Plasma membrane/cell wall perturbation activates a novel cell cycle checkpoint during G1 in *Saccharomyces cerevisiae*

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Edited by Daniel J. Lew, Duke University Medical Center, Durham, NC, and accepted by Editorial Board Member Douglas Koshland April 29, 2016 (received for review December 22, 2015)

Cellular wound healing or the repair of plasma membrane/cell wall damage (plasma membrane damage) occurs frequently in nature. Although various cellular perturbations, such as DNA damage, spindle misalignment, and impaired daughter cell formation, are monitored by cell cycle checkpoint mechanisms in budding yeast, whether plasma membrane damage is monitored by any of these checkpoints remains to be addressed. Here, we define the mechanism by which cells sense membrane damage and inhibit DNA replication. We found that the inhibition of DNA replication upon plasma membrane damage requires GSK3/Mck1-dependent degradation of Cdc6, a component of the prereplicative complex. Furthermore, the CDK inhibitor Sic1 is stabilized in response to plasma membrane damage, leading to cell integrity maintenance in parallel with the Mck1-Cdc6 pathway. Cells defective in both Cdc6 degradation and Sic1 stabilization failed to grow in the presence of plasma membrane damage. Taking these data together, we propose that plasma membrane damage triggers G1 arrest via Cdc6 degradation and Sic1 stabilization to promote the cellular wound healing process.

cdc6 | mck1 | cell wall integrity | sic1 | DNA replication

It is common for cells to suffer from various attacks to their plasma membrane and cell wall, such as physical damage, pathogen invasion, and various environmental perturbations (1). Maintaining structured barriers between the internal and external environment of a cell, upon such challenges as those listed, is critical for the integrity of genetic materials. Therefore, it is advantageous to study cellular wound healing in a simple, genetically tractable model organism such as yeast.

The budding yeast, *Saccharomyces cerevisiae*, responds to cell wall and plasma membrane damage using an evolutionary conserved signaling cascade known as the cell wall integrity (CWI) pathway. The CWI pathway includes a Rho-type GTPase (Rho1) (2, 3) and protein kinase C (Pkc1) (4), both conserved plasma membrane repair proteins, and also includes part of the mitogen-activated protein (MAP) kinase cascade (5). One major consequence of the CWI pathway activation is transcriptional activation of enzymes required for cell wall synthesis (6). The CWI pathway can be studied using chemicals such as sodium dodecyl sulfate (SDS), which perturbs the plasma membrane. Previously, Kono et al. established a laser damage assay that specifically creates acute local damage to the yeast membrane (7). Using this assay, the authors showed that daughter cell growth is temporarily arrested by Pkc1-dependent degradation of cell-polarity regulators (7). Although the growth arrest was suggested to be triggered by plasma membrane damage, how the cell cycle progression was modulated upon local membrane damage remains to be understood.

The cell cycle is a series of events that leads to genome duplication and cell division, producing two daughter cells. In most eukaryotes, cells commit to division in G1, at which point the cells integrate internal and external cues to determine cell fates (8). In budding yeast, the restriction point is called START (9). During pre-Start, cells arrest in response to mating pheromone, whereas post-Start cells are committed to one cell cycle progression (10). G1 progression is triggered by the G1 cyclin Cln3/CDK complex, which phosphorylates and inactivates Whi5, an inhibitor of transcription factor Swi4/Swi6 (SBF) (11). SBF and MBF, an additional transcription factor complex, then activate the transcription of two additional G1 cyclins, Cln1 and Cln2 (10, 12). Cln1 and Cln2 compose a positive feedback circuit via the activation of transcription factors SBF and MBF (13, 14), triggering a genome-wide transcriptional change that promotes the G1/S transition (15, 16). Subsequently, the CDK inhibitor Sic1 is phosphorylated by the G1 cyclins, ubiquitinated, and degraded in a SCF (Skp1–Cullin–F-box)-dependent manner (17, 18). DNA replication is triggered upon Sic1 degradation. DNA replication origins are licensed for subsequent firing by assembly of prereplicative complexes (pre-RCs). Each pre-RC contains the Mcm2-7 helicase loaded onto origin DNA through sequential binding of Orc1-6, Cdc6, and Cdt1 (19). At the onset of S-phase, the pre-RC is acted on by two protein kinases, Dbf4-Cdc7 (DDK) and S-phase Cyclin-Cdk (S-CDK), and then converts into the Cdc45-Mcm2-7-GINS (CMG) replicative DNA helicase (20). This step involves DDK-dependent loading of Sld3 and CDK-dependent assembly of the preloading complex, consisting of Sld2, Dpb11, GINS, and DNA polymerase ε (20, 21). In yeast, three pre-RC components (Cdc6, Mcm2-7, and the ORC) are phosphorylated by Cyclin/CDK to prevent a second round of DNA replication, thereby inhibiting DNA rereplication (22–28).

