DNA Damage Checkpoints Inhibit Mitotic Exit by Two Different Mechanisms[▽]

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Received 16 January 2007/Returned for modification 15 February 2007/Accepted 30 April 2007

Cyclin-dependent kinase (CDK) governs cell cycle progression, and its kinase activity fluctuates during the cell cycle. Mitotic exit pathways are responsible for the inactivation of CDK after chromosome segregation by promoting the release of a nucleolus-sequestered phosphatase, Cdc14, which antagonizes CDK. In the budding yeast *Saccharomyces cerevisiae*, mitotic exit is controlled by the FEAR (for “Cdc-fourteen early anaphase release”) and mitotic exit network (MEN) pathways. In response to DNA damage, two branches of the DNA damage checkpoint, Chk1 and Rad53, are activated in budding yeast to prevent anaphase entry and mitotic exit, allowing cells more time to repair damaged DNA. Here we present evidence indicating that yeast cells negatively regulate mitotic exit through two distinct pathways in response to DNA damage. Rad53 prevents mitotic exit by inhibiting the MEN pathway, whereas the Chk1 pathway prevents FEAR pathway-dependent Cdc14 release in the presence of DNA damage. In contrast to previous data, the Rad53 pathway negatively regulates MEN independently of Cdc5, a Polo-like kinase essential for mitotic exit. Instead, a defective Rad53 pathway alleviates the inhibition of MEN by Bfa1.

Cyclin-dependent kinase (CDK) governs cell cycle progression, and the activity of CDK is tightly regulated during the cell cycle. CDK activity is high during the S and M phases, but cells have to inactivate CDK after chromosome segregation in order to initiate DNA synthesis for the next cell cycle (16, 20). Cdc14, a protein phosphatase, plays an important role in the inactivation of CDK. In budding yeast, Cdc14 is mainly regulated through its cellular localization. Before chromosome segregation, Cdc14 is sequestered within the nucleolus through its association with Net1/Cfi1, a nucleolar protein (26, 32). Cdc14 is released into the nucleus following chromosome segregation and dephosphorylates its substrates, resulting in the degradation of mitotic cyclins and the accumulation of the CDK inhibitor Sic1, both of which contribute to CDK inactivation (31).

In budding yeast, two signal transduction cascades, the mitotic exit network (MEN) and FEAR (for “Cdc-fourteen early anaphase release”) pathways, control mitotic exit by regulating Cdc14 localization (19). The components of the MEN pathway include the protein kinases Cdc5, Cdc15, and Dbf2; a GTPase, Tem1; Cdc14; and a Dbf2 binding protein, Mob1 (11). Tem1 plays a central role in the regulation of MEN. Before anaphase, the spindle pole body (SPB)-localized GTPase Tem1 remains inactive due to its association with Bfa1-Bub2, a GAP complex (12, 21, 35). After one of the SPBs enters the daughter cell, SPB-associated Tem1 encounters its activator, Lte1, which localizes within daughter cells, leading to the activation of the MEN pathway (5). Another layer of regulation is Cdc5-dependent Bfa1 phosphorylation, which frees Tem1 from inhibition by Bfa1-Bub2 (14). The FEAR pathway comprises the Polo kinase Cdc5, the separase Esp1, the kinetochore-associated protein Slk19, and

Spo12 (27). As opposed to MEN, which induces Cdc14 release in telophase, FEAR contributes to the release of Cdc14 from the nucleolus during early anaphase. It is believed that the FEAR-dependent initial Cdc14 release promotes activation of the MEN pathway by dephosphorylating Cdc15.

Recent evidence indicates a negative role of protein phosphatase 2A (PP2A) in mitotic exit. In a genetic screen for genes that are toxic to *cdc5-1* mutants when overexpressed, *CDC55*, *PPH21*, and *PPH22*, which encode the B-regulatory and catalytic subunits of PP2A, respectively, were identified (36). PP2A mutant cells exhibit Cdc14 release in the presence of nocodazole, a microtubule poison (36, 39). Interestingly, Esp1, a component of the FEAR network, promotes Cdc14 release by inactivating PP2A^{Cdc55}. Biochemical evidence indicates that PP2A^{Cdc55} dephosphorylates Net1 and antagonizes its phosphorylation by CDK. Therefore, it is likely that FEAR-dependent inactivation of PP2A^{Cdc55} promotes Net1 phosphorylation and leads to Cdc14 release (3, 22). In addition to the function in mitotic exit, Cdc55 also plays a role in sister chromatid separation (18, 34). We have shown that Cdc55 regulates sister chromatid separation in Pds1-dependent and -independent manners (29).

In response to DNA damage, cells activate DNA damage checkpoints that prevent both anaphase entry and mitotic exit (23). In the budding yeast *Saccharomyces cerevisiae*, the Mec1 DNA damage checkpoint kinase, a member of the conserved family of phosphatidylinositol 3-kinases, phosphorylates its downstream targets, Chk1 and Rad53, in the presence of DNA damage (23, 24). Activated Chk1 kinase phosphorylates the anaphase inhibitor Pds1 and prevents its degradation (33). It is well documented that Pds1 blocks anaphase entry by inhibiting the separase Esp1, which cleaves the cohesin Scc1 and leads to sister chromatid separation (9, 30). In the presence of DNA damage, *chk1Δ* mutants form minicolonies, indicating that the cells are able to exit mitosis. It has been shown that Clb2, the mitotic cyclin of budding yeast, is stable in cells that overproduce nondegradable Pds1 (10). Also, the failure of Pds1 degradation prevents

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[▽] Published ahead of print on 7 May 2007.

