrictive temperature. These experiments revealed that Rad24p colocalized with molecules important for cytokinesis-checkpoint execution and also depended on the scaffold proteins Cdc11p and Sid4p for its localization to the SPB.

Previous studies have demonstrated that fission yeast cells delay in a cytokinesis-competent phase in response to minor perturbations of the cell-division apparatus. In addition, these studies have also suggested that cytoplasmic retention of the Cdc14p family phosphatase Clp1p was important for the checkpoint, although the mechanism of cytoplasmic retention of Clp1p was unknown [1]. In this study, we have shown that the retention of Clp1p in the cytoplasm depends on the 14-3-3

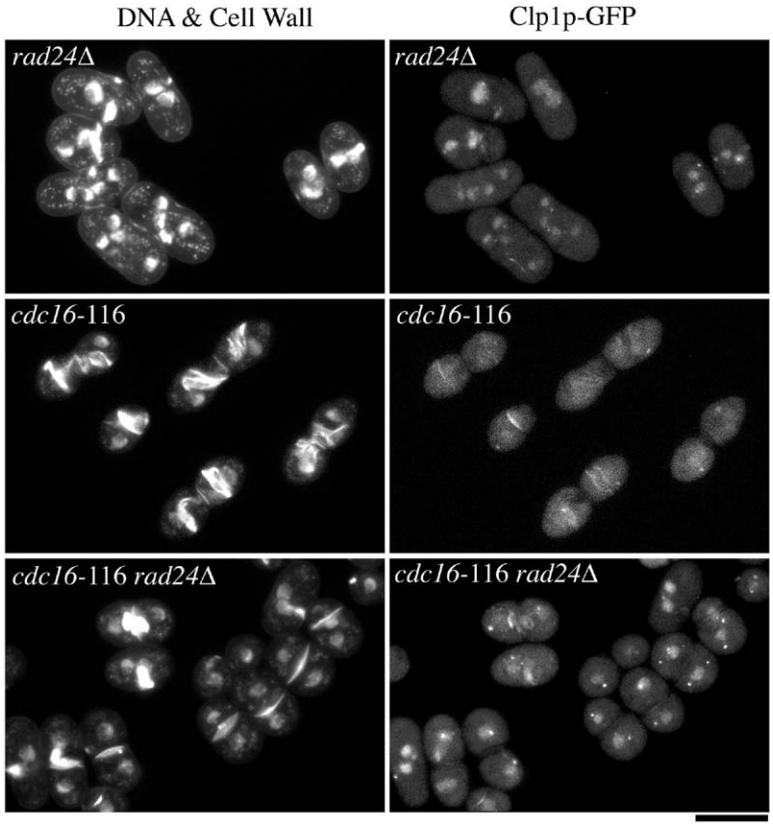

Figure 4. Ectopic Activation of the SIN Compensates for the Loss of Rad24p during Cytokinesis

Cells of the indicated genotype carrying an integrated copy of Clp1p-GFP were grown to mid-log phase at 25°C, shifted to 36°C, and treated with 0.2 μM LatA for 5 hr before fixing and staining with DAPI and aniline blue. Clp1p-GFP localization was monitored by using GFP autofluorescence. Scale bar, 10 μm.

protein Rad24p but not the related 14-3-3 protein Rad25p. We have also shown that Rad24p physically interacts with a phosphorylated form of Clp1p and, thus, the cytoplasmic retention is likely to be mediated by this physical interaction. Given that Cdc14p is member of a highly conserved family of protein phosphatases [15–22], regulation of its localization by 14-3-3 proteins appears to be a strong possibility.