Cdc6 protein degradation ensures DNA replication once and only once during the cell cycle by which Cdc6 requires phosphorylation by CDK for degradation (29). Previously, we demonstrated that the yeast GSK-3 kinase, Mck1, phosphorylates Cdc6 at the GSK-3

Significance

Cells need to rapidly control their cell cycle profile, gene expression, or protein stability in response to the activation of stress-response pathways. One such type of stress results from plasma membrane damage, which can arise because of physical damage or pathogen invasion. Here, we report that plasma membrane stress inhibits polarized cell growth and DNA replication through a novel cell cycle checkpoint pathway in *Saccharomyces cerevisiae*. To our knowledge, this is the first study to link a plasma membrane signaling pathway with the cell cycle and DNA replication control, which is relevant to plasma membrane repair and the role of stress and cell proliferation control in higher eukaryotes.

Author contributions: K.K., A.A.-Z., and A.E.I. designed research; K.K., A.A.-Z., L.S., and A.E.I. performed research; K.K., M.N., and A.E.I. contributed new reagents/analytic tools; K.K., A.A.-Z., M.N., and A.E.I. analyzed data; and K.K., L.S., and A.E.I. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. D.J.L. is a guest editor invited by the Editorial Board.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1523824113/-/DCSupplemental.

consensus site, at Thr-39 and Thr-368, promoting degradation of Cdc6 to guarantee genome integrity (30). Cdc6-Thr368 phosphorylation requires a priming phosphorylation at Ser372 by Cyclin/CDK (31). This double phospho-degron at Cdc6-T368-S372 created by Mck1 and CDK serves as a binding site for the SCF F-box protein, Cdc4 (31).

The yeast Mck1 is a serine/threonine protein kinase homologous to mammalian glycogen synthase kinase-3 (GSK-3) (32, 33). Mck1 also plays a role in the stress response, such that *mck1* deletion cells are sensitive to hot and cold temperatures (34), benomyl (34), and osmotic stress (35). Mck1 also stimulates calcineurin signaling (36–38), and binds stress-response elements to activate transcription (38). We recently showed that GSK-3–dependent Cdc6 degradation plays a role in genome integrity maintenance when cells are exposed to DNA damage (31). Thus, Mck1 ensures proper DNA replication, prevents DNA damage, and maintains genome integrity by inhibiting Cdc6 (31).

Here we provide evidence that plasma membrane damage activates a novel cell cycle checkpoint in G1 through Mck1-depedent Cdc6 degradation and Sic1 stabilization.

Results

Plasma Membrane Stress Inhibits S-Phase Entry. First, we analyzed the cell cycle profile upon plasma membrane stress. Cells were synchronized in G1 by α-factor and then released into media with or without SDS treatment. Wild-type cells, without SDS treatment, entered S-phase 30 min after G1 release (Fig. 1*A*, *Left* column). In contrast, S-phase entry was significantly delayed with SDS treatment (Fig. 1*A*, *Center* column). However, cell cycle progression was completely normal when cells were treated with SDS after the origins have been fired, 20 min after G1 release (Fig. 1*A*, *Right* column). This finding indicates that S-phase entry is inhibited in response to plasma membrane damage. To test which DNA replication step is affected by SDS treatment, we used a *cdc7-4* mutant to arrest the cell cycle. Cdc7 binds to Dbf4 to make the DDK complex, which then phosphorylates and activates Mcm4 to initiate DNA replication after origin licensing (39, 40). *cdc7-4* is a DDK temperature-sensitive mutant that arrests the cell cycle during G1-S phase, after pre-RC formation but with an inactive helicase not yet able to unwind DNA (41). When cells were treated with SDS upon *cdc7-4* block and release, the cell cycle proceeded normally (Fig. 1*B*), indicating that SDS affects the DNA replication step before DDK is activated, probably at pre-RC formation.

S-cyclin/CDK Activity Is Inhibited in Response to Plasma Membrane Damage. Next, we examined cell cycle regulators to test if they play a role in G1 arrest induced by membrane damage. We investigated Sic1 protein expression, the S-phase CDK inhibitor, during cell cycle arrest induced by plasma membrane stress. Sic1 is rapidly degraded at the onset of the G1/S transition in untreated cells and is expressed again 60 min later during the next G1 phase (Fig. 2*A*, *Left*) (18, 42). In contrast, we found the Sic1 protein levels to be more stable in the presence of SDS throughout the time course (Fig. 2*A*, *Right*). Next, we examined protein levels of the S-phase cyclin, Clb5, under plasma membrane stress. Under normal conditions, Clb5 is degraded by the APC^{cdc20} complex during mitosis (43), which we observed 60 min after α-factor block and release in untreated cells (Fig. S1*A*, *Left*). Clb5 expression was observed again 80 min later during the next cell cycle. Under SDS treatment, Clb5 was continuously expressed throughout the time course (Fig. S1*A*, *Right*). Because of stable Sic1 protein level under SDS treatment, CDK activity might be inhibited despite continual Clb5 expression. To test this possibility, we examined the phosphorylation status of a CDK substrate, Sld2, a DNA replication protein phosphorylated by Clb5 (44). In a normal cell cycle progression, Sld2 is phosphorylated during G1/S-phase and dephosphorylated after S-phase (Fig. S1*B*, *Left*). In contrast, the timing of Sld2 phosphorylation was delayed in cells treated with SDS