TABLE 1. Strains used in this study

Strain	Relevant genotype	Source
Y300	<i>MATa trp1-1 ura3-1 his3-11,15 leu2-3,112 ade2-1 can1-100</i>	Laboratory stock
SAY801	<i>MATa cdc14::CDC14-5-GFP-TRP1</i>	A. Toh-e
RJD2862	<i>MATa Δnet1::his5⁺ net1-6Cdk-TEV-myc9::TRP1</i>	R. J. Deshaies
664-17-3	<i>MATa cdc13-1 cdc14::CDC14-5-GFP-TRP1</i>	This study
686-6-1	<i>MATa cdc13-1 Δchk1::HIS3 cdc14::CDC14-5-GFP-TRP1</i>	This study
714-2-1	<i>MATa cdc13-1 Δchk1::HIS3 Δnet1::his5⁺ net1-6Cdk-TEV-myc9::TRP1 cdc14::CDC14-5-GFP-TRP1</i>	This study
736-3-1	<i>MATa cdc13-1 Δchk1::HIS3 cdc15-2 cdc14::CDC14-5-GFP-TRP1</i>	This study
685-3-3	<i>MATa cdc13-1 pds1-m9-URA3 cdc14::CDC14-5-GFP-TRP1</i>	This study
715-3-1	<i>MATa cdc13-1 pds1-m9-URA3 Δnet1::his5⁺ net1-6Cdk-TEV-myc9::TRP1 cdc14::CDC14-5-GFP-TRP1</i>	This study
664-27-2	<i>MATa cdc13-1 Δcdc55::his5⁺ cdc14::CDC14-5-GFP-TRP1</i>	This study
674-5-1	<i>MATa cdc13-1 Δcdc55::his5⁺ Δnet1::his5⁺ net1-6Cdk-TEV-myc9::TRP1 cdc14::CDC14-5-GFP-TRP1</i>	This study
712-7-1	<i>MATa cdc13-1 Δcdc55::his5⁺ cdc15-2 cdc14::CDC14-5-GFP-TRP1</i>	This study
667-12-2	<i>MATa cdc13-1 rad53-21 cdc14::CDC14-5-GFP-TRP1</i>	This study
687-4-1	<i>MATa cdc13-1 rad53-21 Δnet1::his5⁺ net1-6Cdk-TEV-myc9::TRP1 cdc14::CDC14-5-GFP-TRP1</i>	This study
713-3-1	<i>MATa cdc13-1 rad53-21 cdc15-2 cdc14::CDC14-5-GFP-TRP1</i>	This study
666-1-2	<i>MATa cdc13-1 Δdun1::HIS3 cdc14::CDC14-5-GFP-TRP1</i>	This study
663-4-2	<i>MATa cdc13-1 Δbfa1::his5⁺ cdc14::CDC14-5-GFP-TRP1</i>	This study
663-3-1	<i>MATa cdc13-1 Δdun1::HIS3 pds1-m9-URA3 cdc14::CDC14-5-GFP-TRP1</i>	This study
665-11-3	<i>MATa cdc5-2::URA3 cdc14::CDC14-5-GFP-TRP1</i>	This study
738-8-1	<i>MATa cdc5-1 cdc14::CDC14-5-GFP-TRP1</i>	This study
737-3-3	<i>MATa cdc5-1 rad53-21 cdc14::CDC14-5-GFP-TRP1</i>	This study
708-10-3	<i>MATa cdc13-1 rad53-21 cdc5-2::URA3 cdc14::CDC14-5-GFP-TRP1</i>	This study
710-3-3	<i>MATa cdc13-1 BFA1-myc13-his5⁺ TEM1-HA3-TRP1</i>	This study
722-9-4	<i>MATa cdc13-1 rad53-21 BFA1-13myc-his5⁺ TEM1-HA3-TRP1</i>	This study
YFH336	<i>MATa cdc13-1 BFA1-13myc-his5⁺</i>	Laboratory stock
722-1-2	<i>MATa cdc13-1 rad53-21 BFA1-13myc-his5⁺</i>	This study
YYW117	<i>MATa cdc13-1 TUB1-GFP</i>	This study
YYW118	<i>MATa cdc13-1 Δdun1::HIS3 TUB1-GFP</i>	This study
YYW119	<i>MATa cdc13-1 rad53-21 TUB1-GFP</i>	This study
YYW120	<i>MATa cdc13-1 Δchk1::HIS3 TUB1-GFP</i>	This study
YYW121	<i>MATa cdc13-1 Δbfa1::his5⁺ TUB1-GFP</i>	This study
YYW122	<i>MATa cdc13-1 Δcdc55::his5⁺ TUB1-GFP</i>	This study
YYW123	<i>MATa cdc13-1 pds1-m9-URA3 TUB1-GFP</i>	This study
819-5-4	<i>MATa cdc13-1 Δchk1::HIS3 pds1-m9-URA3</i>	This study
817-5-2	<i>MATa cdc13-1 rad53-21 Δdun1::HIS3</i>	This study
820-1-2	<i>MATa cdc13-1 Δchk1::HIS3 rad53-21</i>	This study
822-3-1	<i>MATa cdc13-1 Δchk1::HIS3 Δcdc55::his5⁺</i>	This study
818-8-2	<i>MATa cdc13-1 rad53-21 Δcdc55::his5⁺</i>	This study
824-1-2	<i>MATa cdc13-1 Δdun1::HIS3 BFA1-13myc-his5⁺</i>	This study
815-1-1	<i>MATa cdc13-1 Δdun1::HIS3 BFA1-13myc-his5⁺ TEM1-HA3-TRP1</i>	This study

both Clb2 degradation and Cdc14 release (25). Furthermore, *PDS1* overexpression blocks Net1 phosphorylation, which is required for the dissociation of Cdc14 from Net1, suggesting a negative role of Pds1 in mitotic exit (28). Thus, it is believed that the Chk1-Pds1 pathway controls mitotic exit through Esp1, a component of the FEAR pathway.

