Slt2p phosphorylation induces cyclin C nuclear-to-cytoplasmic translocation in response to oxidative stress

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ABSTRACT **The yeast C-type cyclin represses the transcription of genes required for the stress response and meiosis. To relieve this repression, cyclin C undergoes nuclear-to-cytoplasmic translocation in response to many stressors, including hydrogen peroxide, where it is destroyed by ubiquitin-mediated proteolysis. Before its destruction, cyclin C promotes stress-induced mitochondrial fission and programmed cell death, indicating that relocalization is an important cell fate regulator. Here we show that cyclin C cytoplasmic translocation requires the cell wall integrity (CWI) mitogen-activated protein kinase Slt2p, its pseudokinase paralogue, Kdx1p, and an associating transcription factor, Ask10p. Furthermore, Slt2p and Kdx1p regulate cyclin C stability through different but required mechanisms. Slt2p associates with, and directly phosphorylates, cyclin C at Ser-266. Eliminating or mimicking phosphorylation at this site restricts or enhances cyclin C cytoplasmic translocation and degradation, respectively. Conversely, Kdx1p does not bind cyclin C but instead coimmunoprecipitates with Ask10p, a transcription factor previously identified as a regulator of cyclin C destruction. These results reveal a complex regulatory circuitry involving both downstream effectors of the CWI mitogen-activated protein kinase signal transduction pathway to target the relocalization and consequent destruction of a single transcriptional repressor.**

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INTRODUCTION

All organisms maintain systems for sensing environmental changes such as the absence of nutrients or the presence of cytotoxic agents. This ubiquitous phenomenon is commonly called the stress response and has evolved to combat the harmful effects of a variety of stress conditions. Among the most common stressors are reactive oxygen species (ROS). ROS can be derived from internal (e.g., by-product of respiration) or external sources, such as pro-oxidants and ionizing radiation (reviewed in Perrone *et al.*, 2008). In response to ROS stress, the cell makes a major cell fate decision: stop cell division and repair the damage or induce programmed cell death (PCD). Although much is known about the molecular details of DNA repair and PCD, little is known about how cells decide which one of these programs to initiate after stress. In yeast, the cell wall integrity (CWI) signal transduction pathway responds to a variety of stresses, including ROS (Vilella *et al.*, 2005) and heat shock (Kamada *et al.*, 1995). The CWI pathway senses stress via cell-surface sensors (Wsc1-3p, Mid2p, and Mtl1p; reviewed in Jendretzki *et al.*, 2011), which transmit the signal to a small G protein Rho1p (reviewed in Levin, 2011). Activated Rho1p stimulates protein kinase C (Pkc1p; Nonaka *et al.*, 1995; Kamada *et al.*, 1996) and the mitogen-activated protein kinase (MAPK) module composed of the MAPK kinase (MEK) Bck1p, the redundant MEKs Mkk1p and Mkk2p (Irie *et al.*, 1993), and the MAPK Slt2p/Mpk1p (Lee *et al.*, 1993) or its pseudokinase paralogue, Kdx1p/Mlp1p (Watanabe *et al.*, 1997; Figure 1A).

An important role for signal transduction pathways is to alter gene expression in response to specific environmental cues. In yeast, the CWI pathway stimulates two well-characterized transcriptional activators, Rlm1p and the heterodimeric factor Swi4p-Swi6p (also termed SBF). Slt2p phosphorylates Rlm1p within its transcriptional activation domain to stimulate DNA binding

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Abbreviations used: PCD, programmed cell death; ROS, reactive oxygen species.