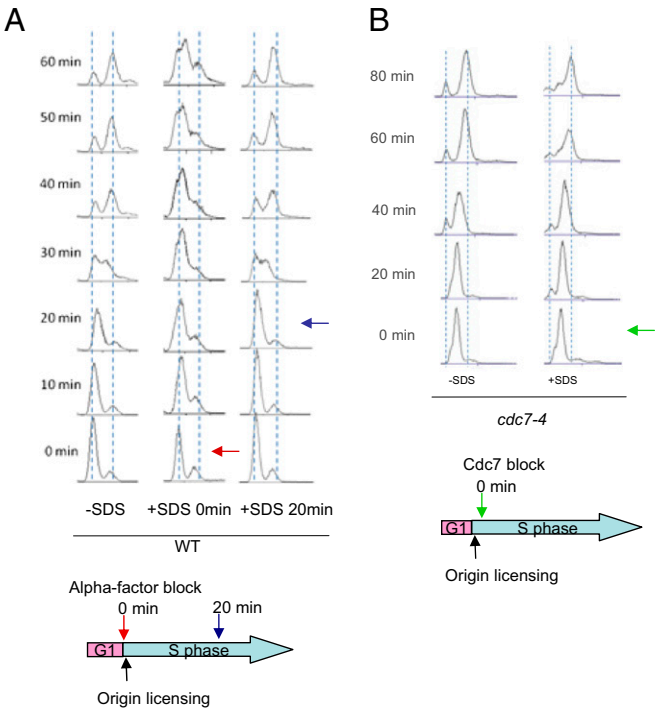

Fig. 1. S-phase progression is inhibited in response to SDS. (*A*) Wild-type cells were arrested and released in G1-phase by α-factor. The cell cycle progression was monitored by FACS analysis in the presence or absence of 0.0075% SDS added at time 0 (red arrow) or 20 min after release (blue arrow). (*B*) First, *cdc7-4* cells were arrested in G1-phase by α-factor at 23 °C and then released at 37 °C, the restrictive temperature, to block the cells after pre-RC formation but with an inactive helicase. Cells were then released into YPD media at 23 °C and collected every 20 min to monitor the cell cycle progression by FACS analysis in the presence or absence of SDS 0.0075% added at time 0 (green arrow). The diagrams below show at what point of the cycle that the cells were in when SDS was added.

(Fig. S1*B*, *Right*). This delay indicates that CDK activity is inhibited in response to plasma membrane stress, which leads to S-phase delay; this was further tested by in vitro kinase assay using Clb5/CDK purified from S-phase cells treated with SDS. We found that CDK activity is greatly reduced after SDS treatment (Fig. 2*B*).

Plasma Membrane Damage Inhibits DNA Replication Through Cdc6 Degradation. Sic1 protein levels were more stable in response to SDS (Fig. 2*A*). The Sic1 deletion strain, *Δsic1*, was sensitive to SDS, indicating that Sic1 may play a role in G1 arrest upon SDS treatment (Fig. S2*A*). We tested if *Δsic1* rescues the S-phase delay caused by SDS and observed that the cell cycle arrest was sustained (Fig. S2*B*), indicating that Sic1 is dispensable for G1 arrest induced by SDS treatment. This result is consistent with previous findings that Sic1 degradation is not a cause but a consequence of commitment to the cell cycle progression (45). Next, we considered the possibility that the cell cycle arrest upon SDS treatment is a result of pre-RC disassembly, because the cell cycle progression had no effect when SDS was added after pre-RC formation (Fig. 1*B*). We analyzed protein levels of pre-RC components after SDS treatment and observed that only Cdc6 was degraded in response to membrane damage triggered by SDS (Fig. 3*A* and Fig. S3*A*). Next, we monitored Cdc6 levels after different stress treatments. In contrast to Cdc6 degradation under SDS treatment (plasma membrane stress), we found that Cdc6 was stable at 40 °C (heat shock) or in the presence of 1 M NaCl (osmotic stress) (Fig. S3*B*). Previously, we showed that Cdc6 degradation is mediated by Mck1 during an unperturbed cell cycle or during a DNA damage response (30, 31). *Δmck1* deletion cells are sensitive to