It is believed that the Rad53 kinase plays a more important role in preventing mitotic exit, as Clb2 protein levels decrease rapidly in *rad53* mutant cells in the presence of DNA damage. Interestingly, *cdc5-1* mutants block the anaphase entry of *rad53* mutants in the presence of DNA damage (23), raising the possibility that Cdc5 is the target of the Rad53-dependent checkpoint. However, Cdc5 kinase activity has been demonstrated to be high in DNA damage-arrested cells (8). Moreover, Bfa1, a substrate of Cdc5, is phosphorylated in DNA damage-arrested cells, which argues against the role of Cdc5 as a target of DNA damage checkpoints.

Here, we have investigated the role of DNA damage check-

point components in preventing mitotic exit. Chk1, Pds1, Rad53, and Dun1 are all required for the nucleolar localization of Cdc14 in DNA damage-arrested yeast cells. Moreover, PP2A and Bfa1, the negative regulators of FEAR and MEN, are also required to prevent mitotic exit in the presence of DNA damage. Rad53 and Dun1 may negatively regulate the MEN pathway in a Cdc5-independent manner. However, the loss of function of Rad53 or Dun1 alleviates inhibition of the MEN pathway by Bfa1. The presence of Chk1, Pds1, or PP2A prevents Net1 phosphorylation and subsequent Cdc14 release in the presence of DNA damage. Therefore, inactivation of both the MEN and FEAR pathways by DNA damage checkpoints contributes to the block of mitotic exit.

MATERIALS AND METHODS

Yeast strains and growth. The yeast strains used in this study are listed in Table 1. All strains are isogenic with Y300, a derivative of W303. Yeast cells were grown in yeast extract-peptone-dextrose (YPD) medium except where indicated. To arrest cells in G₁ phase, 5 μg/ml α-factor was added to the cell culture. After

FIG. 1. DNA damage checkpoint genes are required for mitotic arrest. (A) Microcolony formation of *cdc13-1* single and double mutant cells. Cells with the indicated genotypes were grown at 25°C in YPD to mid-log phase and then plated onto prewarmed YPD plates and incubated at 37°C. After 8 h of incubation, the growth of cells was examined under a microscope. Percentages of cells that formed microcolonies (more than four cells) are shown. The growth of representative cells on YPD plates is shown on the right. (B) Spindle morphology of *cdc13-1* single and double mutants. *TUB1-GFP* strains with the indicated genotypes were arrested in G₁ phase with α -factor at 25°C and then released into YPD medium and incubated at 34°C. The spindle morphology was examined under a fluorescence microscope, and percentages of cells with a short spindle structure are shown. The spindle morphology of some cells after 4 h of incubation is shown on the right. (C) Budding index of *cdc13-1* single and double mutants. Cells with the indicated genotype were G₁ arrested and then sonicated before being released into the cell cycle at 34°C. The percentages of rebudded cells are shown at the top, and representative cells are shown at the bottom.

2 h of incubation, the G₁-arrested cells were washed twice with water and then released into YPD medium to start the cell cycle.

Cytological techniques. Cells with 5-green fluorescent protein (5-GFP)-tagged Cdc14 or GFP-tagged Tub1 were fixed with 3.7% formaldehyde for 5 min at room temperature and then washed twice with 1× phosphate-buffered saline (PBS) buffer and resuspended in 1× PBS buffer for microscopic examination.

For DAPI staining, cells were harvested and fixed with 100% methanol at −20°C for 30 min. Then, the cells were pelleted and resuspended in DAPI (4',6'-diamidino-2-phenylindole) (final concentration, 2.5 μg/ml) for 1 min at room temperature. The cells were finally resuspended in 1× PBS. A fluorescence microscope (Carl Zeiss MicroImaging, Inc.) was used to examine the GFP and DNA.

Protein techniques. For protein immunoprecipitation, 50 ml of cells were harvested and protein extracts were prepared with radioimmunoprecipitation assay (RIPA) buffer supplied with protease and phosphatase inhibitors (37). After incubation with antihemagglutinin (anti-HA) antibody (Covance Research Products, Inc.) at 4°C for 1.5 h, protein A/G plus agarose beads (Santa Cruz Biotechnology, Inc.) were added and the mixture was incubated for 1.5 h at 4°C. After three washes with RIPA buffer, the precipitates were resuspended in equal volumes (20 μ l) of RIPA buffer and sodium dodecyl sulfate (SDS) protein-loading buffer. The samples were then boiled for 5 min and resolved with an 8% SDS-polyacrylamide gel. Proteins were detected by enhanced chemiluminescence (Perkin Elmer LAS, Inc.) after being probed with primary antibody (anti-Myc or anti-HA antibodies; Covance Research Products, Inc.) and horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch, Inc.).