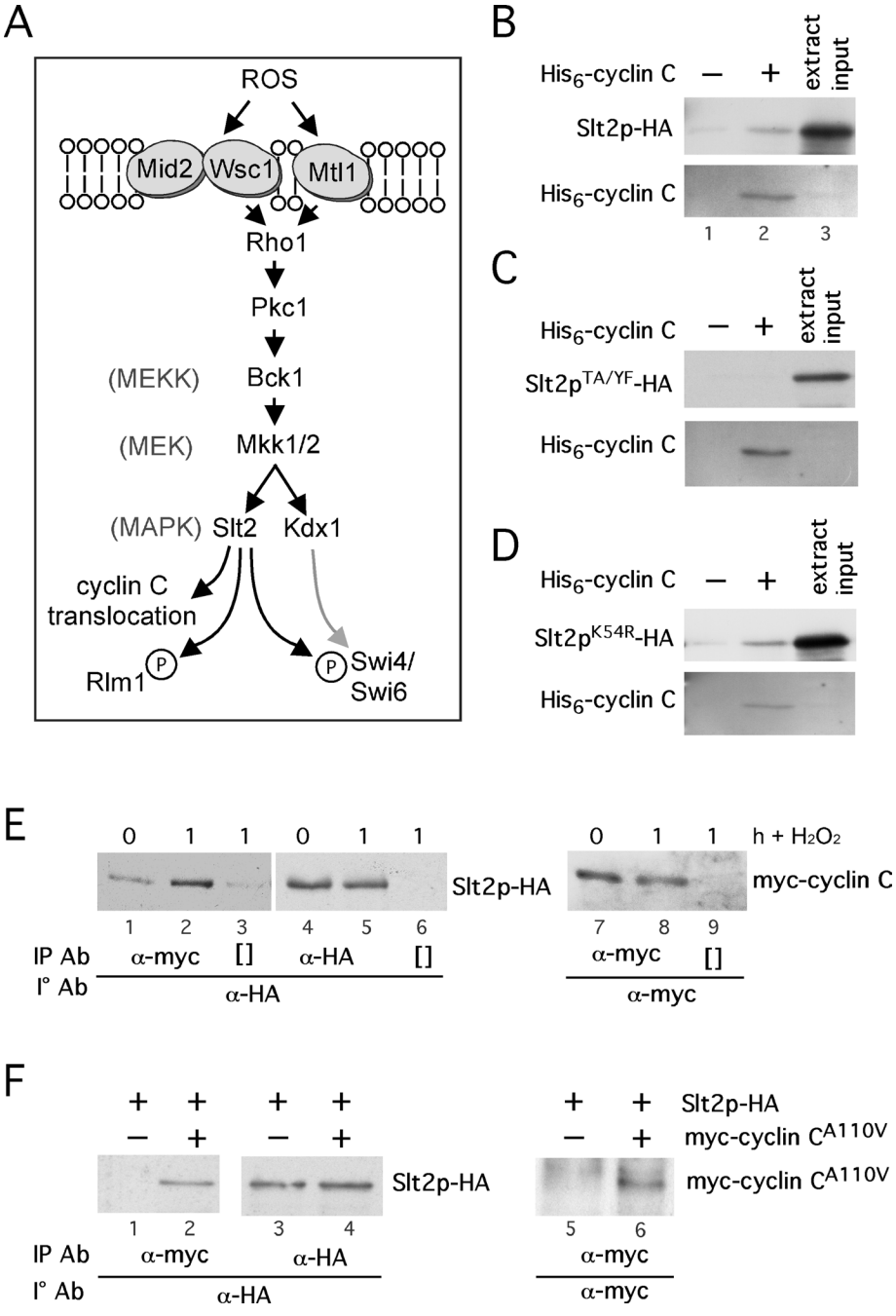

FIGURE 1: Active Slt2p associates with cyclin C. (A) Model of the cell wall signaling pathway. Activation of the pathway results in Slt2p-mediated phosphorylation (P) of the transcription factors Rlm1p and Swi4p/Swi6p and cyclin C translocation and degradation. The pseudokinase Kdx1p also contributes to Swi4p/Swi6p activation through a noncatalytic mechanism (gray arrow). Adapted from Levin (2011) and previous data (Cooper *et al.*, 1997, 2012; Krasley *et al.*, 2006; Jin *et al.*, 2013). (B) In vitro pull-down assays with Slt2p-HA and His_6–cyclin C. Extracts prepared from *slt2Δ* cells harboring Slt2p-HA on a high-copy plasmid were treated with 0.4 mM H_2O_2 for 1 h and then incubated with either Ni-agarose beads (lane 1) or His_6–cyclin C bound to Ni-agarose beads (lane 2). The bound fractions (top) and His_6–cyclin C input (bottom) were visualized by Western blot analysis using either anti-HA or anti-His_6 antibodies. The input (lane 3) of the extract shown is 10% of the amount of extract used for the binding assay. (C, D) Same as B, except that extracts were prepared from a strain expressing the *slt2* T-loop ($Slt2p^{TA/YF}$-HA) or kinase-dead ($Slt2p^{K54R}$-HA) mutation. (E) Coimmunoprecipitation with Slt2p-HA and myc-cyclin C. Extracts prepared from wild-type cells expressing Slt2p-HA from a high-copy plasmid and *ADH1*-myc-*CNC1* before and after addition of 0.4 mM H_2O_2 were immunoprecipitated with the antibodies shown. Brackets, [], indicate the no-antibody control. Lanes 1 and 2 represent the coimmunoprecipitations, lanes 4–9 the inputs and controls. (F) Coimmunoprecipitation of Slt2p-HA with myc-cyclin C^{A110V}. Extracts prepared from *cnc1Δ* cells (RSY391) expressing Slt2p-HA and myc-cyclin C^{A110V} as indicated 1 h after addition of 0.4 mM H_2O_2 were immunoprecipitated with the antibodies indicated. Lanes 1 and 2 represent coimmunoprecipitations; lanes 3–6, inputs.

(Watanabe *et al.*, 1997; Jung *et al.*, 2002). Of interest, although Slt2p phosphorylates Swi6p (Madden *et al.*, 1997), a noncatalytic role for this kinase and Kdx1p for SBF-dependent activation of cell wall stress genes has been described (Kim *et al.*, 2008). A noncatalytic role for transcriptional regulation also extends to elongation, as well as initiation (Kim and Levin, 2011). Of importance, both Slt2p and Kdx1p are activated by phosphorylation on their respective T-loop domains by Mkk1p/Mkk2p (Kim *et al.*, 2008). These results indicate that Slt2p and Kdx1p can play multiple roles in regulating transcription factor activity.