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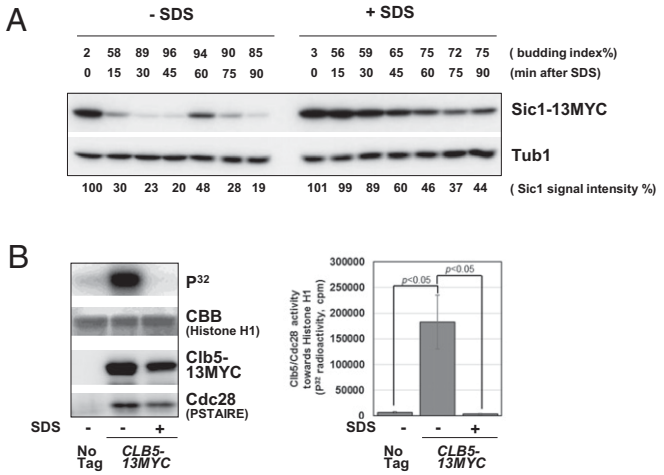

Fig. 2. CDK is inhibited through Sic1 stabilization upon SDS treatment. (*A*) Cells expressing Sic1-13myc (BY4741) from the genomic locus were synchronized by α-factor, and then released into fresh YPD medium in the presence or absence of 0.02% SDS to induce plasma membrane damage. Protein samples were collected every 15 min to observe Sic1-13myc levels by Western blotting. Tub1 was used as a loading control. The budding index is shown as percent of budded cells. (*B*) CLB5-13MYC cells (BY4741) were synchronized in G1 phase by α-factor. The cell cycle block was released and SDS was added to the media. Samples were collected at 20 min after the release. Clb5-13myc was immunopurified using anti-myc (9E10) antibody. Kinase reactions were performed with Histone H1 as a substrate. Total Histone H1 was visualized by Coomassie staining. Western blotting was performed to show the total amount of Clb5 and Cdc28. The graph indicates the averages of four independent experiments. Error bars represent SEM. Statistical significance was determined by Student's *t* test.

various stress stimuli, including plasma membrane stress (Fig. 4*B*), which suggests a link between Mck1-dependent Cdc6 degradation and membrane stress. Therefore, we examined the possibility that Mck1 promotes Cdc6 degradation in the presence of membrane damage. Indeed, Cdc6 degradation was rescued in *Δmck1* deletion cells, indicating that Cdc6 degradation in response to plasma membrane stress requires Mck1 (Fig. 3*A*).

To further understand how Cdc6 is degraded upon plasma membrane perturbation in respect to the cell cycle, Cdc6 protein degradation was monitored using cells synchronized in G1 with low CDK activity or in mitosis with high CDK activity. Cdc6 was degraded after SDS treatment when cells were arrested during G1 phase by α-factor, suggesting that CDK activity is not required for Cdc6 degradation under membrane stress (Fig. 3*B*, *Left*). We also observed Cdc6 degradation during mitotic arrest induced by the nocodazole treatment (Fig. 3*B*, *Right*), suggesting that Cdc6 degradation takes place independent of the cell cycle stages. We conclude that Cdc6 does not require CDK activity for its degradation in response to plasma membrane damage.

Next, the subcellular localization of Cdc6-GFP was monitored under a time-lapse microscope after local plasma membrane laser damage (Fig. 3*C*). Both the control and *Δmck1* cells show an immediate drop in GFP signal that we suspect to be an artifact, possibly because of pH change or protein loss through membrane leakage or photo bleaching. After the initial decline, the Cdc6-GFP signal for both genotypes became relatively stable; however, the GFP signal in the *Δmck1* cells stabilized with a higher intensity than in the wild-type cells. The *Δmck1* cells retained a nuclear Cdc6-GFP signal even after laser damage, confirming the above results that Mck1 is required for Cdc6 degradation after plasma membrane damage (Fig. 3*A*). Thus, Cdc6 is selectively degraded in a Mck1-dependent manner after membrane damage.

Mck1-Dependent Cdc6 Phosphorylation at T39 and T368 Are Responsible for DNA Replication Inhibition Induced by Plasma Membrane Stress. Previously, we showed that Mck1 phosphorylates Cdc6 at T39 and T368 (30, 31). Motivated by these findings, we tested if the Cdc6 phosphorylation mutant, Cdc6-T39A-T368A, is stabilized upon SDS stress. The levels of Cdc6-T39A-T368A protein, even after SDS treatment, remained unchanged (Fig. 4*A*), similar to wild-type Cdc6 protein in *Δmck1* cells (Fig. 3*A*), suggesting that SDS-dependent Cdc6 degradation requires phosphorylation at T39 and T368 by Mck1.

