Protein phosphatase 2A (PP2A) promotes anaphase entry after DNA replication stress in budding yeast

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ABSTRACT **DNA replication stress activates the S-phase checkpoint that arrests the cell cycle, but it is poorly understood how cells recover from this arrest. Cyclin-dependent kinase (CDK) and protein phosphatase 2A (PP2A) are key cell cycle regulators, and Cdc55 is a regulatory subunit of PP2A in budding yeast. We found that yeast cells lacking functional PP2A^{Cdc55} showed slow growth in the presence of hydroxyurea (HU), a DNA synthesis inhibitor, without obvious viability loss. Moreover, PP2A mutants exhibited delayed anaphase entry and sustained levels of anaphase inhibitor Pds1 after HU treatment. A DNA damage checkpoint Chk1 phosphorylates and stabilizes Pds1. We show that *chk1*Δ and mutation of the Chk1 phosphorylation sites in Pds1 largely restored efficient anaphase entry in PP2A mutants after HU treatment. In addition, deletion of *SWE1*, which encodes the inhibitory kinase for CDK or mutation of the Swe1 phosphorylation site in CDK (*cdc28F19*), also suppressed the anaphase entry delay in PP2A mutants after HU treatment. Our genetic data suggest that Swe1/CDK acts upstream of Pds1. Surprisingly, *cdc55*Δ showed significant suppression to the viability loss of S-phase checkpoint mutants during DNA synthesis block. Together, our results uncover a PP2A-Swe1-CDK-Chk1-Pds1 axis that promotes recovery from DNA replication stress.**

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INTRODUCTION

Accurate DNA replication is essential for genome stability, whereas genome instability contributes to cell death or tumorigenesis. In addition to the innate complexity associated with DNA replication, cells face numerous internal and external stressors that may slow or stall DNA replication (Zeman and Cimprich, 2014). External stressors include extreme temperatures, toxin exposure, and mechanical damage, while internal stressors include limited resources, chemical imbalance, dysfunctional DNA replication machinery, and replication errors. Given the importance of genomic integrity maintenance, cells have adapted numerous cell cycle responses to deal with stressful DNA replication (Fulda *et al.*, 2010).

The cell cycle is driven in part by a series of phosphorylation events. The main kinase involved in cell cycle progression is the cyclin-dependent kinase (CDK) (Hartwell *et al.*, 1974; Gómez-Escoda and Wu, 2017). Unlike mammalian cells, the budding yeast *Saccharomyces cerevisiae* has only one CDK, Cdk1 (Cdc28) (Beach *et al.*, 1982; Enserink and Kolodner, 2010). Progression through the cell cycle is driven by Cdk1 in association with different cyclins that are characteristic of each stage of the cell cycle (Andrews and Measday, 1998; Malumbres, 2014). In *S. cerevisiae*, cyclins Clb5 and Clb6 promote S-phase progression; Clb3 and Clb4 associate with early mitotic events; Clb1 and Clb2 are critical for mitosis (Kõivomägi *et al.*, 2011). One CDK inhibitor is Swe1, a protein kinase that phosphorylates tyrosine 19 (Y19) of Cdc28 to inhibit the mitotic activity of Cdk1 (Sia *et al.*, 1996; Liu and Wang, 2006; Hu *et al.*, 2008). The presence of Swe1 delays anaphase onset by phosphorylating Y19 of Cdk1 (Lianga *et al.*, 2013; Leitao *et al.*, 2019). Swe1 has also been

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Abbreviations used: APC, anaphase promoting complex; CDK, cyclin-dependent kinase; HU, hydroxyurea; PP2A, protein phosphatase 2A; SAC, spindle assembly checkpoint ; WT, wild-type; YPD, yeast extract peptone dextrose.

implicated in the coordination of bud formation and cell division in budding yeast by delaying mitotic entry when bud formation is defective (Sia *et al.*, 1996; Barral *et al.*, 1999). Swe1, once phosphorylated by Cdk1, forms a stable complex with Cdk1, which leads to the inhibition of Cdk1 activity (Harvey *et al.*, 2005). However, Cdk1 dephosphorylation leads to further Swe1 phosphorylation by Cdk1, causing dissociation of Swe1 from Cdk1 and subsequent activation of mitotic CDK (Asano *et al.*, 2005).

Cell cycle checkpoints are vital for genome stability. Within a cell cycle, several checkpoints are in place to ensure accurate DNA replication and chromosome segregation (Barnum and O'Connell, 2014; Ovejero *et al.*, 2020). The S-phase checkpoint is a branch of the DNA damage checkpoint that is associated with DNA replication stress. This checkpoint is triggered by a block in DNA replication fork progression or other forms of replication perturbation that lead to accumulation of single-stranded DNA at replication forks (Willis and Rhind, 2009; Pardo *et al.*, 2017). The major components of the S-phase checkpoint in budding yeast include Mec1 and Rad53 kinases whose activation leads to stabilization of stalled DNA replication forks and inhibition of cell cycle progression (Tourrière and Pasero, 2007; Zou, 2013). Mec1, functioning through the mediator of the replication checkpoint (Mrc1), phosphorylates and activates the effector kinase Rad53 (Osborn and Elledge, 2003). Mec1 and Rad53 are also the essential components of the DNA damage checkpoint (Kiser and Weinert, 1996). It has been shown that Rad53 is required to maintain mitotic Cdk1 activity following the activation of the DNA damage checkpoint (Sanchez *et al.*, 1999).

Another well-conserved checkpoint, the spindle assembly checkpoint (SAC), functions to ensure proper kinetochore–microtubule interaction, which is fundamental for accurate chromosome segregation. The major components of the SAC include Mad1, Mad2, Mad3, Bub1, Bub3, and Mps1 (Wang *et al.*, 2014; Stukenberg and Burke, 2015). Once the SAC becomes activated, Mad2 disrupts the interaction between the anaphase promoting complex (APC) and its regulator Cdc20 (Ge *et al.*, 2009). This inhibits the degradation of anaphase inhibitor Pds1 by APC^{Cdc20}, thereby blocking anaphase onset. Anaphase entry is marked by sister chromatid separation. The cohesin complex holds the sister chromatids together until cleaved by separase Esp1. Before anaphase entry, Esp1 is inhibited by forming a complex with securin Pds1 (Ciosk *et al.*, 1998). The Pds1-Esp1 interaction requires Cdk1-mediated Pds1 phosphorylation (Agarwal and Cohen-Fix, 2002). Interestingly, Chk1, a kinase in the DNA damage checkpoint, phosphorylates and stabilizes Pds1 in response to DNA damage by preventing APC^{Cdc20}-mediated Pds1 degradation (Wang *et al.*, 2001).

Protein phosphorylation and dephosphorylation play a key role in cell cycle progression. One of the primary phosphatases critical for the cell cycle is the holoenzyme protein phosphatase 2A (PP2A). In budding yeast, PP2A consists of three subunits: a scaffolding subunit (Tpd3), a regulatory subunit (Cdc55 or Rts1), and a catalytic subunit (Pph21, Pph22, or Pph3) (Sneddon *et al.*, 1990). As a regulatory subunit, Cdc55 governs cell cycle progression through the timed dephosphorylation of downstream targets (Godfrey *et al.*, 2017). One demonstrated function of $PP2A^{Cdc55}$ is to inhibit mitotic exit, a process that inactivates CDK by freeing phosphatase Cdc14 from the nucleolus. $PP2A^{Cdc55}$ dephosphorylates Net1, a nucleolar protein that anchors Cdc14 to the nucleolus, and loss of function of $PP2A^{Cdc55}$ results in premature release of Cdc14 from the nucleolus (Queralt *et al.*, 2006; Wang and Ng, 2006; Yellman and Burke, 2006). Recent evidence also indicates that $PP2A^{Cdc55}$ reverses Cdk1-mediated phosphorylation of Pds1, which is expected to abolish Pds1-Esp1 interaction for anaphase progression (Khondker *et al.*, 2020). PP2A has also been shown to be required for mitotic arrest in response to defective kinetochore–microtubule interaction (Minshull *et al.*, 1996; Wang and Burke, 1997). In addition, $PP2A^{Cdc55}$ promotes Swe1 degradation to facilitate the cell cycle transition from G_2 to M phase (Yang *et al.*, 2000). Although the accumulation of Swe1 and enhanced Cdk1 phosphorylation at Y19 by Swe1 contribute to the abnormal bud morphology and cold sensitivity *cdc55*Δ mutant, the mitotic checkpoint defect in *cdc55*Δ seems to be independent of Swe1-mediated Y19 phosphorylation of Cdk1 (Wang and Burke, 1997). Therefore, $PP2A^{Cdc55}$ plays multiple roles in cell cycle progression, but the detailed molecular mechanisms are not fully understood yet.

While the cell cycle arrest induced by DNA replication stress is well documented, it remains poorly defined how cells recover from this arrest. In this study, we found that loss of function of $PP2A^{Cdc55}$ in budding yeast led to slow growth in the presence of hydroxyurea (HU), a DNA synthesis inhibitor, but the slow growth is not a result of viability loss. Moreover, PP2A mutants exhibited sustained levels of anaphase inhibitor Pds1 along with delayed anaphase entry after treatment with HU, indicating that $PP2A^{Cdc55}$ is required for recovery from DNA replication stress. Interestingly, *chk1*Δ and mutations of the Chk1 phosphorylation sites in Pds1 largely restored efficient anaphase entry in PP2A mutants after HU treatment. In addition, our results indicate that $PP2A^{Cdc55}$ promotes Swe1 degradation and the reversal of Swe1-mediated Cdk1 phosphorylation, which further facilitates Pds1 degradation and anaphase onset. Surprisingly, loss of the S-phase checkpoint failed to suppress the anaphase entry delay in PP2A mutant cells, but the viability loss of S-phase checkpoint mutants in response to DNA synthesis stress is largely suppressed by PP2A mutants, and this suppression likely acts through Swe1 and Pds1. Together, our results uncover the PP2A-Swe1-CDK-Chk1-Pds1 axis that promotes the recovery from cell cycle arrest induced by DNA synthesis inhibition.

