July 2015

Cdc6 degradation requires phosphodegron created by GSK-3 and Cdk1 for SCFCdc4 recognition in Saccharomyces cerevisiae

Amr Al-Zain  
*CUNY Brooklyn College*

Lea Schroeder  
*CUNY Brooklyn College*

Alina Shegllov  
*CUNY Brooklyn College*

Amy E. Ikui  
*CUNY Brooklyn College*

How does access to this work benefit you? Let us know!

Follow this and additional works at: [http://academicworks.cuny.edu/bc_pubs](http://academicworks.cuny.edu/bc_pubs)

**Recommended Citation**

Cdc6 degradation requires phosphodegron created by GSK-3 and Cdk1 for SCF\textsuperscript{Cdc4} recognition in \textit{Saccharomyces cerevisiae}

Amr Al-Zain, Lea Schroeder, Alina Sheglov, and Amy E. Ikui
Department of Biology, Brooklyn College, City University of New York, Brooklyn, NY 11210

**ABSTRACT** To ensure genome integrity, DNA replication takes place only once per cell cycle and is tightly controlled by cyclin-dependent kinase (Cdk1). Cdc6p is part of the prereplicative complex, which is essential for DNA replication. Cdc6 is phosphorylated by cyclin-Cdk1 to promote its degradation after origin firing to prevent DNA rereplication. We previously showed that a yeast GSK-3 homologue, Mck1 kinase, promotes Cdc6 degradation in a SCF\textsuperscript{Cdc4}-dependent manner, therefore preventing rereplication. Here we present evidence that Mck1 directly phosphorylates a GSK-3 consensus site in the C-terminus of Cdc6. The Mck1-dependent Cdc6 phosphorylation required priming by cyclin/Cdk1 at an adjacent CDK consensus site. The sequential phosphorylation by Mck1 and Clb2/Cdk1 generated a Cdc4 E3 ubiquitin ligase–binding motif to promote Cdc6 degradation during mitosis. We further revealed that Cdc6 degradation triggered by Mck1 kinase was enhanced upon DNA damage caused by the alkylating agent methyl methanesulfonate and that the resulting degradation was mediated through Cdc4. Thus, Mck1 kinase ensures proper DNA replication, prevents DNA damage, and maintains genome integrity by inhibiting Cdc6.

**INTRODUCTION**

Initiation of DNA replication requires prior assembly of the prereplicative complex (pre-RC) by Cdc6- and Cdt1-dependent recruitment of the minichromosome maintenance complex (Mcm2–7) to the origin-bound origin recognition complex (Orc1-6) (Araki, 2010). Pre-RC assembly licenses the origin to further recruit the Dbf4-kinase/Cdc7 complex to form a bidirectional replication fork (Araki, 2010). DNA synthesis then occurs through the activity of S-phase/mitotic cyclin-Cdk complexes (Tanaka et al., 2007; Zegerman and Diffley, 2007). In \textit{Saccharomyces cerevisiae}, this includes six B-type cyclins (Clb1–6) and one Cdk1 (Cdc28p) (Nasmyth, 1996).

Cells ensure that DNA replication occurs only once per cell cycle in order to maintain genome integrity. To achieve this, the pre-RC is rapidly disassembled after Cdk1-dependent phosphorylation, and reassembly is inhibited until the following cell cycle. The inhibition of pre-RC assembly is dependent on B-type cyclins (Dahmann et al., 1995; Ikui et al., 2007) and involves multiple overlapping mechanisms. Mcm2–7 is excluded from the nucleus after phosphorylation by the cyclin-Cdk complex (Labib et al., 1999; Nguyen et al., 2000). Orc2 and Orc6 are phosphorylated to inhibit pre-RC loading (Nguyen et al., 2001). To further prevent pre-RC assembly, Clb2 binds to the phosphorylated N-terminal domain of Cdc6 (Mimura et al., 2004), and Clb5p binds to the Arg-X-Leu (RXL) domain in Orc6 to sterically inhibit Cdt1/Mcm2–7 loading (Chen and Bell, 2011; Wilmes et al., 2004). In addition, Cdc6p levels are tightly regulated during the cell cycle. Cdc6 is transcribed in late mitosis and G1 and accumulates throughout G1; then its levels rapidly drop after passage through START (Piatti et al., 1995; Zwerschke et al., 1994). Cdc6 is also regulated through its localization (Honey and Futer, 2007).