In this context, it is worth noting that retention of the mitotic activator Cdc25p in the cytoplasm in response to DNA damage depends on binding of phosphorylated Cdc25p to Rad24p. It has been proposed that Rad24p binding blocks nuclear localization of Cdc25p, which is nuclear at steady state and undergoes shuttling between the nucleus and cytoplasm, thereby leading to progressive accumulation of Cdc25p in the cytoplasm [23, 24]. Given that Clp1p and Rad24p are cytoplasmic during normal cytokinesis and that Rad24p is not enriched in the nucleus upon cytokinesis delays, we prefer the idea that Rad24p prevents nuclear accumulation of Clp1p. This proposal is consistent with the finding that Clp1p fused to a nuclear export sequence was able to partially bypass the checkpoint defect of *rad24*Δ mutants [12]. However, additional studies are required to rule out the possibility that Rad24p promotes nuclear export of Clp1p.

The fact that Clp1p fused to a nuclear export sequence only partially bypasses the checkpoint defect of *rad24*Δ mutants suggests that Rad24p might have additional targets important for cytokinesis regulation. Consistently, we have observed synthetic negative effects upon deletion of *rad24* and *clp1* (Figure S4). A strong possibility is that Rad24p might bind to members of the SIN in order to maintain their cellular locations and/or prolong the duration of activated states of various components of the SIN. In this context, it is interesting that Rad24p colocalizes with SIN components at the SPB and depends on Cdc11p and Sid4p, two known components of the SIN, for its localization. In addition, we have found that Cdc7p and Sid1p physically interact with Rad24p (data not shown). Future studies should clarify the role of Rad24p in regulating SIN function.

We have also shown that Clp1p binding to Rad24p is dependent on the function of the SIN component, Sid2p. The fact that SIN is also important for the cytokinesis checkpoint suggests that SIN-dependent phosphorylation of Clp1p might allow binding to Rad24p, which in turn might prolong the duration of SIN signaling in order to facilitate completion of cytokinesis upon stress to the cell-division structures. We have previously shown by fluorescence-recovery-after-photobleaching (FRAP) experiments that SIN components shuttle between the cytoplasm and the SPB. We therefore imagine that cytoplasmically localized Clp1p might dephosphorylate cytoplasmically localized SIN molecules, which might then remain active over longer periods. The Clp1p/Rad24p-mediated prolonged activation of SIN is consistent with the finding that constitutively active SIN bypasses the requirement for Clp1p [1] and Rad24p (this study) in cytokinesis checkpoint response. Future studies should address the identity of the kinases that