RESULTS

DNA damage checkpoint genes are required to prevent anaphase entry and mitotic exit in response to DNA damage. Both anaphase entry and mitotic exit are blocked by activated DNA damage checkpoints. To gain insights into mitotic exit regulation, we first tested which checkpoint genes are required to prevent cell division in the presence of DNA damage. At the restrictive temperature, budding yeast *cdc13-1* mutants accumulate single-stranded DNA at the telomeres that induces cell cycle arrest by activating the DNA damage checkpoint (17, 38). Microcolony formation was used to examine the cell division of the single and double mutants after 8 h of incubation at 37°C on YPD plates. Most *cdc13-1* single mutants were arrested as large budded cells and failed to form microcolonies (more than four cells). In contrast, mutation in the DNA damage checkpoint genes *CHK1*, *PDS1*, *RAD53*, and *DUN1* allowed cells to form microcolonies (Fig. 1A). Among them, *cdc13-1 rad53-21* mutants exhibited the most dramatic checkpoint defect, as more than 80% of the mutant cells formed colonies. Mutations in *DUN1*, a downstream target of Rad53 (41), and *CHK1* also resulted in microcolony formation, although not as efficient as that with *rad53-21*. Chk1 kinase phosphorylates Pds1 and prevents its degradation. *pds1-m9* mutants that have all of the Chk1 phosphorylation sites mutated to alanine fail to prevent anaphase entry in *cdc13-1*-arrested cells (33). Like *chk1* Δ mutants, some *cdc13-1 pds1-m9* mutant cells were able to form microcolonies. Mutations in Cdc55 and Bfa1, the negative regulators of the FEAR and MEN pathways, respectively, also lead to microcolony formation. The ability to form microcolonies in the presence of DNA damage indicates that mutations in Chk1, Pds1, Cdc55, Rad53, Dun1, and Bfa1 allow both anaphase entry and mitotic exit.

Next, spindle morphology was examined in *cdc13-1 TUB1-GFP* strains incubated at 34°C in the presence or absence of DNA damage checkpoint genes. At 2 h after G₁ release, *cdc13-1* single mutant cells accumulated as large budded cells with a short spindle structure. However, mutation in *CHK1*, *PDS1*, *RAD53*, *DUN1*, *CDC55*, or *BFA1* allowed some cells to enter anaphase, as indicated by the appearance of elongated spindles (Fig. 1B). Some cells exited mitosis, as judged by the disassembly of elongated spindles (Fig. 1B, right). This observation indicates that the tested DNA damage checkpoint mutants are able to exit mitosis in the presence of DNA damage checkpoints.

A new bud appears after a yeast cell initiates a new round of the cell cycle. Thus, we examined the rebudding process of DNA damage checkpoint mutants in *cdc13-1*-arrested cells.

FIG. 2. DNA damage checkpoint genes are required to prevent Cdc14 release from the nucleolus in response to DNA damage. *cdc13-1* single and double mutant cells with the indicated genotype carrying a Cdc14-5-GFP fusion were arrested in G₁ and then released into fresh YPD medium at 34°C. Cells were collected and fixed with 3.7% formaldehyde at the indicated time points for microscopic examination. Budding index (A) and Cdc14 release kinetics (B) in two groups of DNA damage checkpoint mutants are shown. The localization of Cdc14 in *cdc13-1* and *cdc13-1 cdc55Δ* cells at 120 min is shown at the bottom of panel A.

After incubation of *cdc13-1* single mutant cells at 34°C for 4 h, almost all of them were arrested as large budded cells. In contrast, DNA damage checkpoint mutant cells began to rebud after 2.5 h incubation at 34°C (Fig. 1C). Together, the microcolony formation, spindle morphology, and rebudding phenotypes suggest that DNA damage checkpoint genes are required to prevent anaphase entry and mitotic exit in the presence of DNA damage.

Intact DNA damage checkpoints prevent premature Cdc14 release from the nucleolus. The activation of mitotic exit pathways is marked by the release of phosphatase Cdc14 from the nucleolus (27, 32). Hence, we further examined the localization of Cdc14 in various mutants by using a strain with 5-GFP-tagged Cdc14 (40). *cdc13-1* single and double mutants containing Cdc14-5-GFP were synchronized at G₁ phase and then released into 34°C medium. Almost all of the *cdc13-1* single mutant cells showed nucleolar localization of Cdc14, suggesting that the presence of DNA damage inhibits Cdc14 release from the nucleolus. However, some *rad53-21*, *dun1Δ*, *chk1Δ*, and *pds1-m9* mutant cells were unable to sequester Cdc14 within the nucleolus when Rad13 is inactivated (Fig. 2A and B). Among them, *cdc13-1 rad53-21* mutants exhibited the most dramatic phenotype. More than 40% of *cdc13-1 rad53-21* double mutant cells exhibited released Cdc14 after 2 h of incubation.

tion at 34°C. All of these observations suggest that the DNA damage checkpoint components Rad53, Dun1, Chk1, and Pds1 are required to prevent mitotic exit in the presence of DNA damage.

We have shown that Bfa1 and Bub2, the negative regulators of the MEN pathway, are required for mitotic arrest in response to DNA damage (35). PP2A^{Cdc55} also plays a negative role in mitotic exit by dephosphorylating Net1 (22, 36, 39). Both Bfa1 and PP2A have been shown to be required to prevent Cdc14 release from the nucleolus in the presence of nocodazole, a microtubule-depolymerizing drug (35, 36). Thus, we examined Cdc14 localization in *cdc13-1 cdc55Δ* and *cdc13-1 bfa1Δ* mutants. After 2 h of incubation at 34°C, 63% of *cdc13-1 cdc55Δ* and 31% of *cdc13-1 bfa1Δ* mutant cells showed released Cdc14 (Fig. 2A and B). Consistently, more *cdc13-1 cdc55Δ* cells than *cdc13-1 bfa1Δ* cells rebudded. Therefore, the intact DNA damage checkpoint and the negative regulators of FEAR and MEN are all required to prevent mitotic exit in response to DNA damage.

Two parallel DNA damage checkpoint pathways control mitotic exit. In budding yeast, Chk1, Pds1, Rad53, and Dun1 define two parallel checkpoint pathways that control anaphase entry (13, 23). We have shown that mutation in any of the genes allows *cdc13-1* mutant cells to exit mitosis and rebud when incubated at 34°C. It is also possible that these checkpoint genes control mitotic exit with distinct mechanisms. To test this, we performed epistasis analysis of DNA damage checkpoint mutants for the mitotic exit process. *rad53-21 dun1Δ* double mutants did not exhibit an additive mitotic exit phenotype in *cdc13-1*-arrested cells, based on rebudding kinetics, when cells were arrested with *cdc13-1*. A *chk1Δ pds1-m9* double mutant strain exhibited rebudding kinetics similar to those of either single mutant. In contrast, more *rad53-21 chk1Δ* double mutant cells rebudded than did either single mutant (Fig. 3A). Therefore, the Chk1-Pds1 checkpoint acts in parallel to Rad53-Dun1 to prevent mitotic exit in the presence of DNA damage.