Multiple mutations affect rereplication control, including mutation of Orc2 and Orc6 phosphorylation sites (ORC2-ps and ORC6-ps) (Nguyen et al., 2001), forced nuclear localization of Mcm2–7 (MCM7-NLS) (Nguyen et al., 2000), and stabilization through N-terminal truncation of Cdc6p (CDC6\textsuperscript{ΔNT}) (Drury et al., 1997). Combining these mutations is lethal and induces rereplication (Nguyen et al., 2001; Wilmes et al., 2004; Archambault et al., 2005). In addition, each of these individual mutations strongly...
synergizes with ORC6 mutation at the RXL domain (ORC6-ntl) (Wilmes et al., 2004). For instance, an ORC6-ntl CDC6ΔNT double mutant causes slow growth and mitosis, and the functional outcome of causing DNA damage and activating the DNA damage checkpoint (Archambault et al., 2005; Ikui et al., 2007).

We previously found that the ORC6-ntl mutation is synthetically lethal with the deletion of the MCK1 gene, a yeast homologue of glycogen synthase kinase 3 (GSK-3) (Archambault et al., 2005; Ikui et al., 2012). Meiosis and centromere regulatory kinase (Mck1) is a serine/threonine kinase whose catalytic activity requires autophosphorylation on tyrosine residues (Lim et al., 1993; Brazill et al., 1997; Rayner et al., 2002). Mck1p was first found as a dosage suppressor of a centromere mutation that causes chromosomal missegregation (Shero and Hieter, 1991) and as an early regulator of genes required for meiosis and sporulation (Neigeborn and Mitchell, 1991). In addition, it is involved in the stress response by promoting the binding of transcription factors to the promoters of stress-responsive genes (Hirata et al., 2004). Mck1 also phosphorylates the sister cohesion protein Eco1, after Dbf4-kinase/Cdc7 and Cdk1-dependent priming phosphorylation, for ubiquitin-mediated degradation (Lyons et al., 2013).

There are eight CDK consensus sites in the N- and C-termini of Cdc6 that target Cdc6 for ubiquitin-mediated proteolysis (Drury et al., 1997, 2000; Elssasser et al., 1999, Perkins et al., 2001). In mitosis, Cdc6p is degraded through the SCFcdc6 ubiquitin ligase, which is dependent on Cdc6p T368 and S372 (Drury et al., 1997, 2000). Previously, we elucidated a novel function of Mck1 for DNA recombination inhibition (Ikui et al., 2012). We found that Mck1 promotes Cdc6p degradation in a SCFcdc6-dependent manner through the C-terminus. The C-terminal residues T368 of Cdc6 contain a GSK-3 consensus site in the form TTxTxT/Tp. Theoretically, GSK-3 kinase phosphorylates the first S/T after the priming phosphorylation of the S/T residue at the fourth position by another kinase (Fiol et al., 1987). T368 and S372 are important for Cdc6 degradation during mitosis, have been studied as putative Cdk1 phosphorylation sites (Kashi et al., 2007). Mck1 also phosphorylates the sister cohesion protein Eco1, after Dbf4-kinase/Cdc7 and Cdk1-dependent priming phosphorylation, for ubiquitin-mediated degradation (Lyons et al., 2013).

In this article, we provide evidence in vitro and in vivo that Mck1, but not Cdk1, phosphorylates Cdc6 only after priming phosphorylation of S372 by Cib2/Cdk1 to promote Cdc6 degradation during mitosis. The Cdc6 phosphodegron created by Cdk1 and Mck1 is crucial for Cdc4 binding to promote Cdc6 degradation. We also show that GSK3-dependent Cdc6 degradation is augmented after DNA damage stress in order to maintain genome integrity.

**RESULTS**

**GSK-3 consensus site at T368-S372 in Cdc6 is responsible for Cdc6 degradation**

The GSK-3 consensus motif contains S/T-x-x-x-S/T. There are two GSK-3 consensus sites in Cdc6, one at each terminus (Figure 1A). The TPTTS GSK-3 consensus site (T368 to S372) in Cdc6 is overlapped with two CDK sites at TP (368 and 369) and SP (372 and 373) (Figure 1A). We mutated each amino acid from Thr, Ser, or Pro in the GSK-3 consensus site to Ala and examined the stability of Cdc6p. Wild-type Cdc6p was degraded after expression was shut off during mitosis (Figure 1B; Drury et al., 1997, 2000; Perkins et al., 2001). Cdc6-T368A, P369A, S372A, and P373A single mutants or T368A-S372A double-mutant proteins were stabilized, as was Cdc6p in mck1-deletion cells, indicating that the GSK consensus sequence from T368 to S372 is crucial for Cdc6 degradation (Figure 1B; Perkins et al., 2001). Cdc6 stabilization by P369A or P373A mutation indicates that T368 or S372 might be potential Cdk1 phosphorylation sites. A single mutation in Cdc6 at the N-terminus, T39A or S43A, did not alter Cdc6p stability during mitosis (Figure 1B). However, we observed high Cdc6p protein levels at time zero when the two mutations at the GSK-3 consensus sites T39A and T368A were combined (Figure 1B). Consistent with Cdc6 protein stability in CDC6-T368A, T39A double mutants (Figure 1B), we found that GAL-CDC6-T39A, T368A cells showed elongated bud and mitotic arrest (Figure 1D and Supplemental Figure S1). We also observed more stabilized Cdc6p in CDC6-T368A or CDC6-T39A, T368A cells than in Δmck1 cells (Figure 1B). This indicates that Cdc6-T368A site is phosphorylated by multiple kinases.