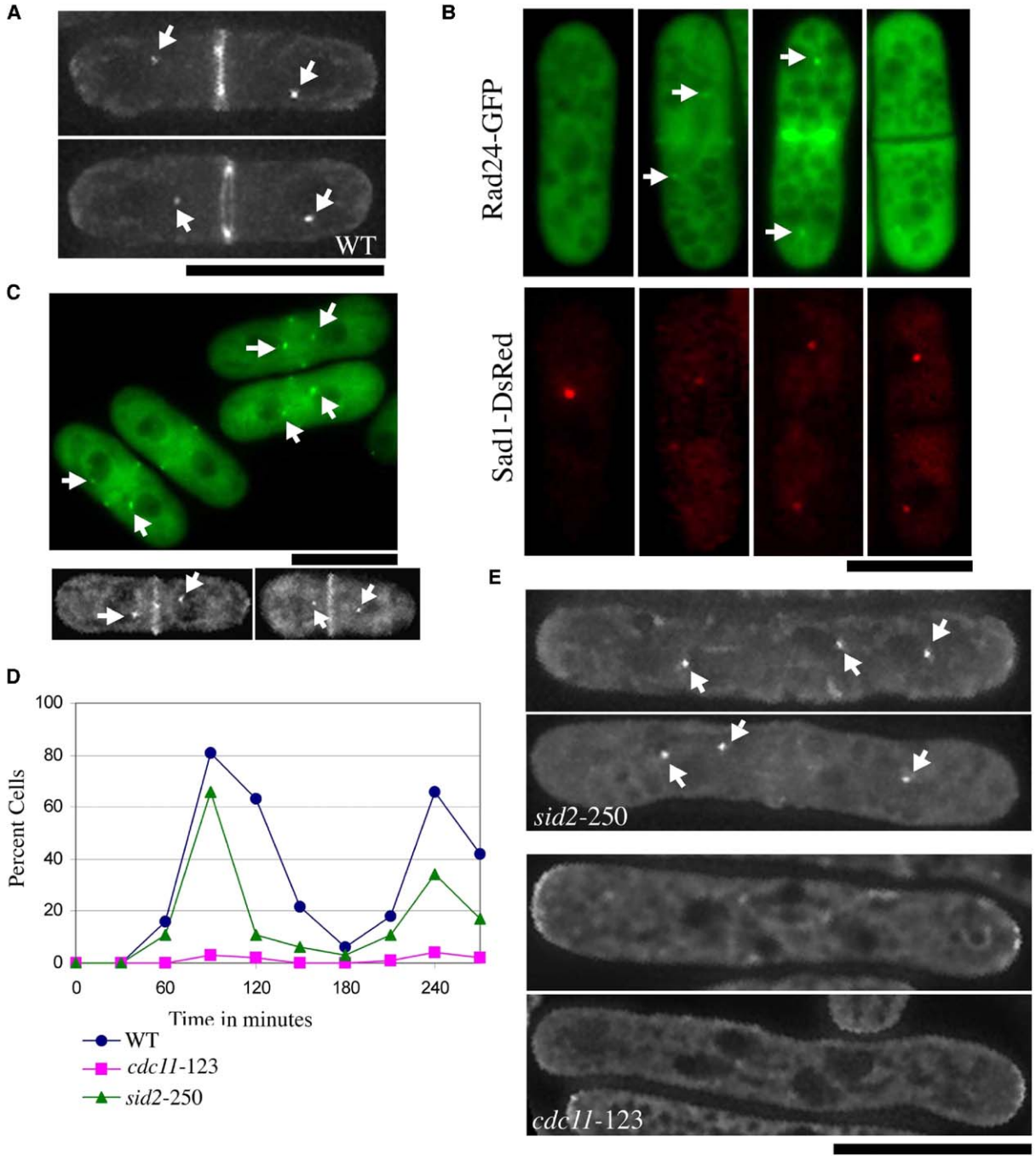

Figure 5. Rad24p Localizes to the Actomyosin Ring and the Spindle Pole Body in Cells Undergoing Mitosis and Cytokineisis in a SIN-Dependent Manner

(A) Deconvolved maximal projections of Rad24-GFP-expressing cells during cytokinesis.

(B) Cells expressing Rad24-GFP and Sad1-DsRed under their native promoters were cultured to mid-log phase at 19°C and observed under microscope. Cells at different stages of the cell cycle are shown. Note that Rad24p-GFP signal is enriched on the SPB and the actomyosin ring in cells undergoing mitosis and persists until cells complete cytokinesis.

(C) *cps1*-191 Rad24p-GFP cells were grown to mid-log phase at 24°C and then shifted to 36°C for 3 hr before images were acquired. Deconvolved maximal projections are shown below.

(D) Cells of the indicated genotype carrying an integrated copy of Rad24p-GFP were grown to mid-log phase at 30°C, synchronized in early G2 by centrifugal elutriation, and then shifted to 36°C. Plot shows percent cells with detectable Rad24p signal at the SPB.

(E) Deconvolved maximal projections of *sid2*-250 and *cdc11*-123 cells shifted to 36°C for 3.5 hr. Note that unlike *sid2*-250 cells, *cdc11*-123 cells do not show any enrichment of Rad24p signal on the SPB in mitotic cells.

directly phosphorylate Clp1p and the mechanism by which cytoplasmically localized Clp1p precisely prolongs the duration of SIN signaling.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://www.current-biology.com/cgi/content/full/15/15/1376/DC1/.

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