RESULTS

PP2A mutants are sensitive to DNA replication stress without viability loss

PP2A is a primary phosphatase critical for cell cycle progression. Previous works have shown that yeast PP2A mutants *cdc55*Δ and *pph21*Δ *pph22*Δ are sensitive to microtubule depolymerizing agents, such as benomyl and nocodazole (Minshull *et al.*, 1996; Wang and Burke, 1997; Wang and Ng, 2006). We wanted to further determine whether loss of function of PP2A in budding yeast also led to sensitivity to other stresses that disrupt cell cycle progression. One such stressor is HU, which impedes DNA replication by inhibiting ribonucleotide reductase and depleting free nucleotides (Koç *et al.*, 2004). Interestingly, yeast mutants in several subunits within the PP2A complex showed slow growth on YPD (yeast extract peptone dextrose) plates containing 100 mM HU. Deletion of the scaffold subunit (*tpd3*Δ) of PP2A caused dramatic HU sensitivity, but this mutant also exhibited obvious slow growth in the absence of HU. Pph21, Pph22, and Pph3 are the three catalytic subunits of PP2A. The *pph21*Δ mutant was not sensitive to HU, but the loss of both Pph21 and Pph22 led to HU sensitivity. For the regulatory subunits, *cdc55*Δ, but not the *rts1*Δ mutant, showed obvious slow growth on HU plates (Figure 1A), indicating that the HU sensitivity is specific to the loss of function of $PP2A^{Cdc55}$.

To determine why PP2A mutants show HU sensitivity, we examined whether these mutants showed a checkpoint defect in response to DNA replication stress, which would result in viability loss. We first analyzed the viability of PP2A mutant cells grown in liquid

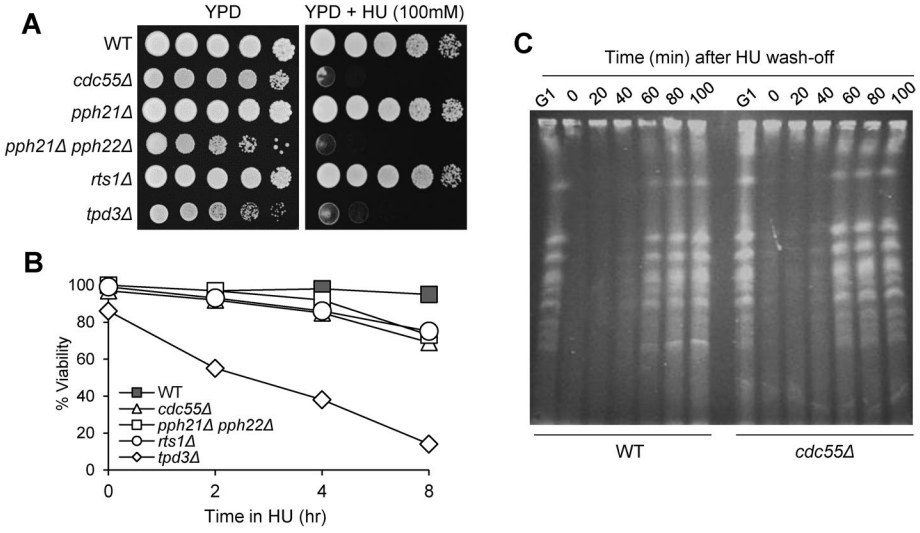

FIGURE 1: PP2A mutants are sensitive to DNA replication stress. (A) PP2A mutants show slow growth in the presence of HU. Saturated cells with the indicated genotypes were 10-fold serially diluted and spotted onto YPD plates with or without 100 mM HU. Growth was analyzed after 2 d on YPD and 3 d on HU plates at 30°C. (B) PP2A mutants do not show significant viability loss when treated with HU. WT and PP2A mutants were grown at 30°C in YPD media until mid–log phase, and then 200 mM HU was added. Samples were collected at 0, 2, 4, and 8 h and spread onto YPD plates to determine the plating efficiency after overnight growth at 25°C ($n \geq 200$). (C) DNA replication kinetics is normal in *cdc55*Δ cells after release from HU arrest. WT and *cdc55*Δ cells were grown at 30°C until mid–log phase and then arrested at G_1 phase with α-factor. G_1-arrested cells were released into YPD with 200 mM HU for 100 min before release into YPD. Cells were collected every 20 min after HU release. All samples were embedded into 1.5% agarose and treated with zymolyase and proteinase K to release genetic material. Samples were analyzed via PFGE and visualized after ethidium bromide staining.

YPD media containing 200 mM HU. Despite the dramatic slow growth of *cdc55*Δ and *pph21*Δ *pph22*Δ mutant cells on HU plates, their viability loss was not significant even after prolonged HU exposure (Figure 1B). This indicates competent checkpoint function in these mutants, which are able to recover from cell cycle arrest after HU is removed. Next, we determined whether the PP2A mutants experienced any DNA replication defect following HU exposure. We analyzed the replication kinetics of *cdc55*Δ cells using pulsed-field gel electrophoresis (PFGE), which separates chromosomes based on their size. Owing to pore size constraints, replicating chromosomes are excluded, allowing only duplicated chromosomes to be visualized by PFGE. Therefore, this method has been used to monitor the kinetics of DNA replication (Liu and Wang, 2006). Strikingly, *cdc55*Δ cells exhibited normal DNA replication kinetics compared with wild-type (WT) cells following release from HU arrest (Figure 1C). On the basis of these results, we conclude that the HU sensitivity of PP2A mutants is not due to the defect in checkpoint arrest or DNA replication.

Anaphase entry is delayed in PP2A mutants following DNA replication stress

One explanation for the HU sensitivity of PP2A mutants might be the delayed recovery from the cell cycle arrest induced by DNA replication stress. To test this idea, we first examined levels of the anaphase inhibitor Pds1 in WT and *cdc55*Δ mutant cells during the cell cycle, because Pds1 degradation marks anaphase entry (Cohen-Fix *et al.*, 1996). *cdc55*Δ and *pph21*Δ *pph22*Δ mutant cells exhibited a clear delay in Pds1 degradation during undisturbed cell cycle, and this is consistent with the persistent appearance of large-budded cells as indicated by the budding index (Figure 2A). Next, we compared Pds1 levels in WT and PP2A mutant cells after release from the cell cycle arrest induced by 200 mM HU. We noticed a significant delay in Pds1 degradation in *cdc55*Δ cells (Figure 2B), indicating that Cdc55 promotes anaphase entry after replication stress. *pph21*Δ *pph22*Δ mutants also showed delayed Pds1 degradation, but the delay was not as dramatic as in *cdc55*Δ mutant cells. Moreover, the delayed Pds1 degradation is consistent with a high percentage of large-budded cells (Figure 2B). These results suggest that the HU sensitivity of PP2A mutants is likely due to the delayed Pds1 degradation that blocks anaphase entry.

We also analyzed nuclear separation kinetics in WT and *cdc55*Δ mutants under normal growth conditions and after HU treatment. Strains containing mApple-tagged histone H2A (*HTA1-mApple*) allow for the fluorescence visualization of chromosome segregation. As expected, *cdc55*Δ and *pph21*Δ *pph22*Δ mutant cells exhibited slightly delayed nuclear division during the normal cell cycle compared with WT cells (Supplemental Figure S1). When comparing nuclear separation kinetics in these cells following release from HU arrest, we noticed a significant nuclear division delay in *cdc55*Δ cells. The delay was also obvious in *pph21*Δ *pph22*Δ cells, although it was less significant than in *cdc55*Δ cells (Figure 2C). Additionally, using a GFP-marked centromere of chromosome IV (*CEN4-GFP*), we observed a significant delay in sister chromatid segregation into two daughter cells in *cdc55*Δ cells following release from HU arrest (Supplemental Figure S2). Together, these results indicate that $PP2A^{Cdc55}$ is required for efficient anaphase entry after DNA replication stress.

The delayed degradation of Pds1 in PP2A mutants might be the result of SAC activation, which prevents anaphase onset in response to kinetochore attachment defects (Wang *et al.*, 2014). Previous research has shown that $PP2A^{Cdc55}$ plays a role in metaphase arrest in response to the disruption of kinetochore–microtubule interaction (Minshull *et al.*, 1996; Wang and Burke, 1997). Interestingly, deletion of *MAD1*, a SAC gene, in *cdc55*Δ cells did not suppress the delayed Pds1 degradation and cell cycle progression after HU treatment (Figure 2D). Moreover, neither *mad1*Δ nor *mad2*Δ suppressed the slow growth phenotype of *cdc55*Δ mutants on HU plates (Figure 2E). The failure to restore the growth of the *cdc55*Δ mutant in the presence of HU indicates that SAC activation is unlikely to play a role in the delayed recovery from DNA replication stress in PP2A mutants.