We previously found that mck1 deletion is synthetically lethal with an ORC6-ntl mutation that disrupts a control for DNA recombination (Ikui et al., 2012). Given the Cdc6p stabilization in mck1-deletion cells (Figure 1B), we tested whether the CDC6 GSK-3 consensus-site mutations that stabilized Cdc6p also induce enhanced synthetic lethality in the ORC6-ntl mutant. Viability of yeast cells that contain various CDC6 mutations and the ORC6-ntl mutation were tested. The GAL-CDC6 ORC6-ntl mutant caused increased lethality on galactose-containing plates compared with controls (Figure 1C). The lethality was exacerbated when cells contained CDC6 mutations (T368A, P369A, S372A, and P373A single mutants or T368A and S372A double mutants) at the GSK-3 consensus sites (Figure 1C, top). ORC6-ntl GAL-CDC6-T39A, T368A cells showed enhanced lethality compared with ORC6-ntl GAL-CDC6-T39A or ORC6-ntl GAL-CDC6-T368A (Figure 1C, bottom). Next the Cdc6-T368A mutation was integrated into the genome locus and was crossed with the ORC6-ntl and ORC6-ntl,ps mutants. The resulting double mutants, ORC6-ntl CDC6-T368A and ORC6-ntl,ps CDC6-T368A, showed slow growth (Supplemental Figure S2).

Mck1 phosphorylates Thr-368 in Cdc6 after priming by Cib2/Cdk1

To identify the Cdk1 or Mck1 phosphorylation sites on Cdc6, we performed an in vitro kinase assay using various Cdc6 synthetic peptides with Mck1p kinase or Cdk1p kinase purified from yeast. Cib2/Cdk1 phosphorylated the wild-type Cdc6 synthetic peptide containing the C-terminal GSK-3 consensus site; however, the phosphorylation was abolished when the Cdc6 peptide contained a phosphate at S372 (Figure 2A, left). This indicates that Cib2/Cdk1 targets the S372 site but not the T368 site. On the other hand, Mck1 did not phosphorylate the wild-type Cdc6 peptide unless the peptide contained a phosphate at S372 (Figure 2A, left). The Mck1-dependent Cdc6 phosphorylation was abolished when we used Cdc6 peptides that contained an alanine mutation at T368 (Figure 2A, left). This result proved that Cdc6-T368 is phosphorylated by Mck1 in vitro. Mck1 weakly phosphorylated the Cdc6 peptide that contained the N-terminal GSK-3 consensus site at T39. However, the phosphorylation efficiency was ~20-fold less than that at the C-terminal T368 (Figure 2A, right). Cib2/Cdk1 did not phosphorylate the Cdc6 peptide that contained the Cdk1 consensus site at T39 and S43 (Figure 2A, right).
FIGURE 1: Analysis of GSK-3 consensus sites in Cdc6. (A) Cdc6 contains two GSK-3 consensus sites, which overlap with two CDK consensus sites. (B) GAL-CDC6-HA strains with various mutations (T368A, P369A, S372A, P373A, T39A, S43A, T39A-T368A, and T368A-S372A) were expressed with galactose-containing medium for 2 h and then blocked with nocodazole for 2 h. Cdc6 expression was then suppressed by adding glucose. Protein extracts were collected every 5 min and subjected to Western blot analysis to observe Cdc6-HA. Pgk1 was used as a loading control. GAL-CDC6-HA in mck1-deletion cells was examined using the same method. Western blotting images for WT, Δmck1, CDC6-T39A, CDC6-T368A, and CDC6-T39A,T368A were quantified. Percentage of Cdc6 protein remaining relative to time zero is shown. Results are the average of three independent experiments, and error bars indicate SD. *p < 0.05. (C) Strains with the indicated genotypes were serially diluted 10-fold, plated on yeast extract/peptone/dextrose or yeast extract/peptone/galactose plates, and incubated at 30°C for 2 d. (D) GAL-CDC6, GAL-CDC6-T368A, or GAL-CDC6-T39A-T368A was grown in raffinose-containing medium first. Cdc6p was expressed with galactose for 3 h.
Next we tested whether Mck1 kinase phosphorylates Cdc6-T368 in vivo, using a phosphospecific antibody against the T368 site. Cdc6 was phosphorylated at the T368 site in wild-type cells when the cell cycle was arrested during mitosis using nocodazole (Figure 2B, left). The Cdc6-T368 phosphorylation was abolished in the Cdc6-T368A mutant as well as in Δmck1 cells. We did not detect T368 phosphorylation when the priming site at S372 was mutated to alanine, indicating that Mck1 phosphorylates T368 only when the S372 priming site is phosphorylated (Figure 2B, left). Furthermore, the T368 site was not phosphorylated in wild-type cells arrested in G1 phase with α-factor (Figure 2B, right). These results support our priming model (Ikui et al., 2012) that Cdc6 requires Cdk1-dependent priming at S372 in order to be phosphorylated by Mck1 at T368 during mitosis.