In addition to stimulating transcription factors, gene activation can also require removal of repressor function. In budding yeast, cyclin C (also known as Ume3p, Srb11p, or Ssn8p) and its cognate cyclin-dependent kinase Cdk8p (Ume5p, Srb10p, or Ssn3p) were identified as repressors of diverse stress-responsive genes (Surosky *et al.*, 1994; Cooper *et al.*, 1997; Holstege *et al.*, 1998; Chi *et al.*, 2001; van de Peppel *et al.*, 2005). This complex associates with the Mediator component of the RNA polymerase holoenzyme (Liao *et al.*, 1995) and can also positively regulate other genes (Hirst *et al.*, 1994; Vincent *et al.*, 2001). In response to H_2O_2 stress (also referred to here as oxidative stress), cyclin C translocates from the nucleus to the cytoplasm, where it is destroyed via the Not4p ubiquitin ligase (Cooper *et al.*, 1997, 2012). Based on use of both loss- and gain-of-function alleles, the CWI pathway was shown to be both necessary and sufficient for cyclin C relocalization and destruction (Krasley *et al.*, 2006; Cooper *et al.*, 2012; Jin *et al.*, 2013). Under low–oxidative stress conditions (0.4 mM H_2O_2), the cell wall sensors Mtl1p and either Wsc1p or Mid2p are required to generate a signal sufficient to induce cyclin C destruction (Jin *et al.*, 2013). However, either Mtl1p or Wsc1p/Mid2p sensor group is sufficient to induce cyclin C destruction in cells exposed to elevated (0.8 mM H_2O_2) reactive oxygen (Jin *et al.*, 2013). These results suggest that a threshold exists that the H_2O_2-induced signal must reach before it triggers cyclin C destruction.

We previously demonstrated that cyclin C is required for normal H_2O_2-induced PCD in yeast (Krasley *et al.*, 2006). Recently we demonstrated that before it is destroyed, cyclin C translocates to the cytoplasm, where it promotes induced mitochondrial fission (Cooper *et al.*, 2014; reviewed in Adachi and Sesaki, 2014; VanHook, 2014). Thus the molecular decision to relocalize cyclin C to the cytoplasm after stress must be carefully regulated so that the PCD pathway is not aberrantly selected. In this study, we show that CWI-dependent regulation of cyclin C is complex and involves both effectors of the CWI MAPK pathway, Slt2p and Kdx1p.

RESULTS

Oxidative stress stimulates Slt2p–cyclin C association

Previously we showed that the CWI signal transduction pathway is required for H_2O_2-induced cytoplasmic relocalization of cyclin C (Cooper *et al.*, 2012; Jin *et al.*, 2013). The CWI pathway uses two MAPKs (Slt2p and its pseudokinase paralogue, Kdx1p) to transmit the stress signal. Because Slt2p activates the transcription factor Swi4 through direct interaction (Madden *et al.*, 1997; Baetz *et al.*, 2001; Kim *et al.*, 2008, 2010), we first asked whether Slt2p could associate with cyclin C using pull-down experiments. Nickel beads with (+) or without (–) hexahistidine (His_6)-tagged cyclin C purified from *Escherichia coli* (see *Materials and Methods* for details) were incubated with extracts prepared from H_2O_2-stressed yeast cultures expressing Slt2p- hemagglutinin (HA). After extensive washing, proteins retained on the beads were subjected to Western blot analysis probing for Slt2p-HA. This experiment revealed that Slt2p-HA was associated with beads bound with His_6-cyclin C but not beads alone (Figure 1B, top). This interaction did not occur in extracts expressing a T-loop mutant ($Slt2p^{TA/YF}$-HA; Figure 1C), indicating that Slt2p must be activated by Mkk1p/Mkk2p (Kamada *et al.*, 1995) before it can bind cyclin C. To address whether Slt2p kinase activity is required for cyclin C binding, we repeated the binding assays with extracts prepared from a *slt2Δ* mutant expressing a kinase-dead Slt2p mutant ($Slt2p^{K54R}$-HA; Zarzov *et al.*, 1996; Madden *et al.*, 1997). This mutant was still able to associate with His_6–cyclin C (Figure 1D). Taken together, these results indicate that kinase activation via T-loop phosphorylation, but not kinase activity, is required for cyclin C–Slt2p interaction.

To confirm these results, we performed coimmunoprecipitation analysis between cyclin C and Slt2p. Extracts prepared from wild-type cells expressing Slt2p-HA and myc–cyclin C before and after H_2O_2 treatment were immunoprecipitated with anti-HA antibodies and the immunoprecipitates probed for the presence of myc–cyclin C. The results show that cyclin C coimmunoprecipitated with Slt2p-HA in extracts both before and after H_2O_2 treatment, with an increased signal under stress conditions (Figure 1E; see Supplemental Figure S1A for additional controls). The increase in interaction between cyclin C and Slt2p is consistent with our finding that T-loop phosphorylation increases Slt2p–cyclin C interaction. Given the transient nature of substrate–enzyme interactions, these experiments were performed with overexpression alleles of both cyclin C and Slt2p. This interaction can also be detected when both cyclin C and Slt2p are expressed from their own promoters (Supplemental Figure S1B). Moreover, this interaction occurs in the nucleus, as it can be detected in *cnc1Δ* cells harboring cyclin C^{A110V} as the only form of cyclin C. Cyclin C^{A110V} is able to repress transcription, remains nuclear after H_2O_2-induced stress (Cooper *et al.*, 2012), and is resistant to H_2O_2-mediated degradation (Cooper *et al.*, 1997). Taken together, these results indicate that, similar to Swi4p (Kim *et al.*, 2008), Slt2p must be in an active conformation but does not require catalytic activity to associate with cyclin C. Although these data cannot determine whether cyclin C directly interacts with Slt2p, they do suggest that the interaction between these proteins occurs in the nucleus.