As *cdc55Δ* mutants also exhibit arrest defects in response to DNA damage, we examined whether Cdc55 functions in the same pathway as either Chk1 or Rad53. A *cdc55Δ chk1Δ* double mutant did not exhibit a more dramatic mitotic exit phenotype than did a *cdc55Δ* single mutant. In contrast, *rad53-21 cdc55Δ* double mutant cells exhibited a more dramatic rebudding phenotype than either single mutant when arrested with *cdc13-1* (Fig. 3A), suggesting that Chk1, Pds1, and Cdc55 act in the same pathway to control mitotic exit in response to DNA damage.

We also compared Cdc14 release kinetics in *pds1-m9* and *dun1Δ* single mutants and *pds1-m9 dun1Δ* double mutants in the presence of DNA damage. Obviously, more *cdc13-1 pds1-m9 dun1Δ* mutant cells exhibited released Cdc14 than did either single mutant. At 90 min after G₁ release, 38% of double mutant cells showed released Cdc14, whereas only 15% of *cdc13-1 pds1-m9* and 18% of *cdc13-1 dun1Δ* cells exhibited released Cdc14. Also, fewer *cdc13-1 pds1-m9 dun1Δ* cells were able to maintain cell cycle arrest, as judged by the more dramatic decrease in large budded cells, than was either single mutant. Moreover, loss of function of both Pds1 and Dun1 resulted in more efficient microcolony formation (Fig. 3B). All of these observations support the conclusion that two DNA

damage checkpoint pathways, Chk1-Pds1-PP2A and Rad53-Dun1, control mitotic exit in response to DNA damage.

Chk1 kinase regulates Cdc14 release by inhibiting the FEAR pathway. In response to DNA damage, Chk1 prevents anaphase entry by stabilizing the anaphase inhibitor Pds1 (23). Our observations indicate that Chk1-dependent Pds1 phosphorylation also plays a role in preventing mitotic exit. Stabilized Pds1 prevents anaphase entry by binding to the separase Esp1 (9). It has been shown that Esp1 facilitates mitotic exit by promoting the phosphorylation of Net1 and that mutation of the phosphorylation sites of Net1 abolishes Esp1-induced mitotic exit (3, 22). Therefore, we tested whether mutations of the Net1 phosphorylation sites are also able to abolish the premature mitotic exit phenotype in *chk1* and *pds1* mutants. We compared cell cycle progression in *cdc13-1*, *cdc13-1 chk1Δ*, and *cdc13-1 chk1Δ net1-6Cdk* mutants incubated at the restrictive temperature. Strikingly, the *net1-6Cdk* mutation, in which six Cdk1 phosphorylation sites were mutated to alanine, suppressed cell division, Cdc14 release, and microcolony formation in *cdc13-1 chk1Δ* mutants (Fig. 4A and C). The *net1-6Cdk* mutation also inhibited premature mitotic exit in *cdc13-1 pds1-m9* mutants (F. Liang, unpublished data). It has been shown that the *net1-6Cdk* mutation delays, but does not block, Cdc14 release (3). The observation that the *net1-6Cdk1* mutation blocks Cdc14 release and microcolony formation is presumably due to the inhibition of the MEN pathway in *cdc13-1*-arrested *chk1Δ* mutants. Thus, we conclude that Chk1 and Pds1 negatively regulate mitotic exit by preventing the phosphorylation of Net1.

To determine whether the premature mitotic exit process in *cdc13-1 chk1Δ* mutants depends on the MEN pathway, a *cdc13-1 chk1Δ cdc15-2* mutant was constructed. Cdc15 kinase is a component of the MEN pathway, and *cdc15-2* cells arrest at telophase when incubated at the nonpermissive temperature (4). To our surprise, introduction of the *cdc15-2* mutation suppressed cell division and microcolony formation of *cdc13-1 chk1Δ* mutants but failed to prevent Cdc14 release (Fig. 4B and C). After the cells were released into 34°C medium from G₁ arrest for 2 h, 24% of *cdc13-1 chk1Δ* and 18% of *cdc13-1 chk1Δ cdc15-2* mutant cells exhibited released Cdc14 (Fig. 4B). We conclude that Chk1 prevents Cdc14 release independently of MEN, but MEN activation is indispensable for mitotic exit.

The *net1-6Cdk* mutation blocks mitotic exit in *cdc13-1 cdc55Δ* cells. More than 60% of *cdc13-1 cdc55Δ* double mutant cells exhibited released Cdc14 after incubation at the nonpermissive temperature (Fig. 2A). Because PP2A^{Cdc55} negatively regulates mitotic exit by dephosphorylating Net1 (22), the hyperphosphorylation of Net1 may contribute to premature Cdc14 release in DNA damage-arrested *cdc55Δ* mutant cells. To test this possibility, we compared the cell cycle progression, microcolony formation, and Cdc14 release in *cdc13-1*, *cdc13-1 cdc55Δ*, and *cdc13-1 cdc55Δ net1-6Cdk* mutant cells. For some unknown reason, *cdc13-1 cdc55Δ net1-6Cdk* cells were not efficiently arrested at 34°C; thus, the experiment was carried out at 37°C. The *net1-6Cdk* mutation not only blocked Cdc14 release but also suppressed microcolony formation in *cdc13-1 cdc55Δ* mutants (Fig. 5A and C), indicating that premature mitotic exit in *cdc13-1 cdc55Δ* mutant cells depends on Net1 phosphorylation.