Next we examined whether Cdc6 degradation is required for viability in the presence of SDS. Indeed, cells with Cdc6-T39A-T368A failed to grow in the presence of SDS, similar to *Δmck1* cells (Fig. 4*B*, *Center*). Moreover, the marginal growth phenotype of Cdc6-T39A-T368A cells and *Δmck1* cells was suppressed by the addition of the plasma membrane-stabilizing reagent, sorbitol (46) (Fig. 4*B*, *Right*), suggesting that the cell lethality in *Δmck1* and *CDC6-T39A-T368A* cells in the presence of SDS was because of cell lysis. We tested if the stabilized Cdc6 bypasses the S-phase delay caused by SDS.

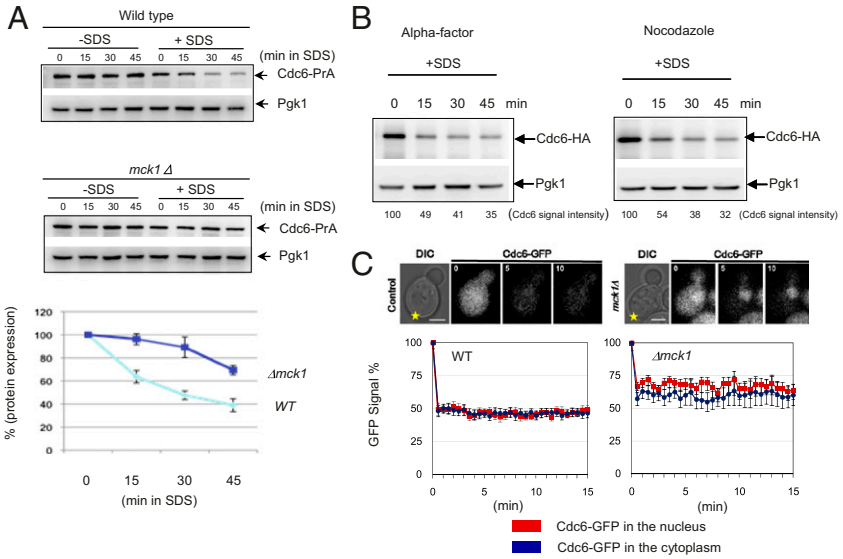

Fig. 3. Cdc6 is degraded in response to plasma membrane stress. (*A*) *CDC6-prA* or *Δmck1 CDC6-prA* cells were grown to log-phase. Samples were collected every 15 min in the presence or absence of 0.0075% SDS. Protein was extracted from each time point for Western blotting to detect Cdc6-prA or Pgk1 as a loading control. The same experiment was repeated three times and the signal was quantified to show the average with SD. (*B*) *GAL-CDC6* cells were incubated in galactose-containing media first. The cell cycle was arrested in G1 by α-factor or in mitosis by nocodazole. The cell cycle block was 86% in G1 and 83% in mitosis. Samples were collected every 15 min in the presence or absence of 0.0075% SDS. Protein was extracted from each time point and subjected to Western blotting. Pgk1 was used as a loading control. (*C*) Wild-type or *Δmck1* cells were damaged with a laser at the location marked with a star. The Cdc6-GFP signal was monitored by live-cell imaging using a fluorescence microscope. The numbers in the upper left corner indicate the time after laser damage (min). (Scale bars, 2 μm.) The graphs show the mean GFP signal intensity after background subtraction (control cells, $n = 15$; *mck1*Δ cells, $n = 11$). The error bars represent SEM.