Slt2p phosphorylation regulates cyclin C stability

Slt2p stimulates the activity of two transcription factors, Rlm1p and Swi6p, by serine or threonine phosphorylation (Madden *et al.*, 1997; Jung *et al.*, 2002; Kim *et al.*, 2008). To determine whether cyclin C is a substrate for Slt2p, we used in vitro kinase assays as previously described (Kamada *et al.*, 1995). Slt2p-HA was immunoprecipitated from extracts prepared from H_2O_2-stressed yeast cultures and incubated with His_6–cyclin C and radioactive ATP (see *Materials and Methods*). A signal was detected that comigrated with His_6–cyclin C, indicating that cyclin C was a substrate of Slt2p (Figure 2A, lane 2). This activity was specific to active Slt2p, as cyclin C was not phosphorylated when the assay was repeated using the kinase-dead Slt2p derivative ($Slt2p^{K54R}$-HA; Zarzov *et al.*, 1996; Madden *et al.*, 1997; Figure 2A, lane 3). Similarly, when the only MAPK recognition site in cyclin C (S/TP) was mutated (Ser-266 to alanine, S266A) cyclin C was no longer an Slt2p substrate (Figure 2A, lane 4). This loss of activity was not due to a failure of Slt2p to interact with cyclin C, as the S266A mutant can still bind the kinase in pull-down experiments (Figure 2B). These results indicate that Slt2p phosphorylates cyclin C on Ser-266.

Our previous studies showed that stress-induced cyclin C destruction requires Slt2p (Krasley *et al.*, 2006). To determine whether Slt2p modification of Ser266 mediates this process, we examined cyclin C^{S266A} turnover in a wild-type strain exposed to H_2O_2. These experiments revealed that cyclin C^{S266A} exhibited slower degradation kinetics ($T_{1/2}$ = 116 min) than did the wild type (34 min; Figure 2C and quantitated in Figure 2D). This rate is similar to the half-life of wild-type cyclin C, which we previously determined to be 90 min (Cooper *et al.*, 1997). Repeating these experiments with a cyclin C derivative carrying a Ser-266–to–glutamic acid (S266E) substitution mutation that often mimics phosphorylation, we obtained the opposite result (half-life, 19 min). However, the turnover rates for cyclin C^{S266E} and cyclin C^{S266A} were enhanced in unstressed cultures compared with wild type (Figure 2E). Taken together, these results suggest that Slt2p phosphorylation plays a role in efficient cyclin C turnover. In addition, our results indicate that cyclin C turnover is sensitive to mutations at Ser-266.

Phosphorylation of cyclin C is required for its cytoplasmic relocalization after H_2O_2 stress

We previously demonstrated that cyclin C degradation requires its cytoplasmic relocalization before Not4p-mediated ubiquitylation (Cooper *et al.*, 2012). Therefore the changes in cyclin C degradation kinetics just described caused by the S266A and S266E mutations should mirror their ability to translocate to the cytoplasm. To test this possibility, we monitored cyclin C^{S266A}–yellow fluorescent protein (YFP) localization by fluorescence microscopy in a *cnc1Δ* strain after H_2O_2 exposure. Consistent with our previously published data (Krasley *et al.*, 2006; Cooper *et al.*, 2012, 2014), wild-type cyclin C–YFP is nuclear before stress but forms cytoplasmic foci after H_2O_2 treatment (Figure 3, A, with quantification in E). This translocation is not dependent on new protein, synthesis as it occurs when the cells are treated with cycloheximide before H_2O_2 addition (Figure 3B). However, cyclin C^{S266A}-YFP remained largely nuclear after 2-h H_2O_2 exposure, with <10% of the culture exhibiting cytoplasmic foci (Figure 3, A and E). Continued exposure for up to 4 h did not induce the formation of cytoplasmic foci (unpublished data), suggesting that the S266A mutation resulted in a defect, and not simply a delay,