Our previous studies indicated that *cdc15-2* mutants block

FIG. 3. Epistasis analysis of DNA damage checkpoint mutants in mitotic exit regulation. (A) *cdc13-1* single and double mutants with the indicated genotypes were arrested in G₁ phase and then released into 34°C medium. The percentage of rebudded cells was counted over time. (B) *pds1-m9* and *dun1Δ* mutants show additive mitotic exit in *cdc13-1*-arrested cells. G₁-synchronized cells were released into 34°C medium. The upper panel shows the budding index and the kinetics of Cdc14 release. Microcolony formation of mutant cells with the indicated genotypes is shown in the lower panel.

mitotic exit in *cdc55Δ* mutants in the presence of nocodazole, a microtubule-depolymerizing drug (36). Thus, we analyzed the effect of *cdc15-2* mutants on the mitotic exit of *cdc13-1 cdc55Δ* mutants. Interestingly, *cdc15-2* did not show any suppression of Cdc14 release in *cdc13-1 cdc55Δ* mutants. In contrast, the suppression of microcolony formation by *cdc15-2* was dramatic, although not complete (Fig. 5B and C). Therefore, the released Cdc14 in *cdc13-1 cdc55Δ cdc15-2* mutants is not sufficient to trigger mitotic exit. Taken together, *net1-6Cdk*

mutants suppress both Cdc14 release and mitotic exit in *chk1*, *pds1*, and *cdc55* mutants in the presence of DNA damage. Inactivation of MEN by the *cdc15-2* mutant fails to restore the nucleolar localization of Cdc14 in the above mutants but is able to suppress cell cycle progression, further confirming the notion that FEAR-induced Cdc14 release promotes mitotic exit by activating the MEN pathway (27).

Rad53 regulates mitotic exit through the MEN pathway. Previous data indicate that Rad53 plays a major role in pre-

FIG. 4. Activated Chk1 kinase inhibits Net1 phosphorylation. (A) *net1-6Cdk* mutants suppress Cdc14 release in *cdc13-1 chk1Δ* mutant cells. Cells with the indicated genotypes were arrested in G₁ at 25°C and then released into 34°C medium. Cells were collected every 30 min and fixed with formaldehyde. The budding index and the percentage of cells with released Cdc14 were determined. (B) Suppression of cell cycle progression in *cdc13-1 chk1Δ* mutants by *cdc15-2*. Cells with the indicated genotypes were treated as described for panel A. (C) Both *net1-6Cdk* and *cdc15-2* mutants suppress microcolony formation in *cdc13-1 chk1Δ* cells. Mid-log-phase cells with the indicated genotypes were plated onto YPD plates and incubated at 37°C for 8 h before the formation of microcolonies was examined.

venting mitotic exit in response to DNA damage (23). To address whether Rad53 inhibits mitotic exit through the FEAR or MEN pathway, we examined the mitotic exit process in *cdc13-1 rad53-21* mutant cells with dysfunctional FEAR or MEN. For this purpose, *cdc13-1 rad53-21 net1-6Cdk CDC14-5-GFP* and *cdc13-1 rad53-21 cdc15-2 CDC14-5-GFP* strains were generated. G₁-arrested cells were released into 34°C YPD medium and then collected for budding index and Cdc14 localization analyses. *cdc15-2* almost completely suppressed cell division and Cdc14 release in *cdc13-1 rad53-21* cells (Fig. 6B). Also, very few *cdc13-1 rad53-21 cdc15-2* cells formed microcolonies after incubation on 37°C plates (Fig. 6C), indicating that the premature mitotic exit in *rad53-21* mutants can be suppressed by inactivation of the MEN pathway. In con-

trast, *net1-6Cdk* delayed but failed to block cell division and Cdc14 release in *cdc13-1 rad53-21* cells (Fig. 6A). Moreover, most *cdc13-1 rad53-21 net1-6Cdk* mutant cells were able to form microcolonies on 37°C plates (Fig. 6C). The data suggest that MEN, but not FEAR, is likely the target of Rad53.

The regulation of Cdc14 release by Rad53 is independent of Cdc5. Previous studies suggested that Cdc5 kinase could be the target of Rad53, as the *cdc5-1* mutant blocks cell cycle progression in *cdc13-1 rad53-21* mutants (23). However, the observation that Bfa1, one of the Cdc5 kinase substrates, appears to be hyperphosphorylated in *cdc13-1*-arrested cells suggests that Cdc5 kinase is active in DNA damage-arrested cells (14). To clarify the role of Cdc5 kinase in Rad53-dependent block of mitotic exit, we constructed *cdc13-1 rad53-21 cdc5-2* mutants.

FIG. 5. The *net1-6Cdk* mutation suppresses Cdc14 release in *cdc5Δ* mutant cells. (A) The *net1-6Cdk* mutation restores the nucleolar localization of Cdc14 in *cdc13-1 cdc5Δ* cells. Cells in mid-log phase were arrested at G₁ phase and then released into 37°C YPD medium. Cells were harvested and fixed with 3.7% formaldehyde at 30-min intervals to count the budding index and examine Cdc14 localization. The percentages of large budded cells and cells with released Cdc14 are shown. (B) The *cdc15-2* mutation failed to restore the nucleolar localization of Cdc14 in *cdc13-1 cdc5Δ* cells. G₁-arrested cells were released into 34°C medium and collected every 30 min to examine budding and Cdc14 localization. (C) Microcolony formation of *cdc5Δ* mutant cells in the presence of DNA damage. Cells with the indicated genotypes were spread onto 37°C YPD plates. The percentage of cells that formed microcolonies was determined after 8 h incubation.