We next tested the cyclin dependence of Mck1-induced Cdc6 phosphorylation at T368. The phosphorylation status of Cdc6-T368 was analyzed in vivo in GAL-CDC6, Δclb2 GAL-CDC6, Δclb4 GAL-CDC6 or Δclb5 GAL-CDC6 cells during mitosis. Cdc6-T368 phosphorylation was dependent on the mitotic cyclin Clb2 (Figure 2C), which is consistent with our in vitro kinase assay results. The phosphorylation was also dependent on the S-phase cyclin Clb5. However, the phosphorylation was still observed in clb4-deletion cells (Figure 2C).

Cdc6 phosphorylation by Mck1 and Cdk1 creates a Cdc4 phosphodegron

We previously proposed a model in which double phosphorylation of Cdc6 by Mck1 and Cdk1 may create a phosphodegron that is recognized by Cdc4 (Ikui et al., 2012). To test this, we created a GAL-CDC6-Δ370 mutant strain lacking one amino acid between the Mck1 and Cdk1 phosphorylation sites and whose expression is controlled under the GAL promoter. Similar to wild-type Cdc6p, the Cdc6-Δ370 protein was still degradable after glucose addition, whereas the Cdc6-Δ370Δ371 protein, lacking two of the three amino acids of the phosphodegron, was more stable than both single mutants (Figure 3A). Cdc6-T370A or Cdc6-T371A proteins were also degraded, signifying that spacing and not the specific
Cdc6 regulation by CDK and GSK-3

2613

We conclude that Cdc4 binds to the Cdc6 phosphodegron at T368 and S372 when there are two or three amino acids between phosphorylations. Only one amino acid between phosphorylations (Cdc6-Δ370Δ371) causes stability because Cdc4 does not recognize it. T368M mutant was used as a negative control (Perkins et al., 2001).

Cdc6 degradation upon DNA damage is mediated by Mck1

It has been shown that the DNA alkylating agent methyl methanesulfonate (MMS) triggers Cdc6 degradation in S. cerevisiae.
that Cdc6 degradation after DNA damage was mediated by Mck1 kinase. We also observed suppression of Cdc6 degradation in the CDC6-T368A mutant, in which T368A mutation was integrated at the endogenous locus (Figure 4C). We conclude that DNA damage triggers Mck1-dependent Cdc6 degradation.

Cdc6 degradation is mediated by the SCF
Cdc4
complex (Drury et al., 1997; Elsasser et al., 1999; Perkins et al., 2001). We found that MMS-induced Cdc6 degradation was suppressed when Cdc4 was defective (Figure 4A), suggesting that Cdc6 degradation after DNA damage was mediated by Mck1 kinase. We also observed suppression of Cdc6 degradation in the CDC6-T368A mutant, in which T368A mutation was integrated at the endogenous locus (Figure 4C). We conclude that DNA damage triggers Mck1-dependent Cdc6 degradation.

Cdc6 degradation is mediated by the SCF
Cdc4
complex (Drury et al., 1997; Elsasser et al., 1999; Perkins et al., 2001). We found that MMS-induced Cdc6 degradation was suppressed when Cdc4 was defective (Figure 5A). Most likely, Cdc6 phosphorylations upon
DNA damage are recognized by the SCF<sup>Cdc6</sup> complex for subsequent Cdc6 ubiquitination. It is known that Cdc6 degradation is also mediated through Tom1 and Dia2 (Hall et al., 2007; Kim et al., 2012). We further tested whether Cdc6 degradation upon MMS treatment is mediated by the Tom1 or Dia2 ubiquitin ligases. We did not observe significant difference in Cdc6 degradation rate between wild-type, Δdia2, or Δtom1 deletion cells after MMS treatment (Supplemental Figure S3).