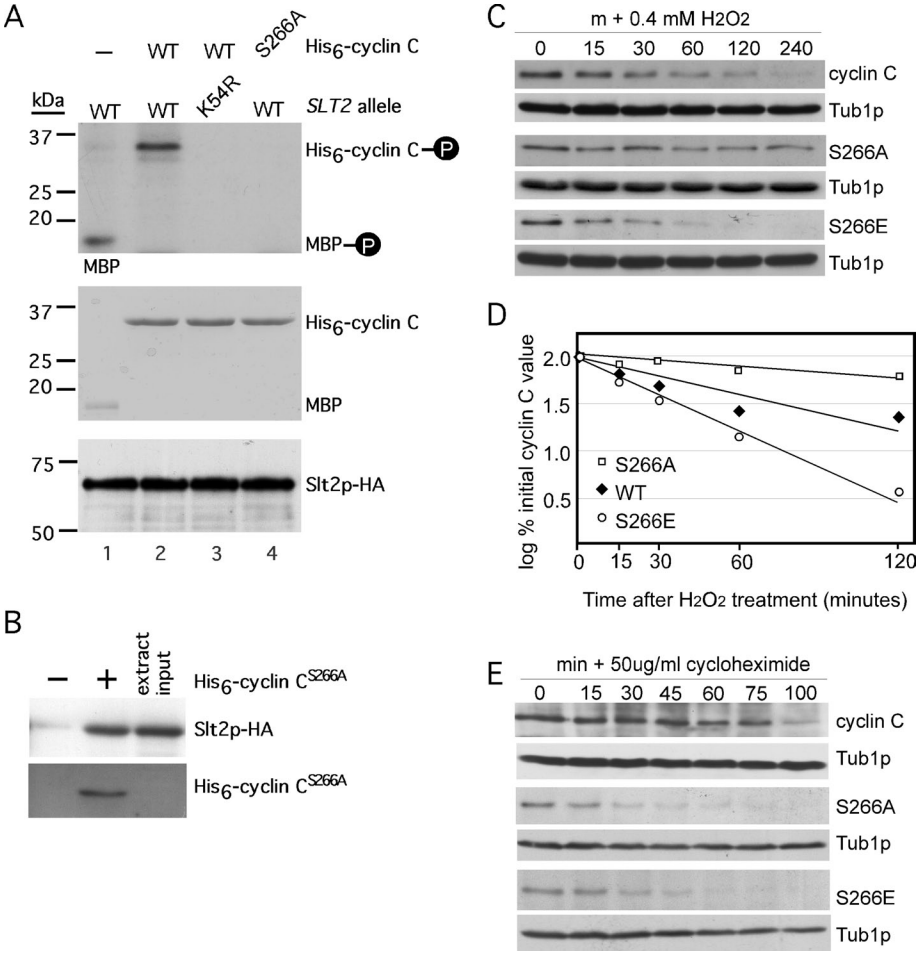

FIGURE 2: Slt2p phosphorylates cyclin C at Ser266. (A) In vitro phosphorylation assays with either wild-type or kinase-dead (Slt2p^{K54R}-HA) and the His$_6$–cyclin C substrate indicated. Myelin basic protein (MBP) was used as a positive control. Kinase reactions were separated by SDS–PAGE and then visualized by autoradiography (top) or to Coomassie staining (middle). Bottom, immunoprecipitation and Western blot analysis of the Slt2p-HA input. (B) In vitro binding assay with Slt2p-HA and His$_6$–cyclin C^{S266A} as described in Figure 1B. (C) Cyclin C levels after H_2O_2 stress (0.4 mM H_2O_2) in a *cnc1Δ* strain expressing wild-type cyclin C or mutant derivatives as indicated. Cyclin C levels were determined by Western blot analysis of immunoprecipitates. (D) Quantification of the results in C using imaging as described in *Materials and Methods*, with Tub1p levels as internal control. (E) Western blot analysis of cyclin C and the indicated mutants after cycloheximide treatment as indicated. Tub1p levels were used as loading control.

in cyclin C relocalization. Consistent with these findings, cyclin C–YFP remained nuclear after oxidative stress in an *slt2Δ* strain harboring the kinase-dead allele of *SLT2* (*slt2*^{K54R}; Figure 3C). These results argue that Slt2p-mediated phosphorylation of cyclin C on Ser266 is required for its cytoplasmic translocation. If Ser266 phosphorylation is necessary for stress-induced relocalization, then the next question is whether this modification is sufficient to induce this process. To address this question, we monitored the localization of cyclin C^{S266E}-YFP in *cnc1Δ* cells before and after oxidative stress. The results show that unstressed cells exhibit a small but reproducible fraction of cytoplasmic cyclin C^{S266E}-YFP (Figure 3, A, with quantification in E). Moreover, stress-induced cytoplasmic relocalization of cyclin C^{S266E} occurred more rapidly in stressed cells (Figure 3, D, with quantification in E), with 35% of the cells showing cytoplasmic foci 30 min after H_2O_2 treatment compared with 5% for wild-type cyclin C (see Supplemental Figure S2A for additional images). However, after 2 h of stress, the number of cytoplasmic foci was similar for cyclin C and cyclin C^{S266E}. Taken together, these findings indicate that Slt2p phosphorylation regulates cyclin C nuclear-to-cytoplasmic relocalization.

Cyclin C–YFP phosphorylation is required for H_2O_2-induced mitochondrial fission

Recently we demonstrated that upon its cytoplasmic translocation, cyclin C associates with the mitochondria and is required for the extensive fragmentation of this organelle in response to multiple stress conditions (Cooper *et al.*, 2014). To determine whether cyclin C phosphorylation is required for this event, we followed mitochondrial morphology in H_2O_2-stressed *cnc1Δ* strains expressing cyclin C, cyclin C^{S266A}, or cyclin C^{S266E} along with the mitochondrial-targeted DsRed (mt-DsRed) expression plasmid. We determined the percentage of cells that displayed short tubules (indicative of mitochondrial fission; Westermann, 2010; Youle and van der Bliek, 2012) versus the branched reticular networks observed under normal growing conditions (example given in Figure 4A). The results show that after peroxide application, ~50% of cells harboring cyclin C or cyclin C^{S266E} underwent fission (Figure 4, B and C). However, <20% of the *cnc1Δ* cells harboring cyclin C^{S266A} exhibited the fragmentation phenotype. These results indicate that cyclin C phosphorylation and its subsequent translocation to the cytoplasm are required for extensive mitochondrial fission in response to H_2O_2 stress. These results appear direct, as cyclin C^{S266E}–YFP associated with sites of fission similar to wild type (arrows, Figure 4C). The reduced levels of cyclin C^{S266E} (Figure 2) required a longer exposure to detect this fusion protein after 2-h H_2O_2 exposure. Taken together, these results are consistent with a model in which H_2O_2-induced activation of Slt2p results in cyclin C phosphorylation, nuclear-to-cytoplasmic translocation, and mitochondrial fission.