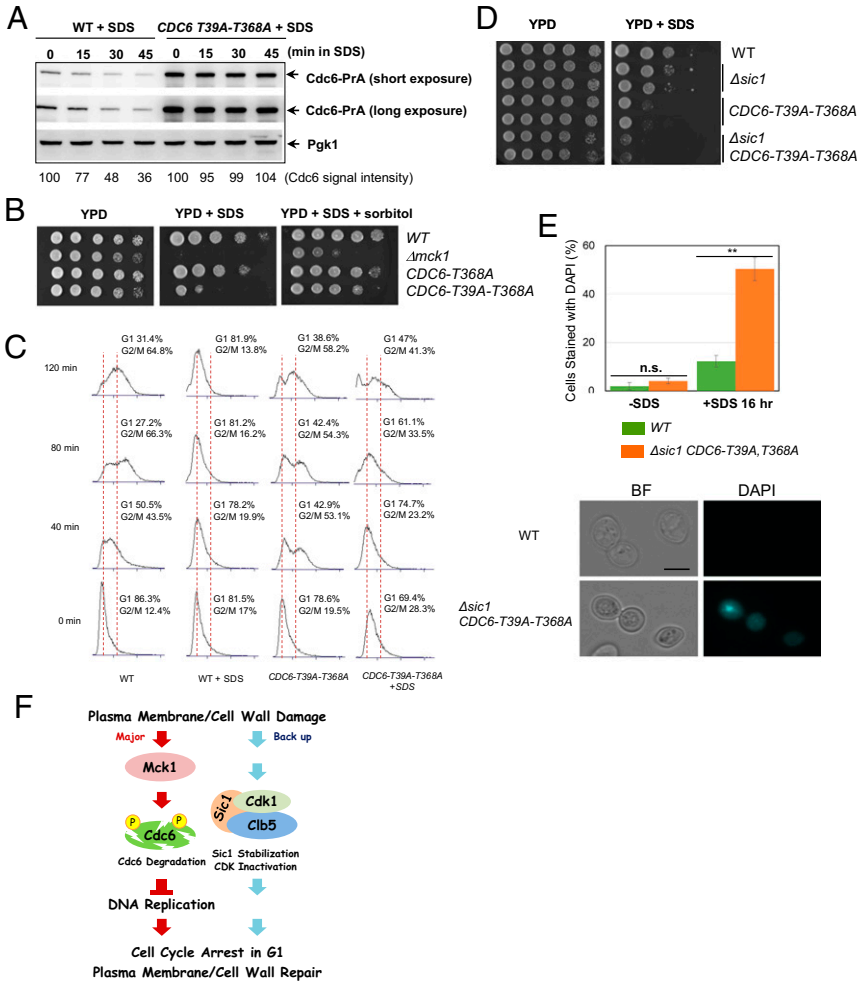

Fig. 4. Phosphorylation of Cdc6-T39 and T368 is required for Cdc6 degradation and cell cycle arrest after plasma membrane damage. (*A*) *CDC6-prA* or *CDC6-T39A-T368A-prA* cells were grown to log-phase. SDS at 0.0075% concentration was added to the media, and samples were collected every 15 min. Protein was extracted from each time point for Western blotting to detect Cdc6-prA or Pgk1 as a loading control. (*B*) Indicated W303 background yeast strains were serially diluted at fivefold, and spotted on YPD, YPD containing 0.005% SDS, or YPD containing 0.005% SDS plus 1 M sorbitol plates. Plates were incubated for 2–3 days. (C) Indicated strains were grown in YPD and were synchronized in G1 phase by α-factor. The G1 block was released into nocodazole-containing YPD media plus 0.0075% SDS at time 0. Samples were collected every 40 min to monitor cell cycle progression via FACS analysis. (*D*) Indicated strains were serially diluted at 10-fold and plated on YPD with or without 0.0075% SDS plates. (*E*) Indicated strains were grown in YPD with or without 0.0075% SDS for 16 h. Cells were washed once with YPD and then stained with DAPI to visualize cells with a ruptured plasma membrane under a fluorescence microscope. Averages of three independent experiments are shown. $n > 100$ per each experiment. Error bars, SD $^{**}P < 0.01$ (Student's t test). (Scale bar, 5 μm.) The images are representations of DAPI stained cells treated with SDS. (*F*) A model of a novel cell cycle checkpoint activated by plasma membrane damage.

CDC6-T39A-T368A cells entered mitosis 40 min after α-factor block and release and showed normal cell cycle progression when cells were untreated (Fig. 4*C*). Wild-type cells arrested their cell cycle during G1-phase during SDS treatment (Fig. 4*C*). Conversely, the *CDC6-T39A-T368A* cells treated with SDS progressed through S-phase and entered mitosis 80 min after α-factor release (Fig. 4*C*). G1-phase in the *CDC6-T39A-T368A* cells was greatly reduced despite the presence of SDS (Fig. 4*C*). We also detected an increase in the sub-G1 population in *CDC6-T39A-T368A* cells treated with SDS after 120 min, suggesting that Cdc6 stabilization after plasma membrane damage causes cell lysis. Thus, upon plasma membrane damage, Mck1-dependent phosphorylation/degradation of Cdc6 is required for cell cycle arrest before the G1/S-transition.

When *CDC6-T39A-T368A* mutations are combined with *Δsic1*, the SDS sensitivity was exacerbated (Fig. 4*D*), suggesting that the Sic1 stabilization and Cdc6 degradation were genetically parallel to maintain cell viability upon SDS treatment. What is the consequence in *Δsic1 CDC6-T39A-T368A* cells after plasma membrane damage? To test whether cells lacking both Cdc6 degradation and Sic1 stabilization mechanisms undergo cell lysis, we examined the plasma membrane's integrity after SDS treatment by using DAPI, as it only stains cells with a ruptured membrane (47). In wild-type untreated cells DAPI does not permeate the cell membrane, therefore there was no DAPI staining observed (Fig. 4*E*). In wild-type cells treated with SDS, 12% of cells showed plasma membrane rupture via DAPI staining (Fig. 4*E*). In contrast, we found that 50% of *Δsic1 CDC6-T39A-T368A* cells underwent plasma membrane rupture under SDS treatment (Fig. 4*E*). Thus, continued DNA synthesis and cell cycle progression in the presence of plasma membrane damage induces plasma membrane rupture and cell lysis, eventually leading to cell death.