Cells with stabilized Cdc6 are susceptible to DNA damage

The homologous recombination protein Rad52 localizes to sites of double-stranded DNA breaks (DSBs) (Lisby et al., 2001). MMS increases the frequency of DSBs in budded cells, as detected by the formation of Rad52-YFP foci (Lisby et al., 2003), likely due to an increased frequency of replication fork collapse (Tercero and Diffley, 2001). To test the functional significance of Mck1-mediated Cdc6 degradation during DNA damage stress, we counted the frequency of Rad52-YFP foci in wild-type cells or Δmck1 cells after MMS treatment. In unbudded cells during G1 phase, we did not observe a significant difference in the frequency of Rad52-YFP foci between wild-type and Δmck1 cells after MMS treatment (Figure 5B). Consistent with previous work, however, more Rad52-YFP foci were formed in wild-type budded cells, both with and without MMS treatment (Figure 5B; Lisby et al., 2003). In untreated Δmck1 cells, we observed a statistically higher rate of Rad52−yellow fluorescent protein (YFP) foci formation (Figure 5B). The Rad52-YFP foci frequency was increased after MMS treatment in both wild-type and Δmck1 cells, but more strikingly in Δmck1 cells (Figure 5B). Furthermore, cells expressing GAL-CDC6-T368A, which contains a mutation at the Mck1 phosphorylation site, had an increase in the frequency of Rad52-YFP foci formation after MMS treatment when grown in galactose-containing but not glucose-containing media compared with GAL-CDC6 cells (Figure 5C).

We further obtained evidence that the Cdc6-T368 site is more phosphorylated after MMS treatment (Figure 5D). This result supports our conclusion that Cdc6 is degraded after phosphorylation at the T368 site through Mck1 kinase. To test whether Mck1 is regulated in a cell cycle–specific manner. These reports, together with our findings, strongly suggest that there might be more GSK-3 substrates that are targeted in a Cdc4-dependent manner.

DISCUSSION

The mechanism of Cdc6 protein degradation is complex. Cdk1-dependent Cdc6 phosphorylations and degradations have been extensively studied in order to understand the molecular mechanism of Cdc6 control. The Cdc6 phosphorylation sites have been studied based on the CDK consensus motif ST or TP (Drury et al., 2000; Perkins et al., 2001; Boronat and Campbell, 2007; Honey and Futcher, 2007). There are two GSK-3 consensus sites in Cdc6 (Figure 1A). Both of the GSK-3 sites in Cdc6 overlap with two CDK consensus sites; the second of each CDK site, at S43 or S372, is conserved, as it contains a lysine at the fourth position, K46 and K375, respectively (Figure 1A). In this study, we found that the phosphorylation site at T368 in Cdc6, which was previously studied as a CDK site, is actually a GSK-3 phosphorylation site (Figure 2). GSK-3 kinase requires priming phosphorylation (Lee et al., 2012; Lyons et al., 2013). Therefore we hypothesized that within the GSK-3 consensus site in Cdc6 (TPTTS), S372 would serve as the priming site for T368 phosphorylation by Mck1 (Ikiu et al., 2012). Because S372 occurs within a conserved Cdk1 site (T/SPxR/K), we predicted that Cdc6 would provide this priming phosphorylation (Ikiu et al., 2012). We showed that T368 is phosphorylated by Mck1 (Figure 2A). The Cdc6-S372 site, followed by a basic residue at the fourth position, was efficiently phosphorylated by Cdc4, which may phosphorylate the S43 site. This would allow cell cycle–dependent Cdc6 degradation whose timing relies on Cdc4 priming. The GSK-3 consensus sequence, S/T-x-x-x-S/T, contains three amino acids between the priming and phosphorylation sites. Together, they create a phoshodegron for Cdc6-dependent ubiquitination (Ikiu et al., 2012; Lyons et al., 2013). The GSK-3 sites at the N-terminus and C-terminus of Cdc6 coincide with a binding site for the Cdc4 E3 ubiquitin ligase (Perkins et al., 2001). It was shown that the protein binding between Cdc4 and Cdc6 is abolished when there is a mutation at T368 or S372 (Perkins et al., 2001). Here, we show that the double phosphorylations created by Cdk1 at S372 and Mck1 at T368 serve as a phoshodegron for Cdc4 binding. The spacing of two or three, and not one, amino acids between the two phosphorylations is important for Cdc4 recognition (Figure 3B). This is consistent with a previous report for Eco1p (Lyons et al., 2013). Mck1 requires a priming phosphorylation. For example, Eco1 must be primed by Dbf4 and Cdc1 for cohesion function regulation (Lyons et al., 2013) and Rpr53 must be primed by Nsn1 in the TOR signaling pathway before Mck1 can phosphorylate either substrate (Lee et al., 2012). Possibly, Mck1 substrates are favored targets of Cdc4 (Mizunuma et al., 2001; Kishi et al., 2007; Ikiu et al., 2012; Lyons et al., 2013; Edenberg et al., 2014), because the GSK-3 consensus motif and the priming model can determine precise Cdc4 specificity. These reports, together with our findings, strongly suggest that there might be more GSK-3 substrates that are targeted in a Cdc4-dependent manner.