Phosphorylation of cyclin C is required for programmed cell death

Cyclin C is required for normal H_2O_2-induced programmed cell death (Krasley *et al.*, 2006; Cooper *et al.*, 2014). The results described in the foregoing section indicate that Slt2p phosphorylation is required for nuclear-to-cytoplasmic translocation of cyclin C. Therefore we asked whether this modification was important for cyclin C–dependent PCD. Two assays were performed to test for the presence of signature PCD markers in a *cnc1Δ* strain expressing wild-type cyclin C or the S266 mutants. Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assays indicated that cyclin C^{S266A}-expressing strains reduced cell death to an extent similar to the vector control (Figure 5A and Supplemental Figure S2B). Conversely, strains expressing cyclin C^{S266E} or wild-type cyclin C

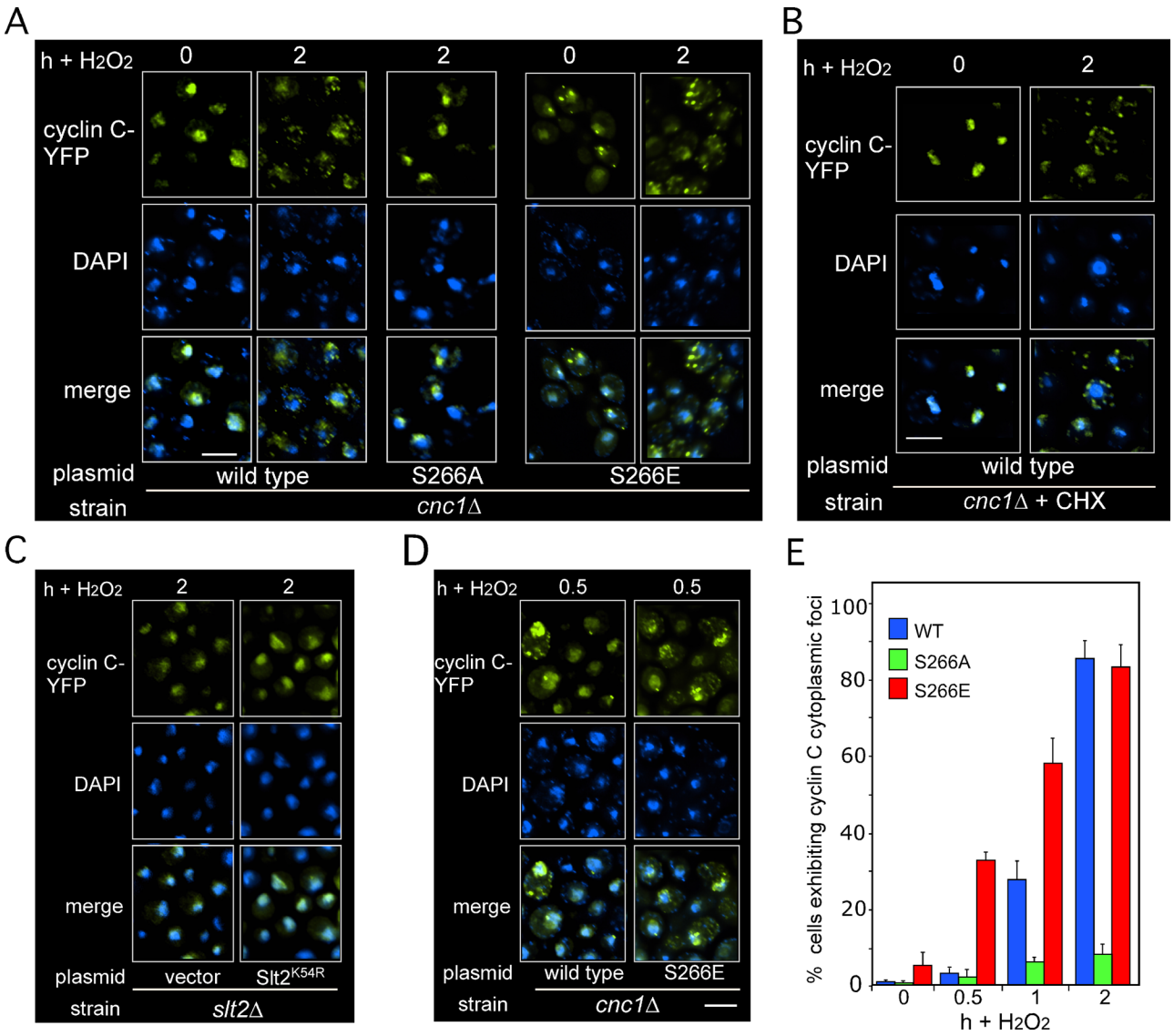

FIGURE 3: Phosphorylation of cyclin C by Slt2p regulates nuclear export of cyclin C in response to H_2O_2 stress. (A) Fluorescence microscopy of mid–log phase *cnc1Δ* cells harboring the cyclin C–YFP plasmids before (0 h) and after (2 h) 0.4 mM H_2O_2 treatment. (B) Fluorescence microscopy of mid–log phase *cnc1Δ* cells harboring the cyclin C–YFP plasmid after 1-h cycloheximide (CHX) treatment before (0 h) and after (2 h) 0.4 mM H_2O_2 addition. (C) Fluorescence microscopy of cyclin C–YFP in a *slt2Δ* mutant expressing $Slt2^{K54R}$ (or vector control) 2 h after 0.4 mM H_2O_2 treatment. (D) Fluorescence microscopy of *cnc1Δ* cells harboring the cyclin C–YFP expression plasmids indicated after 0.5 h 0.4 mM H_2O_2 treatment. In all images, the cells were fixed and stained with DAPI to visualize the nucleus. All images are collapsed, deconvolved 0.2-μm optical slices. Bar, 5 μm. (E) Quantification of A and D and Supplemental Figure S2A. The percentage of the population displaying at least three cyclin C–YFP foci in the cytoplasm is given (mean ± SEM). At least 200 cells were counted per time point from three individual isolates.