Cdc6 degradation is mediated through the SCF^{Cdc4} complex (22–24). Previously we showed that methyl methanesulfonate (MMS)-induced Cdc6 degradation was rescued when Cdc4 was defective (31). To test if Cdc6 degradation induced by SDS is also mediated through Cdc4, we monitored the Cdc6 protein levels in a *cdc4-1* mutant. Cdc6 was stabilized in the presence of SDS when the *cdc4-1* temperature-sensitive mutant was incubated at the nonpermissive

temperature (36 °C) (Fig. S4). Thus, Cdc4 is likely to be involved in the degradation of Cdc6 during plasma membrane damage.

Our results imply that inhibition of DNA replication is crucial when cells encounter plasma membrane damage. Aberrant DNA replication under membrane stress may lead to cell death because cells have no chance to repair membrane damage. To assess this hypothesis, we tested if cells that rereplicate DNA are sensitive to plasma membrane damage induced by SDS. DNA rereplicating cells such as *ORC6-rxl GAL-CDC6* or *ORC6-rxl GAL-CDC6-T368A*, grown on galactose-containing plates, were sensitive to SDS, but not to high temperatures (Fig. S5). These results are consistent with our understanding that continuous DNA replication in the presence of plasma membrane damage leads to lethality. This genetic evidence supports the biological importance of DNA replication control under plasma membrane stress.

Discussion

In this work, we revealed a novel cell cycle checkpoint activated by plasma membrane damage. This checkpoint arrests the cell cycle after Start but before DNA replication initiation. DNA replication is inhibited upon plasma membrane damage, primarily via Mck1-dependent Cdc6 degradation. In addition to the primary pathway, Sic1 stabilization also plays a role in CDK inhibition, to promote plasma membrane healing and protect cells from cell lysis (Fig. 4*F*).

What is the molecular mechanism of Cdc6 degradation caused by membrane stress? Cdc6 phosphorylation at T368 by Mck1 requires a priming phosphorylation at S372 by CDK. Considering that CDK activity is inhibited by Sic1 in response to plasma membrane stress, it is possible that Cdc6 uses a different priming kinase other than CDK that enables cells to promote Cdc6 degradation independently from cell cycle progression but in response to plasma membrane stress. In fact, Cdc6 was still degraded in G1 cells when CDK activity is low (Fig. 3*B*). It is of interest to study if the kinases involved in the CWI pathway phosphorylate Cdc6 at its priming site, which allows Cdc6 to create a "stress-responsive" phosphodegron to ensure timely protein degradation in response to stress. It is worth noting that *Δmck1* cells did not rescue G1 arrest induced by SDS (Fig. S6), which is probably because of other unknown functions of Mck1 during the G1-S transition. Furthermore *CDC6-T39A-T368A* mutant cells partially but not completely rescued the G1 arrest (Fig. 4*C*), which indicates that there might be a Mck1-independent Cdc6 regulation mechanism involved in the G1 arrest caused by SDS. It is also possible that other DNA replication proteins and steps are regulated during S-phase in response to stress.

What is known about DNA replication control in response to stress in general? DNA replication is linked to the osmotic stress-response pathway. Hog1 (stress-activated protein kinase, SAPK) phosphorylates Mrc1 to delay origin firing in response to osmotic stress, resulting in the maintenance of genome integrity in yeast (48). Mrc1 phosphorylation by Hog1 is independent of that by the DNA damage checkpoint; therefore, Hog1 and Mrc1 play a role in a novel S-phase checkpoint upon osmotic stress (48). Taken together, these data show that cells use distinct kinases and downstream cell cycle targets to respond to various environmental stresses and maintain genome integrity.

The sustained Cdc6 expression in *CDC6-T39A-T368A* mutants led to S-phase entry even in the presence of membrane damage caused by SDS, leading to cell lethality (Fig. 4 *B* and *D*). A *CDC6-T368A* single mutation did not cause severe cell growth defects in the presence of SDS (Fig. 4*B*). It might be interesting to study the role of the Cdc6-T39 phosphorylation site, which might have prominent role in the stress response.

Our results also explain previous observations that cells defective in SCF function (*cdc53-1* and *cdc34-2* mutants) show SDS sensitivity (49). First, Cdc6 phosphorylation is recognized by the SCF^{CDC4} complex for its degradation (22, 23, 29, 31), suggesting that the SCF mutants cause Cdc6 stabilization. Furthermore, Sic1 is also targeted for its protein degradation by the SCF complex (18). Therefore, Sic1 is ectopically accumulated in cells defective in SCF function. Thus, SCF function seems to be critical for cell cycle arrest as well as for initiating a wound-healing response.