The presence of a separate Mck1-dependent degradation mechanism of Cdc6 raised the question of why such a mechanism is needed. First, we tested whether Mck1 is regulated in a cell cycle–dependent manner. Mck1 protein was stable throughout the cell cycle, as were its localization and kinase activities (Supplemental Figure S5). Mck1 was shown to promote protein degradation in response to stress (Andoh et al., 2000). We therefore reasoned that...
FIGURE 5: Cdc6 degradation upon DNA damage is mediated through the ubiquitin pathway. (A) CDC6-prA or cdc4-1 CDC6-prA cells under the endogenous promoter were grown to log phase at 26°C first and then the temperature was increased to 36°C for 1.5 h. Then MMS was added (0.1% final). Samples were collected after 0, 30, or 60 min. The same experiment was performed three times, and Cdc6 protein levels were quantified. Error bars represent SD. (B) RAD52-YFP or Δmck1 RAD52-YFP cells were grown to log phase in low-fluorescence medium. MMS was added (0.05% final) for 90 min. Rad52-YFP foci were visualized under a fluorescence microscope to assay for double-stranded DNA breaks before and after treatment with MMS. Percentage of cells containing Rad52 foci was determined in unbudded or budded cells. One hundred cells were counted for each sample. Percentage is average from three independent experiments, and error bars represent the SD. *p < 0.05. (C) GAL-CDC6 or GAL-CDC6-T368A cells were grown to log phase in raffinose-containing medium first, and then either glucose or galactose was added for 3 h of incubation. Finally, MMS was then added (0.05% final) for 1 h. Rad52-YFP foci formation was determined with or without MMS.
the Mck1-dependent Cdc6p degradation could be involved in the cell’s response to stress. The Cdc6 degradation in response to MMS was dependent on Mck1 kinase (Figure 4, A and C). Furthermore, MMS-induced Cdc6 degradation was mediated through SCF F-box protein (Cdc4) (Figure 5A). The continuous Mck1 activity throughout the cell cycle may help to phosphorylate and degrade Cdc6p when cells are exposed to DNA damage agent.

We also tested whether hydroxyurea (HU) or benomyl promotes Cdc6 degradation, since Δmck1-deletion cells are sensitive to these reagents (Shero and Hieter, 1991). However, Cdc6 was stable after HU or benomyl treatment (Supplemental Figure S6). These results indicate that Mck1-dependent Cdc6 degradation is triggered by specific DNA damage through alkylating agents. It has been reported that Sld3 is phosphorylated and inhibits late origin firing when cells are treated with MMS (Lopez-Mosqueda et al., 2010; Zegerman and Dillfey, 2010). The inhibition of late origin firing is dependent on Rad53 (Lopez-Mosqueda et al., 2010; Zegerman and Dillfey, 2010). Mck1-dependent Cdc6 degradation may serve as an additional mechanism to block origin firing, and it would be of interest to know whether the Mck1-dependent mechanism inhibits early and/or late origin firing. It remains to be investigated whether Mck1 is activated upon DNA damage directly. We observed that Cdc6 is more stabilized in CDC6-T368A than in Δmck1 (Figure 1B). This result raised the possibility that Cdc6p may be phosphorylated through another kinase, such as Rad53 or Mec1.

Cells undergoing S phase are more prone to DNA damage by MMS (Lisby et al., 2003). We also observed that Δmck1 cells accumulate more DNA damage in budded cells, which includes S-phase cells, than in wild-type cells (Figure 5C). We conclude that DNA replication has to be controlled and inhibited in early S phase by inhibiting Cdc6p so that cells do not proceed through S phase when cells are exposed to DNA damage agents. Continuous initiation of DNA replication during S phase under DNA damage stress will lead to accumulation of DNA damage and lethality.