Mutation of Chk1 phosphorylation sites in Pds1 suppresses the HU sensitivity of PP2A mutants

In response to DNA damage, Pds1 becomes stabilized after phosphorylation by Chk1, which blocks anaphase entry (Wang *et al.*, 2001). DNA replication stress induced by HU treatment might also trigger Pds1 phosphorylation by Chk1 to prevent anaphase entry. Previous research has mapped Chk1 phosphorylation sites within Pds1, resulting in the generation of *pds1-m8* mutants (S37A, S121A,

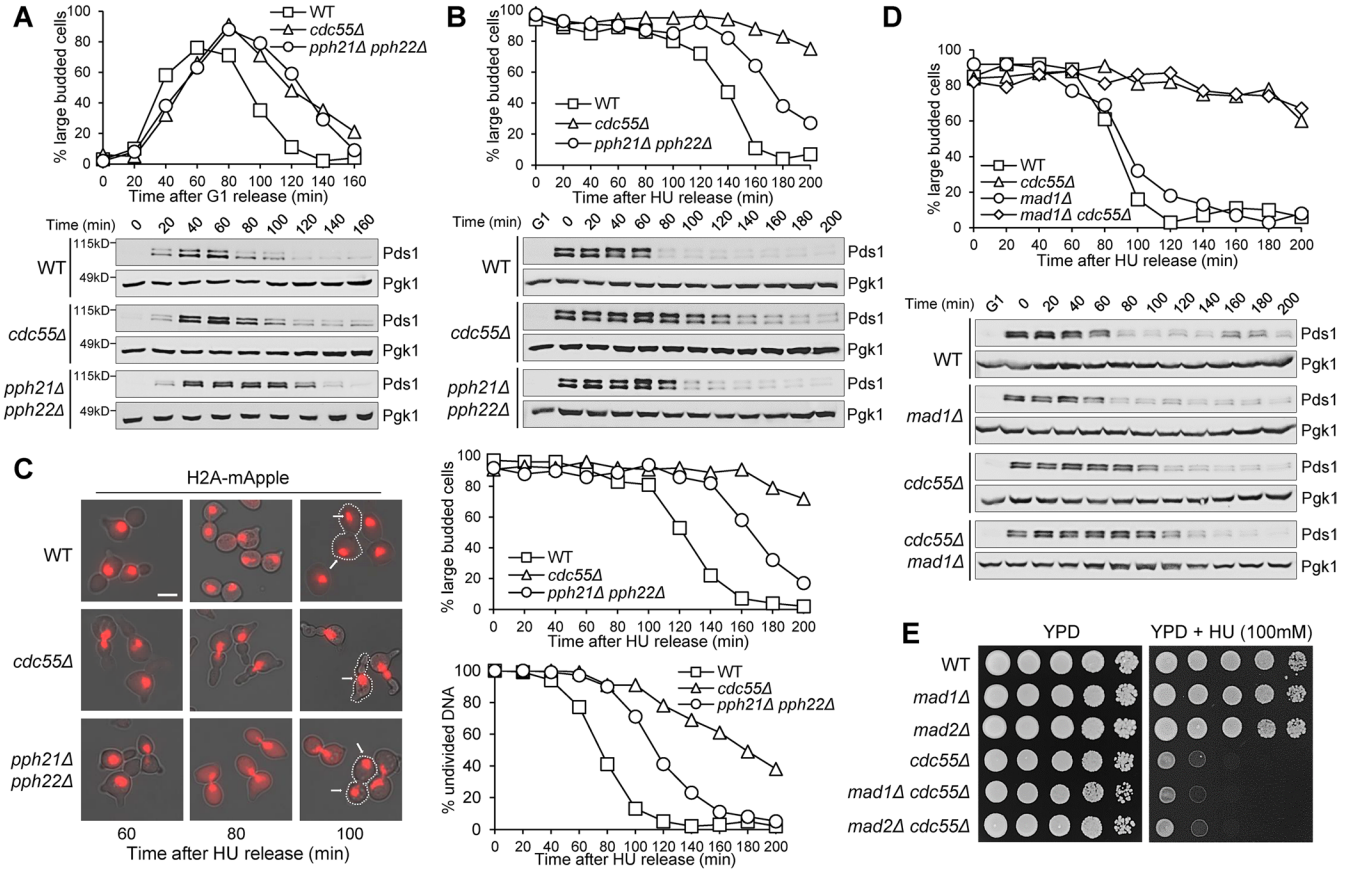

FIGURE 2: Anaphase entry is delayed in PP2A mutants after HU exposure. (A) Pds1 levels during the cell cycle in WT and PP2A mutant cells. WT, *cdc55*Δ, and *pph21*Δ *pph22*Δ cells with Pds1-18myc were grown at 30°C in YPD media until mid–log phase and then they were arrested at G_1 with α-factor. G_1 cells were released into YPD at 30°C with samples collected every 20 min. After G_1 release for 40 min, α-factor was added back to block the following cell cycle. Budding index and Pds1 protein levels are shown. Pgk1, loading control. (B) Pds1 protein remains stabilized in PP2A mutants following HU exposure. G_1-arrested cells were released into YPD with 200 mM HU for 2 h at 30°C. After HU was washed off, the cells were released into YPD, and α-factor was added to block the following cell cycle. Samples were collected every 20 min after HU release. Budding index and Pds1 protein levels are shown. Pgk1, loading control. (C) PP2A mutants show significant delay in nuclear division following HU treatment. G_1-arrested WT, *cdc55*Δ, and *pph21*Δ *pph22*Δ cells containing mApple-tagged H2A were released into YPD with 200 mM HU for 2 h. Then cells were washed and released into YPD at 30°C, and α-factor was added to block the following cell cycle. Every 20 min, cells were collected and counted for budding index and percentage of cells with undivided DNA using fluorescence microscopy. Budding index and the kinetics of nuclear division are shown in the right panel. Nuclear divisions (H2A-mApple) in some representative cells are shown in the left panel. Arrows: the nucleus. Scale bar, 5 μm. (D) The SAC mutant *mad1*Δ fails to rescue the delayed recovery of *cdc55*Δ cells following HU arrest. Cells with the indicated genotypes were treated as described in panel B. Budding index and Pds1 protein levels are shown. Pgk1, loading control. (E) *mad1*Δ and *mad2*Δ mutants do not suppress the HU sensitivity of *cdc55*Δ mutant cells. Saturated cells with the indicated genotypes were 10-fold serially diluted and spotted onto YPD plates with or without 100 mM HU. Growth was analyzed after 2 d on YPD and 3 d on HU plates at 30°C.

S132A, S139A, S158A, S170A, S213A, and S212A) with mutated Chk1 phosphorylation sites (Wang *et al.*, 2001). *pds1-m9* contains an additional mutated threonine (T289A), but both *pds1-m8* and *pds1-m9* mutants exhibit a defective DNA damage checkpoint. Interestingly, we found that the elimination of Chk1 phosphorylation sites in Pds1 rescued the HU sensitivity of *cdc55*Δ mutants based on the growth on YPD plates containing 100 mM HU (Figure 3A).

We further analyzed the nuclear division kinetics of *cdc55*Δ and *cdc55*Δ *pds1-m8* mutants containing H2A-mApple under normal growth conditions and after HU exposure. Under normal growth conditions, the slight delay of nuclear division in *cdc55*Δ cells was suppressed by *pds1-m8* (Supplemental Figure S3A). After release from treatment with 200 mM HU, a dramatic nuclear division delay was observed in *cdc55*Δ mutants, but *cdc55*Δ *pds1-m8* exhibited a nuclear division kinetics similar to that of WT cells (Figure 3B). We also analyzed the viability of *cdc55*Δ *pds1-m8* cells after treatment with HU, but no discernible viability loss was observed for both *cdc55*Δ single and *cdc55*Δ *pds1-m8* double mutants (Supplemental Figure S3B). These results indicate that Pds1 phosphorylation by Chk1 likely contributes to the anaphase entry delay in PP2A mutants after HU treatment.

Given the dramatic suppression of the HU sensitivity of PP2A mutants by *pds1-m8* and *pds1-m9* mutants that lack Chk1 phosphorylation sites, we hypothesized that the elimination of Chk1 would have a similar suppression. Interestingly, *CHK1* deletion in *cdc55*Δ mutants only partially suppressed the HU sensitivity