The sensitivity of the *CDC6-T39A-T368A* mutant to SDS was more severe than that in *Δsic1*, indicating that Cdc6 degradation might be the primary mechanism to arrest the cell cycle during G1 (Fig. 4*D*). DNA rereplication could trigger chromosome instability, which is a hallmark of tumorigenesis in higher eukaryotes (50). The DNA replication protein Cdc6 has to be degraded after origin licensing to prevent DNA rereplication in yeast (24, 51–53). In human, Cdc6 is overexpressed in brain tumors (54), lung carcinomas (55), and lymphomas (56), indicating the importance of Cdc6 protein levels in tumorigenesis. An ectopic expression of CDC6 induces DNA replication in quiescent cells (57) and DNA rereplication in tumor cells (58). To our knowledge, this is the first evidence suggesting that plasma membrane damage promotes a checkpoint-like mechanism inhibiting DNA replication. Given that the players involved in plasma membrane damage-dependent inhibition of DNA replication are evolutionarily conserved, the cell cycle and DNA replication control under plasma membrane stress should be investigated in higher eukaryotes. Thus, linking the stress response to DNA replication and cell cycle control will offer insights into the mechanism for control of cancers.

Materials and Methods

Plasmids and Strains. Standard methods were used for mating, tetrad dissection, and transformation. All strains listed in Table S1 are congenic with W303 unless noted.

Cell Cycle Block and Release. Log-phase cells (OD = 0.3–0.4) were blocked by incubating for 2 h with the addition of α-factor (10^{-7} M). Cells were washed three times with YPD and then placed into fresh YPD media to release the block. For the *cdc7-4* strain, cells were incubated to log-phase at 23 °C and blocked with α-factor, as described above. The temperature was shifted to 36 °C for 1 h upon α-factor release to create an additional block. The cell cycle was released again by shifting the temperature back to 23 °C. To induce mitotic block, cells were treated with nocodazole at the concentration of 15 μg/mL for 2 h.

SDS Experiment. SDS at the concentration of 0.0075% was used unless indicated. BY4741 is less sensitive to SDS and was treated at 0.02% in Fig. 2.

Western Blotting. Cells were lysed by agitation in SDS sample buffer with glass beads using a FastPrep (MP Biomedicals) for 20 s, twice, at speed 6. The protein was separated by SDS/PAGE with 10% (wt/vol) polyacrylamide gel. Western blot analysis was performed using an anti-cMYC antibody 9E10 (Sigma) at 1:2,000 dilution, anti-cMYC antibody A-14 (Santa Cruz) at 1:1,000 dilution, anti-Cdc2 (PSTAIRE) antibody (Santa Cruz) at 1:1,500 dilution, anti-Pgk1 antibody (Life Technologies) at 1:2,000 dilution as a loading control, anti-Tub1 antibody (AbD Serotec) at 1:5,000 dilution as an additional loading control. Protein-A–tagged proteins were probed using HRP-conjugated anti-rabbit IgG antibody (Sigma) at 1:5,000 dilution.

Fluorescence Microscopy. The laser-damage experiment was performed as previously described (7), with modifications as follows. A yeast culture grown overnight was refreshed and incubated for an additional 2–6 h until OD_{600} reached 0.1–0.3. Cells were then spotted onto an agarose bed (SD medium + 1.2% agarose) on glass slides. An LSM 780 NLO confocal laser scanning microscopy (Carl Zeiss) was used to create the damage and to monitor the fluorescent and bright-field images. Signal quantification was performed using FIJI software.

Kinase Assay. Cdc28/Clb5-13myc was immunopurified using anti-myc (9E10) antibody. Kinase reactions were performed as described previously (59). Histone H1 (Roche) was used as a substrate.

Plasma Membrane Integrity Assay. Yeast strains were grown to early-log phase (0.1–0.2 OD_{600}) in YPD media, transferred to YPD supplemented with 0.0075% SDS for 16 h. OD_{600} = 1.5 equivalent of cells were collected and washed once with YPD, then resuspended in YPD including DAPI. Cells were then observed under a fluorescence microscope.

ACKNOWLEDGMENTS. We thank Atsuya Nishiyama, Nube Ramon, and Hsin-I Wen for technical support. This study was supported in part NIH Grant 5SC3GM105498 (to A.E.I.); PSC CUNY enhanced award (to A.E.I.); and JSPS Grants 25112520 (to K.K.) and 26250027 (to M.N.). A portion of this work was conducted in the Institute of Transformative Bio-Molecules at Nagoya University, supported by the Japan Advanced Plant Science Network.

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