As reported previously, the *cdc5-2* mutant delayed chromosome segregation and cell division in *cdc13-1 rad53-21* mutant cells (Fig. 7A and B). But *cdc13-1 rad53-21 cdc5-2* cells exhibited Cdc14 release kinetics similar to those of *cdc13-1 rad53-21* mutants (Fig. 7B). Also, more *cdc5-1 rad53-21* mutant cells showed released Cdc14 than did the *cdc5-1* single mutant (Fig. 7C), suggesting that Rad53 acts downstream or in parallel with Cdc5 to regulate mitotic exit. The impaired function of Cdc5 in chromosome segregation, such as the phosphorylation of cohesin by Cdc5 kinase, may become more important and delay anaphase entry in *cdc13-1 rad53-21* mutants (2). Thus, we believe that the impaired function in chromosome segregation of Cdc5, instead of MEN function, delays mitosis in *cdc13-1 rad53-21* mutants. Moreover, the function of Bfa1 could be regulated at multiple layers. In addition to Cdc5-dependent inhibitory phosphorylation, Bfa1 could also be a target of the DNA damage checkpoint (see next section).

Bfa1-Tem1 association decreases in *rad53-21* mutants. Before chromosome segregation, the Bfa1-Bub2 GAP complex binds to the GTPase Tem1 and keeps Tem1 in an inactive form (21). Since *cdc13-1*-arrested cells show nucleolar localization of Cdc14, while Bfa1 is required for mitotic arrest, we hypothesize that Tem1 associates with its inhibitor, Bfa1-Bub2, in DNA damage-arrested cells. If Rad53 negatively regulates the MEN pathway, it is possible that the association between Bfa1 and Tem1 is reduced in *rad53* mutants. To test this, we examined Bfa1-Tem1 interaction in *cdc13-1* and *cdc13-1 rad53-21* mutants by using a coimmunoprecipitation approach. G₁-arrested cells were released into the cell cycle at 34°C. After being released for 1, 2, and 3 h, 50 ml of cells were collected for the preparation of protein extracts. HA-tagged Tem1 protein was pulled down, and the Bfa1 protein levels in the immunoprecipitates were examined. In *cdc13-1 rad53-21* mutant cells, Tem1-associated Bfa1 protein levels were obviously reduced

FIG. 6. Rad53 regulates mitotic exit through the MEN pathway. (A) The *net1-6Cdk* mutation fails to suppress mitotic exit in *cdc13-1 rad53-21* cells. Cells with the indicated genotypes were treated as described in the legend to Fig. 4. The percentages of large budded cells and cells with released Cdc14 are shown. (B) The *cdc15-2* mutation suppresses mitotic exit in *cdc13-1 rad53-21* mutants. Cells were treated as described in the legend to Fig. 4, and the percentages of large budded cells and cells with released Cdc14 are shown. (C) Microcolony formation of *rad53-21* mutant cells in the presence of DNA damage. Cells with the indicated genotypes were spread onto 37°C YPD plates and treated as described.

after 2 h of incubation at 34°C compared to *cdc13-1* single mutants (Fig. 8A). We also examined the role of Dun1 in regulating Bfa1-Tem1 interaction with the approach described above and found that Bfa1-Tem1 interaction is reduced in *cdc13-1 dun1Δ* mutants (Fig. 8B). Therefore, activated Rad53-Dun1 might be required to maintain or facilitate Bfa1-Tem1 interaction. Given the fact that Dun1 is a substrate of Rad53 kinase (41), Dun1 may play a more direct role in regulating Bfa1-Tem1 interaction. As we found that *rad53* mutants can partially suppress the Cdc14 release defects in *cdc5-1* mutants in the absence of DNA damage (Fig. 7C), it is possible that Rad53-Dun1 also regulates Bfa1-Tem1 interaction during the normal cell cycle.

DISCUSSION

We systematically analyzed the regulation of mitotic exit in response to DNA damage. When DNA damage is introduced by incubating *cdc13-1* mutant cells at 34°C (17), the phosphatase Cdc14 is sequestered within the nucleolus, indicating that mitotic exit pathways are inactive in the presence of DNA

damage. All of the DNA damage checkpoint genes tested are required for the inactivation of mitotic exit pathways in response to DNA damage. Among them, Rad53 seems to play a more important role in preventing mitotic exit, as more *cdc13-1 rad53-21* mutant cells exhibited Cdc14 release and finished cell division when incubated at the nonpermissive temperature.

Rad53 kinase phosphorylates Dun1 directly and is responsible for the activation of Dun1 (6, 7, 15). As both Rad53 and Dun1 negatively regulate mitotic exit and Dun1 acts downstream of Rad53, we reason that Dun1 plays a more direct role in regulating MEN. Our previous data indicate that Bfa1 exhibits a Rad53- and Dun1-dependent, but Chk1-independent, band shift in response to DNA damage (14). Thus, one possibility is that Dun1 kinase phosphorylates Bfa1 and prevents the dissociation of Bfa1 from Tem1. To support this, we observed decreased Bfa1-Tem1 association in *rad53-21* and *dun1Δ* mutants in the presence of DNA damage. Further experiments are required to test this possibility in detail.