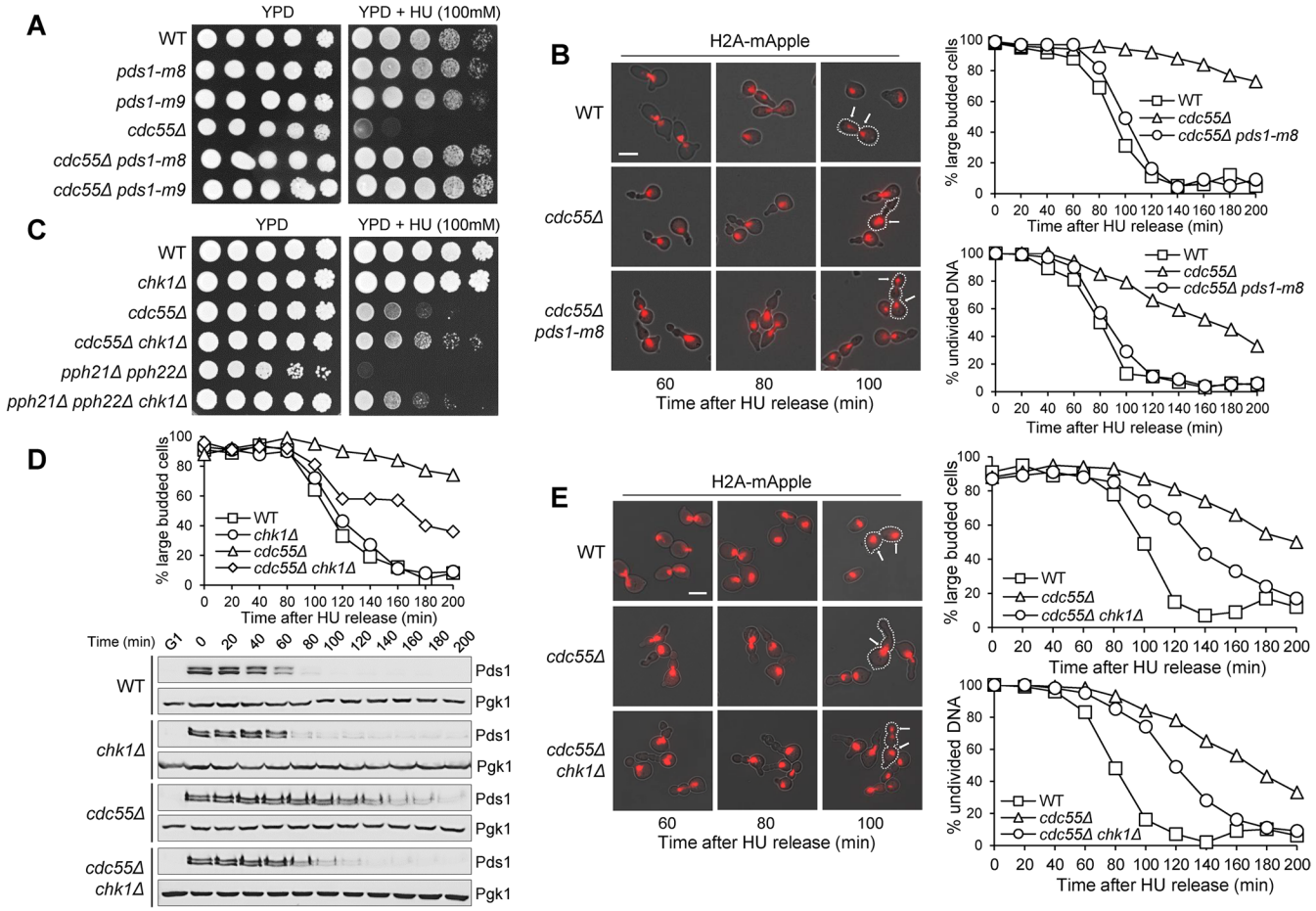

FIGURE 3: Mutation of Chk1 phosphorylation sites in Pds1 suppresses the HU sensitivity of *cdc55*Δ mutants. (A) Elimination of Chk1 phosphorylation sites in Pds1 (m8 and m9) restores the growth of *cdc55*Δ cells on HU plates. Saturated cells with the indicated genotypes were 10-fold serially diluted onto YPD plates with or without 100 mM HU. Growth was analyzed after incubation for 2 d (YPD) or 3 d (HU plates) at 30°C. (B) The *pds1-m8* mutation suppresses the nuclear division delay in *cdc55*Δ mutants after HU release. G_1-arrested WT, *cdc55*Δ, and *cdc55*Δ *pds1-m8* cells containing H2A-mApple were released into YPD with 200 mM HU for 2 h. After HU washout, the cells were released into YPD medium at 30°C, and α-factor was added to block the following cell cycle. Samples were collected every 20 min. Budding index and the percentage of cells with undivided DNA were determined using fluorescence microscopy. Nuclear division in some representative cells is shown in the left panel. Arrows: the nucleus. Scale bar, 5 μm. (C) The *chk1*Δ mutation partially suppresses the HU sensitivity of PP2A mutants. Saturated cells with the indicated genotypes were 10-fold serially diluted onto YPD plates with or without 100 mM HU. Growth was analyzed after 2 d on YPD and 3 d on HU plates at 30°C. (D) The *chk1*Δ mutant partially suppresses the delayed anaphase entry in *cdc55*Δ cells after HU release. G_1-arrested cells were released into YPD with 200 mM HU for 2 h at 30°C before release into YPD. After release from HU, α-factor was added to block the following cell cycle. Samples were collected every 20 min after HU release. Budding index and Pds1 protein levels are shown. Pgk1, loading control. (E) The *chk1*Δ mutation partially rescues the nuclear division defect in *cdc55*Δ cells after HU release. WT, *cdc55*Δ, and *cdc55*Δ *chk1*Δ cells containing H2A-mApple were treated as described in panel B. Cells were imaged via fluorescence microscopy to follow nuclear division (left). Scale bar, 5 μm. Cells were counted at each time point for budding index and the percentage of cells with undivided DNA (right).

(Figure 3C). Similarly, *chk1*Δ showed partial suppression of the HU sensitivity of *pph21*Δ *pph22*Δ mutants. A partial rescue by *chk1*Δ was also observed with regard to cell cycle progression, nuclear separation, and Pds1 degradation in *cdc55*Δ mutant cells after release from HU arrest (Figure 3, D and E). Because *pds1-m8* and *pds1-m9* mutants exhibit stronger suppression of the HU sensitivity of *cdc55*Δ cells than *chk1*Δ mutants, it is likely that the phosphorylation of Pds1 by a kinase other than Chk1 also contributes to the HU sensitivity of PP2A mutants.

Our results suggest that Chk1-independent Pds1 phosphorylation may also regulate replication stress recovery. In addition to Chk1, Cdk1 also phosphorylates Pds1, which facilitates the interaction of Pds1 with separase (Esp1) for its nuclear accumulation (Agarwal and Cohen-Fix, 2002). A recent study indicates that PP2A directly reverses Cdk1-mediated Pds1 phosphorylation (Khondker *et al.*, 2020). Therefore, Cdk1-dependent Pds1 phosphorylation might play a role in replication stress recovery. To test this idea, we acquired the *pds1-38* strain from the Cohen-Fix laboratory, wherein the three Cdk1 phosphorylation sites (S277A, S292, and T301A) were mutated to alanine. However, the phosphodeficient *pds1-38* mutant did not show any rescue of the HU sensitivity of *cdc55*Δ mutants; instead, more dramatic slow growth on HU plates was

observed for *cdc55Δ pds1-38* double mutants (Supplemental Figure S4A). Like Pds1, separase Esp1 is also a substrate of both Cdk1 and $PP2A^{Cdc55}$, and Cdk1-dependent Esp1 phosphorylation promotes anaphase entry (Lianga *et al.*, 2018). To test whether Esp1 phosphorylation plays a role in replication stress recovery in PP2A mutants, we constructed double mutants of *cdc55Δ* in combination with either the phosphomimetic *esp1-3D* or the phosphodeficient *esp1-3A*. Although *esp1-3A* showed a slight rescue for the HU sensitivity of *cdc55Δ* mutants, *esp1-3D* exhibited a similar rescue (Supplemental Figure S4, B and C), indicating that the reversion of Cdk1-dependent Esp1 phosphorylation is unlikely to play a major role during the recovery from HU arrest. Taken together, our results indicate that Chk1 and an additional kinase act together to phosphorylate Pds1 and prevent anaphase onset after DNA replication stress, but the reversal of this phosphorylation is required for stress recovery.

Defective S-phase checkpoint fails to suppress the delayed recovery from DNA replication stress in PP2A mutants

One major function of a checkpoint is to delay cell cycle progression after its activation, allowing cells more time to fix cell cycle problems. Thus, the S-phase checkpoint might be required for Pds1 stabilization, which contributes to the anaphase entry delay after DNA replication stress in PP2A mutants. If that is the case, a defective S-phase checkpoint would abolish this delay. Within the S-phase checkpoint pathway, Mec1 and Rad53 kinases are the key components, and their function is essential for cell cycle delay as well as the stabilization of replication forks in response to DNA replication stress (Kiser and Weinert, 1996; Pardo *et al.*, 2017).

Because both kinases are essential for viability, we utilized *mec1-1* and *rad53-21* point mutants to test their potential suppression of the stress recovery defect in PP2A mutants (Desany *et al.*, 1998). *cdc55Δ, mec1-1, rad53-21* single mutants and double mutants carrying both *cdc55Δ* and *mec1-1/rad53-21* were first arrested in G_1 and then released into HU to examine Pds1 protein levels. Pds1 accumulation was observed in all of these single and double mutant cells after release into HU medium (Figure 4, A and B), which is consistent with a previous study (Palou *et al.*, 2017). For these mutants, we further examined their recovery process following HU treatment. Neither *mec1-1* nor *rad53-21* was able to restore Pds1 degradation in *cdc55Δ* mutants after HU release. In contrast, a higher Pds1 level was detected in *mec1-1, rad53-21* single mutants as well as double mutants with *cdc55Δ* after HU release (Figure 4, C and D). Our explanation is that the collapsed replication forks in S-phase checkpoint mutants after HU treatment likely block DNA replication and anaphase entry that is marked by Pds1 degradation. Therefore, we conclude that a defective S-phase checkpoint does not abolish the accumulation of anaphase inhibitor Pds1 in PP2A mutants after DNA replication stress.

Although S-phase checkpoint mutants do not suppress the anaphase entry delay in *cdc55Δ* cells after HU treatment, surprisingly, *cdc55Δ* drastically increased the viability of *mec1-1* and *rad53-21* mutants following exposure to HU at different concentrations, 50, 100, and 200 mM (Figure 4E). A further question is how loss of function of PP2A rescues the viability of S-phase checkpoint mutants treated with HU.

Swe1 stabilization contributes to the recovery defect from HU arrest in PP2A mutants

Three branches of the DNA damage/replication checkpoint have previously been identified, including the Chk1, Rad53, and Swe1/Cdk1 branches (Sanchez *et al.*, 1999; Palou *et al.*, 2015). Therefore, we further explored whether Swe1-dependent Cdk1 phosphorylation played role in the delayed recovery following HU exposure in PP2A mutants. Previous studies indicate that $PP2A^{Cdc55}$ is required for the degradation of Swe1, the inhibitory kinase for Cdk1 (Yang *et al.*, 2000; Liu and Wang, 2006). We first examined the Swe1 protein levels in WT and *cdc55Δ* and *pph21Δ pph22Δ* mutants during the normal cell cycle and confirmed that Swe1 levels remained relatively high in PP2A mutants compared with WT cells, with *cdc55Δ* cells exhibiting a more dramatic phenotype. Moreover, both *cdc55Δ* and *pph21Δ pph22Δ* mutants exhibited higher level of slow-migrating Swe1 compared with WT cells, and this phenotype was also more pronounced in *cdc55Δ* cells (Figure 5A). Swe1 phosphorylation likely contributes to this slow migration (Sreenivasan and Kellogg, 1999). We also examined Swe1 levels in these cells after their release from HU arrest. Increased Swe1 stability was detected in *cdc55Δ* and *pph21Δ pph22Δ* mutant cells. Similarly, *cdc55Δ* cells showed more dramatic Swe1 stabilization than *pph21Δ pph22Δ* cells after HU release (Figure 5B).