were similarly sensitive to H_2O_2. Similar results were obtained with caspase activity assays (Figure 5B). These results demonstrate a direct correlation between the phosphorylation status of cyclin C and its ability to induce cell death. Finally, a slight but reproducible increase in TUNEL-positive cells was observed in cultures expressing cyclin C^{S266E} compared with cells expressing either wild-type or cyclin C^{S266A} (Figure 5C). These results support our previous model (Cooper *et al.*, 2012, 2014) that cyclin C relocalization to the cytoplasm is required for normal PCD execution. In addition, these results indicate a statistical difference between the percentages of TUNEL- or caspase-positive cells in the wild-type and cyclin C^{S266E}–expressing cells. These results may indicate that the aberrant mislocalization of cyclin C to the cytoplasm increases cellular sensitivity to H_2O_2-induced stress. In case of the "unstressed" cells in Figure 5C, the elevated TUNEL signal may indicate individuals in the population that are experiencing oxidative stress due to aging or mitochondrial dysfunction.

Ser-266 integrity is not required for cyclin C repressor function

An alternative explanation to our results just described is that the effect of S266A or S266E mutations on mitochondrial dynamics and cell viability are due to defects in the ability of cyclin C to regulate transcription. To test this possibility, we performed two experiments.

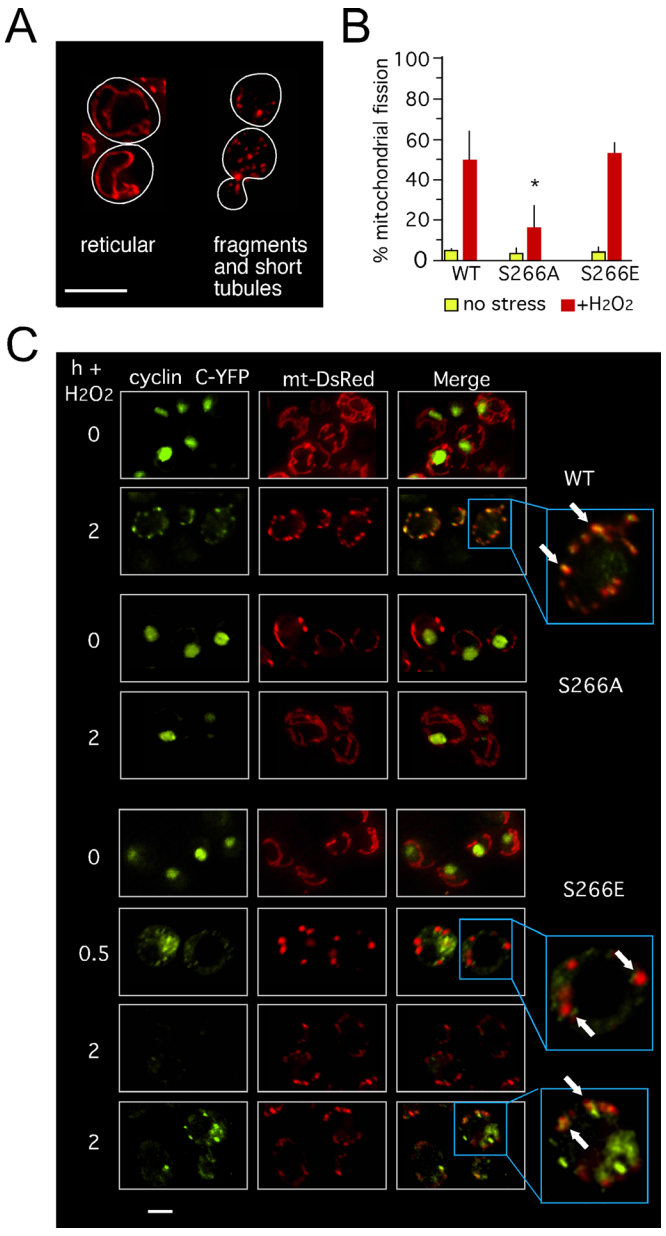

FIGURE 4: Cyclin C^{S266A} is defective in H_2O_2-induced mitochondrial fission. (A) Representative images of reticular or fragmented mitochondria. Bar, 10 μm. (B) Percentage of cells (mean ± SEM) within the population displaying mitochondrial fission before and after H_2O_2 (0.4 mM) treatment for 2 h. *$p < 0.05$ difference from wild type. (C) Fluorescence microscopy of living mid–log phase cells expressing cyclin C–YFP and mt-DsRed before (0 h) and after 0.4 mM H_2O_2 treatment as indicated. Blue boxes indicated enlarged regions of the image. Arrows indicate colocalization sites of cyclin C–YFP and mitochondria. The bottom panel required longer exposure to detect cyclin C^{S266E}–YFP than the wild-type control. Bar, 5 μm.