If Rad53 regulates mitotic exit through Dun1, why do

FIG. 7. Rad53 regulates Cdc14 release independently of Cdc5. (A) The *cdc5-2* mutation delays anaphase entry in *cdc13-1 rad53-21* cells. Cells with the indicated genotypes were arrested at G₁ phase with α -factor at 25°C and then released into 34°C YPD medium. Cells were collected every 30 min and fixed for DAPI staining. The percentages of cells with segregated nuclei are shown. The right panel shows the nuclear morphology of cells at 180 min. (B) The *cdc5-2* mutation fails to block Cdc14 release in *cdc13-1 rad53-21* mutant cells. G₁-arrested cells with the indicated genotypes were released into 34°C YPD medium for the analysis of budding index and Cdc14 localization. The bottom panel shows the localization of Cdc14-GFP in cells collected at 120 min. (C) The *rad53-21* mutation partially suppresses the mitotic exit defects in *cdc5-1* mutants. G₁-arrested *cdc5-1* and *cdc5-1 rad53-21* mutant cells with 5-GFP-tagged Cdc14 were released into 37°C YPD medium. Cells were collected every 30 min for the analysis of budding index and Cdc14 localization.

cdc13-1 rad53-21 mutant cells exhibit a more dramatic checkpoint defect than *cdc13-1 dun1* mutant cells? One possibility is that Rad53 kinase negatively regulates mitotic exit through other pathways. For example, it has been shown that Rad53

also phosphorylates Pds1 and prevents its association with the degradation machinery (1). Thus, activated Rad53 kinase may prevent mitotic exit through both Dun1 and Pds1.

In response to DNA damage, Chk1 phosphorylates and sta-

FIG. 8. (A) Bfa1-Tem1 interaction is decreased in *rad53-21* mutants in the presence of DNA damage. Cells with the indicated genotypes were arrested in G₁ phase at 25°C and then released into the cell cycle at 34°C. After incubation for 1, 2, and 3 h, protein extracts from 50 ml of cells were prepared and used for immunoprecipitation analysis with anti-HA antibody. Bfa1-myc and Tem1-HA levels were analyzed after Western blotting. (B) Bfa1-Tem1 interaction is decreased in *dun1 Δ* mutants in the presence of DNA damage. The same protocol was used to examine the interaction between Bfa1 and Tem1 in the presence or absence of Dun1. (C) Model for the regulation of mitotic exit by DNA damage checkpoints.

bilizes Pds1, which binds to the separase Esp1 and prevents anaphase entry (23, 33). Here we have demonstrated that Chk1 and Pds1 are also required to prevent mitotic exit in DNA damage-arrested cells. Mutations of the CDK phosphorylation sites on Net1 (*net1-6Cdk*) prevent mitotic exit in *cdc13-1 chk1 Δ* and *cdc13-1 pds1-m9* mutants. As Esp1 promotes mitotic exit by facilitating Net1 phosphorylation (22), the negative role of the Chk1-Pds1 DNA damage checkpoint in mitotic exit is to prevent Net1 phosphorylation by inhibiting Esp1.

Cdc55, the B-regulatory subunit of PP2A, is also shown to be required for efficient mitotic arrest in response to DNA damage. Like *chk1* and *pds1* mutants, *net1-6Cdk* also suppressed Cdc14 release and advanced cell cycle progression in *cdc13-1 cdc55 Δ* mutants, supporting the notion that PP2A^{Cdc55} dephosphorylates Net1 and prevents Cdc14 release (22). All of these observations suggest that Chk1, Pds1, and Cdc55 function in the same pathway and that the Chk1-Pds1 checkpoint negatively regulates mitotic exit by preventing the inactivation of PP2A^{Cdc55}.

An interesting observation is that the *cdc15-2* mutant suppresses the cell division of *chk1 Δ* , *pds1-m9*, and *cdc55 Δ* mutants but fails to block Cdc14 release in these mutants in the presence of DNA damage. Strikingly, *cdc13-1 cdc55 Δ cdc15-2* cells failed to exit mitosis, although more than 60% of the mutant cells exhibited released Cdc14 (Fig. 5B and C). A similar observation has been made with metaphase-arrested *cdc15-2 cdc55 Δ* mutant cells (22, 39). Why is the released Cdc14 in these cells unable to promote mitotic exit? It is likely that the role of FEAR in mitotic exit is to activate the MEN

pathway. Consistent with this notion, it has been shown that FEAR pathway-dependent Cdc14 release leads to dephosphorylation of Cdc15, which may promote MEN activation (27). In *cdc15* mutant-arrested cells, a FEAR pathway-dependent transient Cdc14 release was observed (27); why, then, is Cdc14 not resequestered into the nucleolus in *cdc55 Δ* mutants in the presence of DNA damage? Our result reveals relatively high levels of Clb2 in *cdc13-1 cdc55 Δ* mutant cells (Liang, unpublished data), raising the possibility that active Clb2-Cdk1 may prevent the resequestering of Cdc14. However, overexpression of Sic1, the Cdk1 inhibitor, failed to induce Cdc14 nucleolar localization (Liang, unpublished data). Thus, an unidentified mechanism contributes to the redistribution of Cdc14 after FEAR-induced Cdc14 release.

In summary, our data indicate that yeast cells prevent mitotic exit by inhibiting both the FEAR and MEN pathways in response to DNA damage (Fig. 8C). The activated Chk1-Pds1 pathway inhibits Cdc14 release by preventing Esp1-dependent inactivation of PP2A^{Cdc55}. In contrast, Rad53-Dun1 may directly regulate MEN by preventing the dissociation of Bfa1 from Tem1 in a Cdc5-independent manner. Together, activated Chk1-Pds1 and Rad53-Dun1 checkpoint proteins keep the FEAR and MEN pathways inactive in response to DNA damage.

ACKNOWLEDGMENTS

We thank S. J. Elledge, A. Toh-e, D. Clarke, and R. J. Deshaies for plasmids and strains. We thank Akash Gunjan for reading the manu-

script. We also thank Tuen-Yung Ng for preparing media and buffers for the experiments.

This work was supported by an American Heart Association Scientist Development grant and by a grant from the James and Esther King Biomedical Research Program (04NIR13) from the Florida State Department of Health to Y.W.

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