After establishing the stabilization of Swe1 in PP2A mutants following HU release, we next tested whether the elimination of Swe1 was able to suppress the HU sensitivity of PP2A mutants. We found that *swe1Δ* showed clear suppression of the slow growth phenotype of *cdc55Δ* mutants on plates containing 100 mM HU, although to a lesser extent compared with *pds1-m8* and *pds1-m9* (Figure 5C). Furthermore, we used the H2A-mApple strains to examine the nuclear division kinetics in *cdc55Δ* and *cdc55Δ swe1Δ* cells after release from HU arrest. The nuclear division kinetics was completely restored in the double mutants (Figure 5D). Together, these results indicate that Swe1 stabilization also contributes to the delayed recovery from DNA replication stress in PP2A mutants.

Our results show that *swe1Δ* and *pds1-m8* mutants suppress the anaphase entry delay in *cdc55Δ* mutants after release from HU arrest. *cdc55Δ* mutants are also sensitive to microtubule depolymerizing agents, such as nocodazole and benomyl, because of the failure of mitotic arrest (Minshull *et al.*, 1996; Wang and Burke, 1997; Yellman and Burke, 2006). Thus, we further tested whether *swe1Δ* or *pds1-m8* was able to suppress the sensitivity of *cdc55Δ* to spindle poison benomyl, but no suppression was observed (Supplemental Figure S5), indicating that PP2A likely regulates the response to stressful DNA replication and disrupted kinetochore attachment through different mechanisms.

We noticed that the *pds1-m8* mutant shows stronger suppression for the HU sensitivity of the *cdc55Δ* mutant than *chk1Δ* and *swe1Δ*. Then, we tested whether *swe1Δ chk1Δ* double mutants exhibited stronger suppression than each single mutant. Surprisingly, *swe1Δ chk1Δ cdc55Δ* triple mutants showed slow growth on HU plates, like the *cdc55Δ* single mutant (Supplemental Figure S6A). It is possible that Swe1 and Chk1 play additional roles in response to DNA replication stress, and the slight HU sensitivity of *swe1Δ chk1Δ* mutants supports this speculation. We also compared the viability of *cdc55Δ* single and double/triple mutants in combination with *chk1Δ, swe1Δ,* or *swe1Δ chk1Δ* after HU exposure. All these mutants did not show significant viability loss (Supplemental Figure S6B). Therefore, *swe1Δ, chk1Δ,* and *pds1-m8* mutants restore the efficient recovery of *cdc55Δ* mutants from HU arrest without causing viability loss.

Elimination of Swe1-dependent Cdk1 phosphorylation suppresses the HU sensitivity of the *cdc55Δ* mutant

Swe1 inhibits Cdk1 kinase activity by phosphorylating tyrosine 19 (Y19) on Cdc28 in budding yeast (Booher *et al.*, 1993). Thus, Cdc28-Y19 phosphorylation might play a role in the delayed

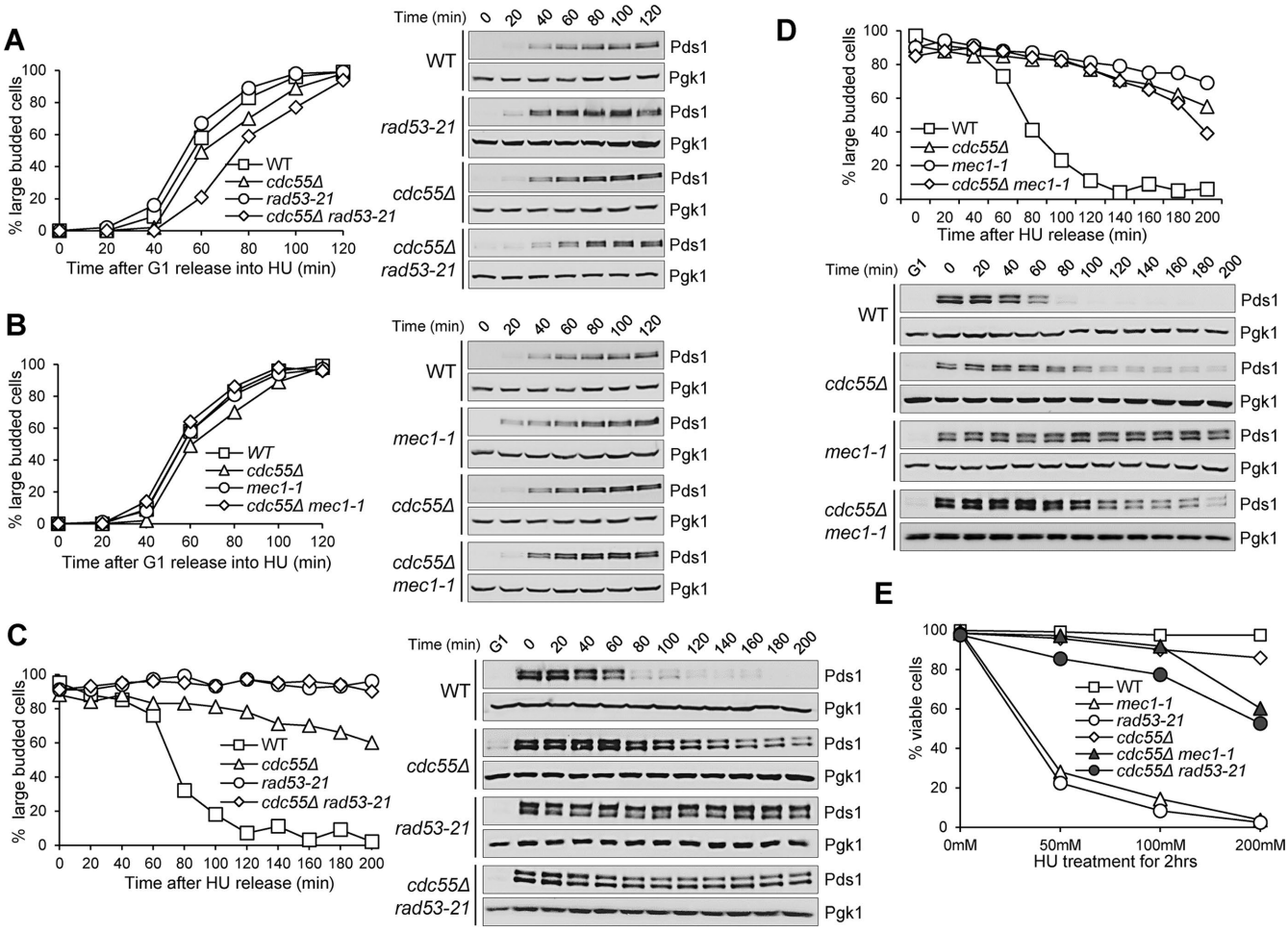

FIGURE 4: Genetic interactions between *cdc55*Δ and S-phase checkpoint mutants. (A) Pds1 levels in *cdc55*Δ, *rad53-21*, and *cdc55*Δ *rad53-21* mutants treated with HU. G_1-arrested cells were released into YPD with 200 mM HU at 30°C. Samples were collected every 20 min after G_1 release. Budding index and Pds1 protein levels are shown. Pgk1, loading control. (B) Pds1 levels in *cdc55*Δ, *mec1-1*, and *cdc55*Δ *mec1-1* mutants treated with HU. Cells were treated as described above. Budding index and Pds1 protein levels are shown. Pgk1, loading control. (C) The *rad53-21* mutant fails to suppress the anaphase entry delay in *cdc55*Δ cells after HU release. G_1-arrested cells were released into YPD with 200 mM HU for 2 h before release into YPD medium at 30°C. After release from HU arrest, α-factor was added to block the following cell cycle. Samples were collected every 20 min after HU release. Budding index and Pds1 protein levels are shown. Pgk1, loading control. (D) The *mec1-1* mutant fails to suppress the anaphase entry delay in *cdc55*Δ cells after HU release. The cells were treated as described above. Budding index and Pds1 levels are shown. (E)The *cdc55*Δ mutant suppresses the viability loss of S-phase checkpoint mutants after HU treatment. WT and mutant cells were grown at 30°C in YPD media until mid–log phase. Cultures were split four ways with HU concentrations at 0, 50, 100, and 200 mM. Samples were collected after 2-h treatment and spread onto YPD plates to determine the plating efficiency after overnight growth at 25°C ($n \geq 200$).

recovery from HU-induced cell cycle arrest in PP2A mutants. To test this idea, we used the phosphodeficient *cdc28F19* mutant, in which tyrosine 19 was replaced with phenylalanine to abolish Cdk1-Y19 phosphorylation by Swe1 kinase (Amon *et al.*, 1992). We found that *cdc28F19* largely suppressed the slow growth phenotype of *cdc55*Δ cells on HU plates (Figure 6A). Following release from HU-induced arrest, *cdc28F19 cdc55*Δ cells exhibited kinetics for Pds1 degradation and nuclear segregation similar to those of WT cells (Figure 6, B and C). In addition, *cdc28F19 cdc55*Δ cells did not show increased viability loss after HU treatment compared with *cdc55*Δ cells (Figure 6D). Together, our results suggest that Swe1-dependent Cdk1-Y19 phosphorylation plays a major role in the delayed recovery from HU-induced arrest in PP2A mutants.