It will be also interesting to identify novel Mck1 substrates. We searched for proteins that contain a GSK-3 consensus motif similar to that in Cdc6, S/T-P-X-S/T-P-X-R/K. Using the MOTIF search database (www.genome.jp/tools/motif/MOTIF2.html), we identified 17 candidate proteins that contain a similar GSK-3 consensus sequence, including three DNA replication proteins, Cdc6, MCM3, and Sld2. Although we do not yet have evidence that Mck1 targets these proteins, it would be interesting to study whether Mck1 targets other proteins besides Cdc6p.

The Δmck1 cells are not sensitive to MMS (Figure 5E), which suggests that the DNA damage checkpoint might be activated and protects Δmck1 cells even after the accumulation of DNA damage. Deletion of a DNA damage checkpoint gene enhanced Δmck1 sensitivity to MMS, supporting the hypothesis (Figure 5E). Desany et al. (1998) reported that overexpression of MCK1 suppresses lethality in rad53 mutants, indicating that Mck1p has a role in DNA damage repair. We propose a model in which Cdc6 inhibition by Mck1 under DNA damage stress ensures genome integrity and maintenance of proper cell proliferation. We also observed that overexpression of CDC6 but not the CDC6-T368A mutant partially rescues MMS sensitivity in the mre11-deletion strain (Figure 5E, bottom). Mcm complex plays a role in the DNA damage-induced signaling that controls DNA replication (Cortez et al., 2004). Overexpression of Cdc6 helps Mcm complex to be loaded on DNA (Frigola et al., 2013), which may enhance the DNA damage signaling pathway to rescue the Δmck1 lethality to MMS.

In humans, deregulation of replication factors, such as Cdc6, has been observed in many cancers. For instance, Cdc6 overexpression has been associated with brain tumors (Ohta et al., 2001), cervical cancer (Murphy et al., 2005), and lung carcinomas (Karakiados et al., 2004). Given the implication of Cdc6 up-regulation in human cancers (Borlado and Mendez, 2008), it would be of considerable interest to study whether mammalian GSK-3 kinase plays a role in Cdc6 degradation. Cdc6 degradation during alkylating DNA damage is conserved between humans and yeasts (Hall et al., 2007). Therefore it is possible that human GSK-3 kinase, similar to Mck1, might promote Cdc6 degradation. A link between mammalian GSK-3 kinase and the DNA damage response has been reported. GSK-3 kinase phosphorylates the oncogenic metazoan transcription factor c-Myc after DNA damage triggered by ultraviolet light. The phosphorylated c-Myc is targeted for ubiquitination by SCF<sup>βTrcp</sup>, the human homologue of Cdc4 (Popov et al., 2007). Of interest, c-Myc was shown to have a nontranscriptional role in the initiation of DNA replication (Dominguez-Sola et al., 2007). Although it is not clear whether the c-Myc degradation during DNA damage is associated with the replication function of c-Myc, it would support the general idea that DNA replication is inhibited during DNA damage through degradation of replication proteins.

**MATERIALS AND METHODS**

**Plasmids and strains**

Standard methods were used for mating, tetrad dissection, and transformation. All strains listed in Supplemental Table S1 are congenic with W303. p305-based GAL-CDC6-HA plasmid was a gift from Stephen P. Bell (Massachusetts Institute of Technology, Cambridge, MA). GAL-CDC6-HA strains were made as described previously (Wilmes et al., 2004). Briefly, GAL-CDC6-HA plasmid was linearized with Stul and integrated at the URA locus in the wild-type strain. The copy number of the integrated GAL-CDC6 was determined by real-time PCR. GAL-CDC6 mutations were made by site-directed mutagenesis (QuikChange II XL mutagenesis kit; Agilent, Santa Clara, CA) using the GAL-CDC6-HA plasmid as a template. The mutated GAL-CDC6 plasmid was integrated at the URA locus in wild-type cells, and the copy number was determined as described. MCK1-9MYC strain was constructed as described previously (Knop et al., 1999). CDC6-T368A and CDC6-T39A, T368A strains were generated by two-step PCR in order to integrate the mutation into the genomic locus (Figures 4C and 5F; Toulmay and Schneiter, 2006).
Cell cycle block experiments
Mitotic cell cycle arrest was achieved using 15 μg/ml nocodazole for 2 h at 30°C. GAL-CDC6-HA strains were grown to log phase in medium containing 3% raffinose. Galactose was then added to induce CDC6-HA expression for 2 h, followed by incubation in nocodazole for another 2 h. Glucose was then added to shut off the expression. For the α-factor arrest experiments, log-phase cells were arrested in G1 using 100 nM α-factor for 2 h at 30°C.