Swe1 acts upstream of Pds1 to regulate the recovery from DNA replication stress

Our results indicate that Swe1 and Pds1 act downstream of PP2A in the recovery from cell cycle arrest induced by HU. Next, we assessed the relationship between Swe1 and Pds1 in this recovery process. For this purpose, we first examined whether Swe1 stabilization in PP2A mutants was dependent on Pds1 phosphorylation. WT, *cdc55*Δ, and *cdc55*Δ *pds1-m8* mutants with Swe1-myc were arrested with HU, and Swe1 protein levels were determined after release from HU arrest. As expected, *cdc55*Δ cells showed delayed Swe1 degradation. Strikingly, we observed an even stronger Swe1 stabilization in *cdc55*Δ *pds1-m8* cells compared with *cdc55*Δ cells after HU release, although the accumulation of large-budded cells was abolished by *pds1-m8* (Figure 7A). Therefore, *cdc55*Δ *pds1-m8*

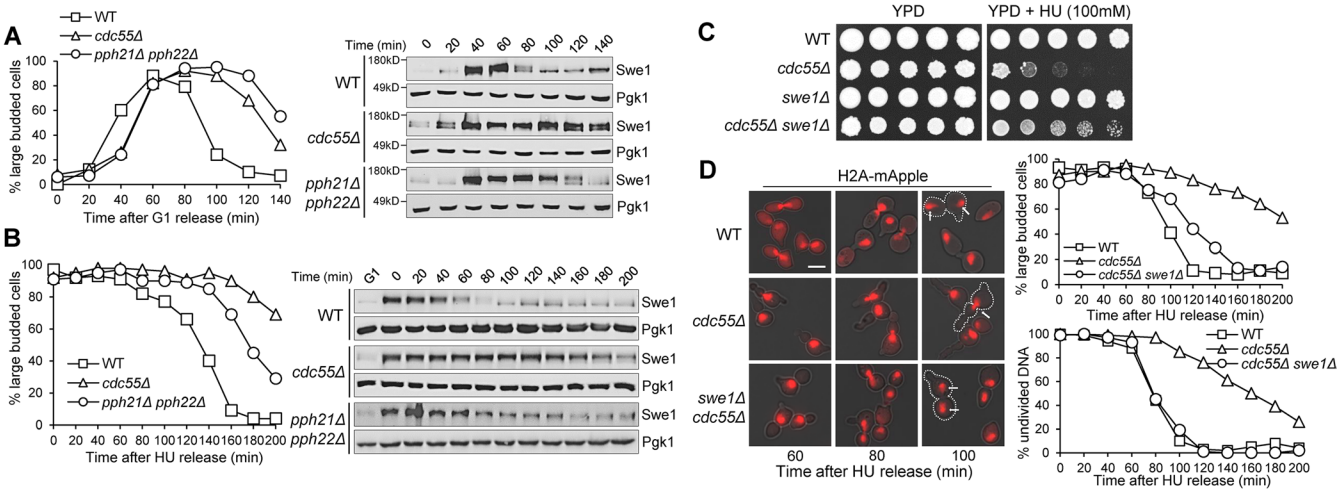

FIGURE 5: Swe1 stabilization contributes to the delayed recovery from HU arrest in PP2A mutants. (A) Swe1 levels are higher in PP2A mutants during the normal cell cycle. WT, *cdc55*Δ, and *pph21*Δ *pph22*Δ cells with Swe1-myc were grown at 30°C in YPD media until mid–log phase and then arrested at G_1 with α-factor. Cells in G_1 were released into YPD at 30°C with samples collected every 20 min. α-factor was added back after release for 40 min to block the following cell cycle. Budding index and Swe1 protein levels are shown. Pgk1, loading control. (B) PP2A mutants show defective Swe1 degradation following release from HU arrest. G_1-arrested WT, *cdc55*Δ, and *pph21*Δ *pph22*Δ cells expressing Swe1-myc were released into YPD with 200 mM HU for 2 h at 30°C before release into YPD. After release from HU arrest, α-factor was added to block the following cell cycle. Samples were collected every 20 min after HU release. Budding index and Swe1 protein levels are shown. Pgk1, loading control. (C) *swe1*Δ suppresses the HU sensitivity of *cdc55*Δ cells. Saturated cells with the indicated genotypes were 10-fold serially diluted onto YPD plates with or without 100 mM HU. Growth was analyzed after 2 d on YPD and 3 d on HU plates at 30°C. (D) *swe1*Δ rescues the nuclear division defect in *cdc55*Δ mutants after HU release. WT, *cdc55*Δ, and *swe1*Δ *cdc55*Δ cells containing H2A-mApple were synchronized at G_1 with α-factor and then released into YPD with 200 mM HU for 2 h. The cells were released into YPD at 30°C with samples collected every 20 min. After HU release, α-factor was added to block the following cell cycle. Cells were imaged via fluorescence microscopy to follow nuclear division (left). Arrows: the nucleus. Scale bar, 5 μm. Cells were counted at each time point for budding index and the percentage of cells with undivided DNA (right).

cells still show impaired Swe1 protein degradation despite the restoration of efficient anaphase entry in these cells after HU treatment. To further analyze the relationship between Swe1 and Pds1, we performed a reciprocal experiment, in which Pds1 levels were tracked in *cdc55*Δ *swe1*Δ mutant cells. Cells were first arrested with HU and then released. Pds1 levels were stabilized in *cdc55*Δ cells after HU release, but this stabilization was largely diminished in *cdc55*Δ *swe1*Δ cells. *swe1*Δ also restored cell cycle progression in *cdc55*Δ mutants after HU release based on the budding index (Figure 7B). The abolished Pds1 stabilization in *cdc55*Δ *swe1*Δ cells after HU release suggests that Pds1 likely acts downstream of Swe1 in the process of recovery from HU arrest.

An interesting finding is that the *cdc55*Δ mutant largely suppresses the viability loss of the S-phase mutants *rad53-21* and *mec1-1* following HU exposure (Figure 4E). Because our data indicate that Swe1 and Pds1 act downstream of PP2A, we further examined whether *swe1*Δ or *pds1-m8* could abolish this suppression by *cdc55*Δ. Given that *cdc55*Δ, *mec1-1*, and *rad53-21* mutants are all HU sensitive, and *swe1*Δ or *pds1-m8* suppresses the HU sensitivity of *cdc55*Δ, the strain construction would be challenging. Therefore, we first inserted a *Sphis5*$^+$ marker in a chromosome locus close to either *rad53-21* or *mec1-1* to generate *rad53-21-Sphis5*$^+$ and *mec1-1-Sphis5*$^+$ strains. With these strains, we first generated *rad53-21-Sphis5*$^+$ *cdc55*Δ *pds1-m8* and *rad53-21-Sphis5*$^+$ *cdc55*Δ *swe1*Δ strains. Interestingly, introduction of the *pds1-m8* or *swe1*Δ mutant into *rad53-21-Sphis5*$^+$ *cdc55*Δ cells caused viability loss after HU treatment (Figure 7C). We were unable to construct a *mec1-1-Sphis5*$^+$ *cdc55*Δ *swe1*Δ strain, indicating synthetic lethality. This lethality is likely a result of disruption of many cell cycle events caused by the loss of function of $PP2A^{Cdc55}$, Mec1, and Swe1 (Palou *et al.*, 2015). However, the *mec1-1-Sphis5*$^+$ *cdc55*Δ *pds1-m8* mutant was constructed successfully, and viability loss induced by HU treatment was restored in this triple mutant (Figure 7D). All these results suggest that the suppression of HU-induced viability loss in S-phase checkpoint mutants by *cdc55*Δ depends on the PP2A downstream targets Swe1 and Pds1. Taken together, these results support the conclusion that $PP2A^{Cdc55}$ promotes anaphase entry after DNA replication stress through the sequential reversal of Swe1-mediated Cdk1 phosphorylation and Chk1-dependent Pds1 phosphorylation. Therefore, our data revealed the PP2A-Swe1-CDK-Chk1-Pds1 axis that is critical for the recovery from DNA replication stress (Figure 7E).

DISCUSSION

DNA replication stress activates the S-phase checkpoint that delays cell cycle progression and stabilizes stalled replication forks. This checkpoint has been studied extensively because of its critical role in genome stability. However, much less is known about the recovery process following the removal of DNA replication stress. Here we report that a protein phosphatase, PP2A, plays a critical role in replication stress recovery. First, we found that the PP2A holoenzyme containing B-regulatory subunit Cdc55 is required for efficient anaphase entry after DNA replication stress. Moreover, our results indicate that $PP2A^{Cdc55}$-dependent degradation of anaphase inhibitor Pds1 and CDK inhibitory kinase Swe1 is important for cell cycle progression after DNA replication stress. We further found that the phosphorylation of Cdk1 at Y19 by Swe1 and the phosphorylation of Pds1 by Chk1 contribute to anaphase entry delay in PP2A mutants after DNA replication stress. Finally, Swe1

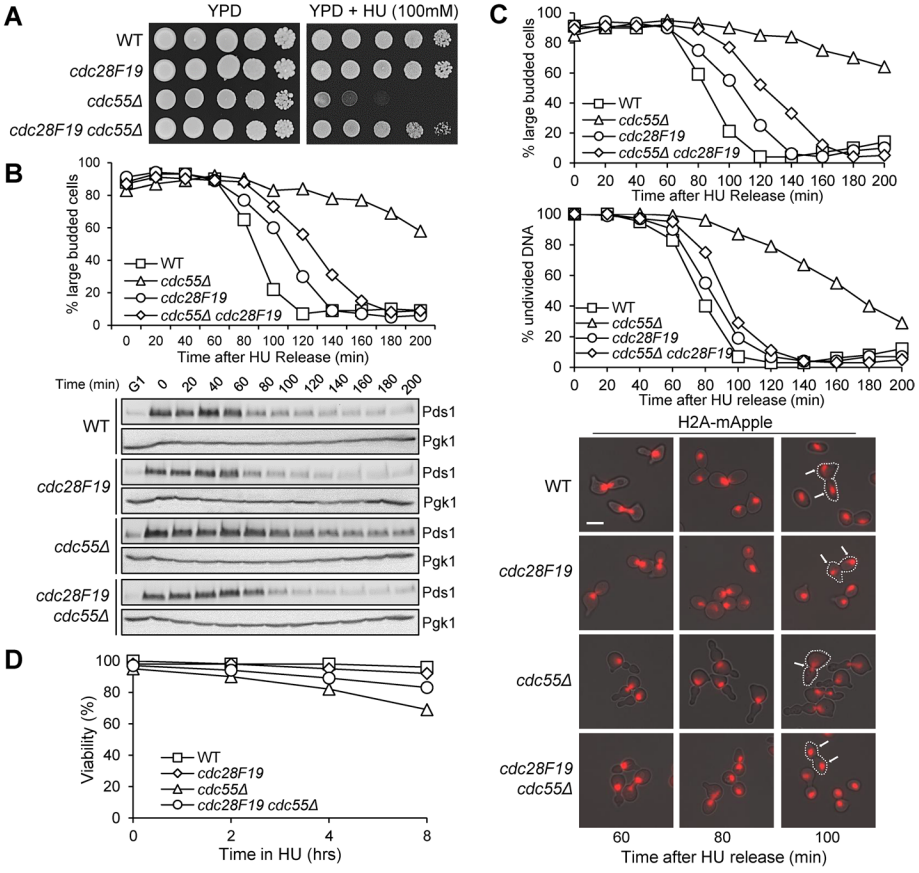

FIGURE 6: Abolishment of Swe1-mediated Cdk1 phosphorylation suppresses the HU sensitivity of *cdc55*Δ mutants. (A) The *cdc28F19* mutation suppresses the HU sensitivity of *cdc55*Δ. Saturated cells with the indicated genotypes were 10-fold serially diluted and spotted onto YPD plates with or without 100 mM HU. Growth was analyzed after incubation for 2 d (YPD) or 3 d (HU plates) at 30°C. (B) *cdc28F19* largely suppresses the delayed anaphase entry in *cdc55*Δ cells after HU release. G_1-arrested cells were released into YPD with 200 mM HU for 2 h at 30°C before release into YPD. After HU release, α-factor was added to block the following cell cycle. Samples were collected every 20 min after HU release. Budding index and Pds1 protein levels are shown. Pgk1, loading control. (C) *cdc28F19* rescues the nuclear division defect in *cdc55*Δ mutants after HU release. WT, *cdc55*Δ, *cdc28F19*, and *cdc28F19 cdc55*Δ cells containing H2A-mApple were synchronized at G_1 and then released into YPD with 200 mM HU for 120 min. Cells were then released into YPD at 30°C with samples collected every 20 min. After HU release, α-factor was added to block the following cell cycle. Cells were counted at each time point for budding index and the percentage of cells with undivided DNA (top). Cells were also imaged via fluorescence microscopy to follow nuclear division (bottom). Arrows: the nucleus. Scale bar, 5 μm. (D) *cdc28F19 cdc55*Δ mutants do not show viability loss after HU exposure. WT, *cdc55*Δ, *cdc28F19*, and *cdc28F19 cdc55*Δ cells were grown at 30°C in YPD media until mid–log phase, and then 200 mM HU was added. Samples were collected at 0, 2, 4, and 8 h and spread onto YPD plates to determine the plating efficiency after overnight growth at 25°C ($n \geq 200$).

degradation likely acts upstream of Pds1 to regulate the recovery from HU arrest. Interestingly, the loss of function of PP2A restores the viability of S-phase checkpoint mutants under DNA replication stress, and this restoration is abolished by either *swe1*Δ or *pds1-m8*. These results reveal an unappreciated PP2A-Swe1-CDK-Chk1-Pds1 pathway required for the recovery from DNA replication stress (Figure 7E).

Although our results support the critical role of $PP2A^{Cdc55}$ in the recovery from DNA replication stress, it remains unclear which protein is directly dephosphorylated by PP2A to promote this recovery process. The known $PP2A^{Cdc55}$ substrates are involved in cellular processes such as mitosis and cytokinesis (Moyano-Rodriguez and Queralt, 2020). Previous studies indicate that PP2A along with Cdc14 counteracts CDK-imposed phosphorylation (Godfrey *et al.*, 2017). Interestingly, a recent work showed increased Pds1 phosphorylation in *cdc55*Δ cells and that $PP2A^{Cdc55}$ directly dephosphorylates Pds1 to reverses Cdk1-mediated phosphorylation (Khondker *et al.*, 2020). However, it is unlikely that $PP2A^{Cdc55}$ promotes cell cycle recovery from replication stress by directly reversing CDK-dependent Pds1 phosphorylation, because the *pds1-38* mutant lacking the CDK phosphorylation sites was unable to suppress the HU sensitivity of PP2A mutants (Supplemental Figure S4A). Similarly, it is unlikely that PP2A promotes anaphase entry after DNA replication stress by counteracting CDK-dependent phosphorylation of separase Esp1 based on our observation that the suppression of the HU sensitivity of *cdc55*Δ by phosphodeficient *esp1-3A* was not significant (Supplemental Figure S4B).

In addition to Pds1, another downstream target of $PP2A^{Cdc55}$ is Swe1, which phosphorylates and inhibits CDK (Sia *et al.*, 1996). PP2A-dependent Swe1 degradation has been shown to facilitate the transition from G_2 to M phase (Yang *et al.*, 2000). We detected delayed Swe1 degradation in PP2A mutant cells (Figure 5A). Because Cdc5, Cla4, and other kinases phosphorylate Swe1 to promote its degradation (Sreenivasan and Kellogg, 1999; Sakchaisri *et al.*, 2004; Liu and Wang, 2006), PP2A may promote Swe1 degradation by up-regulating these kinases. In addition, Mec1 kinase phosphorylates the SQ motif of Swe1 after S-phase checkpoint activation (Palou *et al.*, 2015); thus it will be interesting to test whether PP2A reverses Mec1-dependent Swe1 phosphorylation for its degradation. Swe1 is also phosphorylated by Cdk1 to regulate Cdk1-Swe1 association (Harvey *et al.*, 2005), but $PP2A^{Cdc55}$ opposes Swe1 phosphorylation by Cdk1, which limits Swe1 activity and allows mitotic CDK activation (Harvey *et al.*, 2011). Because we observed that *cdc28-F19* suppresses the delayed recovery from HU arrest in PP2A mutants, we speculate that one important role of $PP2A^{Cdc55}$ in the anaphase entry after DNA replication stress is to activate mitotic CDK by reversing Swe1-mediated Cdk1 phosphorylation. However, we cannot exclude the possibility that $PP2A^{Cdc55}$ directly dephosphorylates other CDK substrates to promote the recovery from DNA replication stress.

We found that deletion of *SWE1* largely abolished the delay in Pds1 degradation in *cdc55*Δ mutant cells after HU release. In contrast, the *pds1-m8* mutant lacking Chk1 phosphorylation sites had no effect on Swe1 degradation in *cdc55*Δ mutant cells after HU release (Figure 7). Therefore, we conclude that Swe1 acts upstream of Chk1-dependent Pds1 phosphorylation to regulate anaphase entry. Because Swe1 phosphorylates Cdk1 to inhibit its mitotic activity