Western blotting
Cells were lysed by agitation in SDS sample buffer with glass beads using FastPrep (MP Biomedicals, Santa Ana, CA) for 20 s, twice, at speed 6. Proteins were separated by SDS–PAGE with 10% polyacrylamide gel, except for the samples in Figure 2, which were separated using SDS–PAGE with Novex 4–20% Tris-glycine polyacrylamide gel (Invitrogen, Life Technologies, Carlsbad, CA). Western blot analysis was performed using anti-hemagglutinin (HA) antibody 3F10 (Roche, Penzberg, Germany) at 1:2000 dilution, anti-CIB2 antibody (a generous gift from Frederick Cross, Rockefeller University, New York, NY), anti–phospho Cdc6–T368 antibody at 1:1000 dilution (custom-made antibody by 21st Century Bio, Marlboro, MA), anti-CMYC antibody 9E10 (Sigma-Aldrich, St. Louis, MO) at 1:2000 dilution, and anti-Pgk1 (Life Technologies, Carlsbad, CA) at 1:2000 as a loading control. Protein A–tagged proteins were probed using horseradish peroxidase–conjugated anti-rabbit immunoglobulin G (Sigma-Aldrich) at 1:5000.

To detect phospho–Cdc6–T368, CDC6 was immunoprecipitated by HA-affinity purification first in order to enhance the signal (Ikui et al., 2012).

Kinase assay
Cib2-Cdk1 and Mck1 proteins were purified from yeast cells expressing TAP-tagged proteins as previously described (Lyons and Morgan, 2011). The TAP-tagged plasmid for Cib2 and Mck1 were kindly provided by David O Morgan (University of California, San Francisco, San Francisco, CA; Lyons et al., 2013). Peptide kinase assay was performed as described (Lyons et al., 2013) at room temperature for 30 min using purified Mck1 or Cib2-Cdk1 with 5 μM peptides (synthesized by NeoBioLab, Woburn, MA) in a 15-μl total reaction, with 0.6 μCi of [γ-32P]ATP (3000 μCi mmol−1; PerkinElmer, Melville, NY). After stopping the reaction and washing, radioactivity was measured using a scintillation counter (Tri-Carb 2800; PerkinElmer; Puig et al., 2001).

Microscope
Cells were grown in low-fluorescence medium, which was prepared as described previously (Sheff and Thorn, 2004). Cells were imaged on an Eclipse 90i microscope (Nikon, Tokyo, Japan) with a total internal reflection fluorescence 60×/1.45 numerical aperture Plan Apo chromatic objective lens (Nikon) fitted with a cooled Clara interline charge-coupled device camera (Andor, Belfast, United Kingdom) and using an Intensilight Ultra High Pressure 130-W mercury lamp (Nikon) as an illumination source. Images were acquired using the NiS Element BR software (Nikon). Image acquisition times for green fluorescent protein, YFP, 4′,6-diamidino-2-phenylindole (DAPI), and differential interference contrast (DIC) were 300, 200, 50, and 50 ms, respectively. All images were processed using ImageJ software (National Institutes of Health, Bethesda, MD; Schneider et al., 2012). To visualize RAD52–YFP foci, the DIC image was acquired, followed by seven YFP images at 0.5-μm intervals along the z-axis. YFP image stacks were combined through maximum intensity projection in order to count Rad52–YFP foci along the entire z-stack for each field. The corresponding DIC images were used as a reference to determine the budding of the cells. Log-phase MCK1-GFP cells were lightly fixed in 4% paraformaldehyde and then stained with DAPI.

The fixed cells were imaged using the relevant wavelengths, and the images were pseudocolored and processed using ImageJ.

Yeast two-hybrid assay
The pBTM116 constructs containing Cdc6 is a generous gift from J. Diffley's lab (Francis Crick Institute, London, UK; Perkins et al., 2001). The CDC6–pBTM116 plasmid was subjected to site-directed mutagenesis to create the various Cdc6 mutants (QuikChange Lightning Kit; Agilent). The CDC4–pACT plasmid and each of the various CDC6–pBMT116 plasmids were cotransformed into L40 strain and plated on synthetic defined-Leu/Trp plates, and β-galactosidase activity was measured as described previously (Ikui et al., 2012).

ACKNOWLEDGMENTS
We gratefully acknowledge David Morgan for plasmids and yeast strains used in Figure 2. We also thank John Diffley and Stephen Bell for the BTM116-Cdc6 and GAL-Cdc6 constructs, respectively. CDC6–T368A and CDC6–T369A, T368A mutants were created by Shoily Khondker. A.E.I. was supported by National Institutes of Health Grant 5SC3GM105498 and a Professional Staff Congress–City University of New York Enhanced Award.

REFERENCES