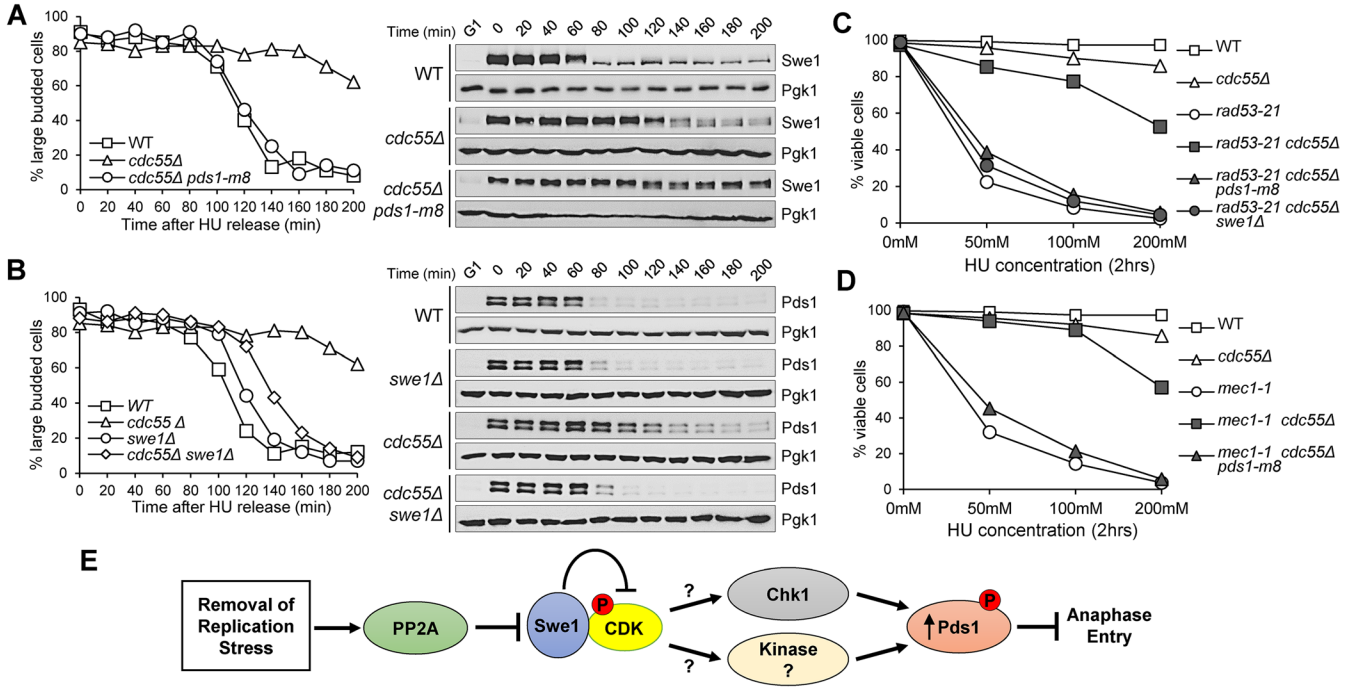

FIGURE 7: As the downstream targets of PP2A, Swe1 acts upstream of Pds1 to regulate cell cycle recovery from HU arrest. (A) Swe1 levels remain high in *cdc55*Δ mutant cells expressing phosphodeficient Pds1 (*pds1-m8*). G_1-arrested cells were released into YPD medium containing 200 mM HU for 2 h at 30°C before release into YPD. After HU release, α-factor was added to block the following cell cycle. Samples were collected every 20 min after HU release. Budding index and Swe1 protein levels are shown. Pgk1, loading control. (B) The *swe1*Δ mutant abolishes delayed Pds1 degradation in *cdc55*Δ cells after HU release. Cells with the indicated genotypes were treated as described above. Budding index and Pds1 protein levels are shown. Pgk1, loading control. (C) *swe1*Δ and *pds1-m8* mutants restore the HU-induced viability loss in *rad53-21 cdc55*Δ. Mid–log phase cell cultures of WT and mutants were split four ways with HU concentrations at 0, 50, 100, and 200 mM. Samples were collected after 2-h treatment and spread onto YPD plates to determine the plating efficiency after overnight growth at 25°C ($n \geq 200$). (D) The *pds1-m8* mutant restores the HU-induced viability loss in *mec1-1 cdc55*Δ mutants. Cells were treated as described above. The plating efficiency is shown. (E) Proposed model for $PP2A^{Cdc55}$-Swe1-CDK-Chk1-Pds1 pathway that promotes the recovery from DNA replication stress.

(Harvey *et al.*, 2005; Lianga *et al.*, 2013), it is likely that mitotic CDK negatively regulates Chk1-mediated Pds1 phosphorylation to promote anaphase entry (Figure 7E). In support of this possibility, evidence from human cells shows that CDK phosphorylates Chk1 for its nuclear export, which promotes mitotic entry (Enomoto *et al.*, 2009). In budding yeast, DNA damage checkpoint protein Rad9 is phosphorylated by CDK, and this phosphorylation exhibits dual regulation of the Rad9-Chk1 interaction (Abreu *et al.*, 2013). One possibility is that S-phase CDK-mediated Rad9 phosphorylation enhances Rad9-Chk1 interaction for checkpoint activation, but further Rad9 phosphorylation by mitotic CDK decreases Rad9-Chk1 interaction, which down-regulates checkpoint activity for anaphase onset. Further experiments are needed to test these ideas. We also showed that mutations of Chk1 sites on Pds1 (*pds1-m8* and *pds1-m9*) resulted in a nearly complete rescue of the HU sensitivity of *cdc55*Δ mutants, but the rescue by *chk1*Δ was only partial. This indicates that some of the identified Chk1 phosphorylation sites on Pds1 are also phosphorylated by another kinase, and it will be our future interest to identify this kinase.

One interesting observation from this study is that *cdc55*Δ mutants drastically rescue the viability of S-phase checkpoint mutants, *mec1-1* and *rad53-21*, after exposure to HU (Figure 4E). These two mutants are extremely sensitive to even low concentrations of HU because of their roles in dNTP synthesis and DNA replication fork stabilization (Desany *et al.*, 1998; Zhao *et al.*, 1998; Gupta *et al.*, 2013; Rodriguez and Tsukiyama, 2013). One explanation is that the delayed anaphase onset in *cdc55*Δ mutants allows S-phase checkpoint mutants to fix the problems caused by DNA replication stress. Another explanation for this rescue could be the increased dNTP pool or stabilized replication forks in *cdc55*Δ cells. Indeed, in human cells, active PP2A induces Cdc45 to decouple from the replisome, resulting in the collapse of replication forks (Perl *et al.*, 2019). Interestingly, we observed that *swe1*Δ and *pds1-m8* restored the viability loss in *mec1-1 cdc55*Δ and *rad53-21 cdc55*Δ (Figure 7, C and D). Therefore, an important open question is how the PP2A-Swe1-CDK-Chk1-Pds1 axis modulates the DNA replication process that contributes to the viability loss in S-phase checkpoint mutants after HU exposure.

In summary, we show that PP2A facilitates the recovery from HU-induced cell cycle arrest and the likely downstream targets of PP2A involved in this recovery process include Swe1 and Pds1. Another interesting observation in this research is that defective PP2A suppresses the viability loss of S-phase checkpoint mutants after DNA replication stress. Given that PP2A is a well-conserved holoenzyme across eukaryotic species, it will be important to understand whether the function of PP2A in response to replication stress is also conserved (Orgad *et al.*, 1990). In the fission yeast *Schizosaccharomyces pombe*, PP2A regulates the phosphorylation of Wee1, the Swe1 homologue (Lucena *et al.*, 2017). As described above, human PP2A destabilizes the replisome by dephosphorylating Cdc45

(Perl *et al.*, 2019). Given that PP2A dysregulation has been found in many solid tumors and leukemia (Cristóbal *et al.*, 2011; Seshacharyulu *et al.*, 2013), the functional studies of PP2A in the DNA replication stress response may provide new therapeutic strategies.

MATERIALS AND METHODS

Request a protocol through *Bio-protocol*.

Yeast strains and growth conditions

The relevant genotypes and the sources of the strains used in this study are listed in Supplemental Table S1. All of the strains listed are isogenic to Y300, a derivative of W303. The *pds1-38* strain was a gift from the Cohen-Fix laboratory. The *pds1-m8* and *pds1-m9* strains were from the Elledge laboratory. The *esp1-3A* and *esp1-3D* strains were a gift from the Rudner laboratory. Yeast cells were grown in YPD medium. To arrest cells in G_1 phase, 5 μg/ml α-factor was added into cell cultures. After incubation for 120 min, the G_1-arrested cells were washed twice with water and then released into fresh YPD medium to start the cell cycle. For HU treatment, G_1-arrested cells were released into YPD containing 200 mM HU (Sigma). After incubation for 120 min, the S-phase arrested cells were washed twice with water and then released into fresh YPD medium to start the cell cycle.

H2A-mApple strain construction

The H2A-mApple strain was constructed using a PCR-based method. The template plasmid pHG72 was provided by Hong-Guo Yu, and primers are listed in Supplemental Table S2. PCR products were transformed into Y300 WT cells, and the cells were spread onto histidine dropout plates and incubated at 30°C. Colonies were confirmed via colony PCR and fluorescence microscopy.

Construction of *mec1-1* and *rad53-21* strains conjugated with *Sphis5*$^+$

A PCR-based method was used to insert the *Sphis5*$^+$ marker into a chromosome locus close to either *mec1-1* or *rad53-21*. A template plasmid (pFA6aHis3MX6) containing *Sphis5*$^+$ was used for the PCR (Longtine *et al.*, 1998). The primers used for this PCR are listed in Supplemental Table S3. The PCR products were transformed into either AY202 (*rad53-21*) or AY203 (*mec1-1*) yeast strains. Cells were spread onto HIS dropout plates and incubated at 30°C to select transformants. Colonies were confirmed via colony PCR.

Western blot analysis

We collected 1.5 ml of yeast cell culture, the cell pellets were resuspended in 100 ml of H_2O, and then 100 ml of 0.2 M NaOH was added. The mixture was left at room temperature for 5 min. After centrifugation, the pellets were resuspended in the 1× loading buffer. For Pds1-18myc protein detection, we used 10% acrylamide gels for SDS–PAGE. For Swe1-myc protein detection, we used 8% acrylamide gels. The anti-myc antibody (9E10) (Covance Research Products) was used at 1:1000 dilution. Phosphoglycerate kinase 1 (Pgk1) antibody (Molecular Probes, Eugene, OR) was used at 1:5000 dilution. Proteins were detected with ECL (Perkin-Elmar-Cetus, Norwalk, CT). After enhanced chemiluminescence (ECL), the Western blot membranes were imaged using Bio-Rad ChemiDoc.

PFGE analysis

Collected yeast cells were washed once with water and then fixed with 70% ethanol for 1 h at room temperature. To remove cell walls, cells were resuspended in LiSorb buffer (100 mM lithium acetate, 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM sorbitol) and treated with zymolyase at 37°C for 1 h. Then cells were resuspended in TE buffer (10 mM Tris, 10 mM EDTA, pH 8.0). An equal volume of 1% melted agarose (after cooling down to 50°C) was added into cells. After agarose was solidified, cells embedded in agarose blocks were subject to digestion with lysis buffer (100 mM EDTA, 10 mM Tris, 1% sarkosyl, 100 μg/ml proteinase K, pH 8.0) overnight at 50°C. After that, the agarose blocks were washed with TE buffer twice and were ready for PFGE analysis. The CHEF-DR II pulsed-field electrophoresis system (Bio-Rad, Richmond, CA) was used. The running time was 20 h at 6 V/cm with a 60–120-s switch time ramp (14°C).

Fluorescent signal analysis

Strains containing mApple-labeled H2A (*HTA1-mApple*) or the GFP-marked centromere of chromosome IV (*CEN4-GFP*) and Tub1-mCherry were collected and fixed with 3.7% formaldehyde at room temperature for 5 min. The cells were then washed with water and resuspended in 1× phosphate-buffered saline. The fluorescent signal was analyzed over time after G_1 or S-phase release to examine nuclear division using a fluorescence microscope (Keyence).

ACKNOWLEDGMENTS

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