First, the ability of cyclin C^{S266A} to associate with its partner Cdk8p was tested using coimmunoprecipitation studies. Similar to wild type, cyclin C^{S266A} was able to associate with Cdk8p in unstressed cultures (compare lanes 6 and 4, Figure 6, A and B, respectively). In cultures exposed to H_2O_2, wild-type cyclin C levels are reduced as it translocates to the cytoplasm. This result, combined with our previous observation that Cdk8p remains nuclear in H_2O_2-stressed cells (Cooper *et al.*, 2012), significantly reduces its interaction with Cdk8p. In contrast, cyclin C^{S266A} and Cdk8p can still efficiently coimmunoprecipitate 1 h after addition of H_2O_2 (Figure 6B, lane 5). This result was confirmed using binding assays (Supplemental Figure S3) and is consistent with our findings that cyclin C^{S266A} does not leave the nucleus and is not destroyed upon H_2O_2-induced stress. The second set of experiments used quantitative PCR strategies to monitor the mRNA levels of two genes, *SPO13* (Strich *et al.*, 1989) and *DDR2* (Cooper *et al.*, 2012), that are repressed by cyclin C–Cdk8p. As previously observed, loss of cyclin C function results in twofold to fourfold increase in mRNA levels for these genes (Figure 6C). Although we did not analyze every gene regulated by cyclin C–Cdk8p, these experiments do suggest that mutating Ser-266 does not grossly alter the transcriptional repression ability of cyclin C. Therefore the effect of mutating Ser-266 on mitochondrial fission and cell death is most likely the result of differences in subcellular localization rather than changes in transcriptional regulation.

Kdx1 is required for oxidative stress–induced cyclin C destruction

We previously found that similar to H_2O_2-mediated stress (Cooper *et al.*, 1999), ectopic activation of the CWI pathway is sufficient to induce cyclin C destruction in the absence of stress (Cooper *et al.*, 2012; Jin *et al.*, 2013). Further, cyclin C is protected from degradation in *slt2Δ* mutants exposed to 0.4 mM H_2O_2 (Krasley *et al.*, 2006). If the H_2O_2 stress signal were being transduced solely through Stl2p to cyclin C, then deleting *SLT2* should protect the cyclin from destruction in response to CWI activation. To test this possibility, we monitored cyclin C levels in unstressed *slt2Δ* cultures expressing constitutively active alleles of *BCK1* (*BCK1-20*) or *RHO1* (*RHO1*^{G19V}; Lee and Levin, 1992). Rho1p is a small GTPase that, among its many roles, stimulates Pkc1p and the CWI pathway (Figure 1A). Western blot analysis revealed significant reduction in cyclin C levels in the presence of either activated allele even in the absence of Slt2p (Figure 7A, left). These results indicate the existence of an Slt2p-independent pathway to trigger cyclin C destruction.

Previous studies identified a role for Kdx1p/Mlp1p, the noncatalytic pseudokinase paralogue of Slt2p, in mediating the CWI response (Kim *et al.*, 2008, 2010; Kim and Levin, 2011; Truman *et al.*, 2009; Figure 1A). To determine whether Kdx1p also plays a role in transmitting the H_2O_2-induced stress destruction signal, we repeated the experiments just described in a *kdx1Δ* strain. Similar to the *slt2Δ* strain, Western blot analysis revealed that cyclin C levels were severely reduced compared with vector control (Figure 7A, middle). Finally, we tested the possibility that both Slt2p and Kdx1p regulate cyclin C destruction by repeating the experiment in a *kdx1Δ slt2Δ* double mutant (Figure 7A, right). Of interest, cyclin C levels were reduced in the Rho1p^{G19V}-expressing strain, but the reduction was not as severe as for either single mutant. However, little or no reduction in cyclin C levels was observed in the double mutant when Bck1p was constitutively active. These results indicate that, in the presence of a constitutively active CWI pathway, either Slt2p or Kdx1p is sufficient to transmit the destruction signal to cyclin C.

We next determined whether Kdx1p was required for cyclin C destruction in response to H_2O_2. Western blot analysis revealed that cyclin C was protected from destruction in *kdx1Δ* cells exposed to 0.4 mM H_2O_2 (Figure 7B). These results are consistent with our model that both Slt2p and Kdx1p are required for cyclin C destruction. However, we were unable to demonstrate an association between these two proteins in either pull-down experiments (Figure 7C) or coimmunoprecipitation analysis (Supplemental Figure S4A). Although negative results, these findings suggest that Kdx1p may be regulating cyclin C destruction through an indirect mechanism.

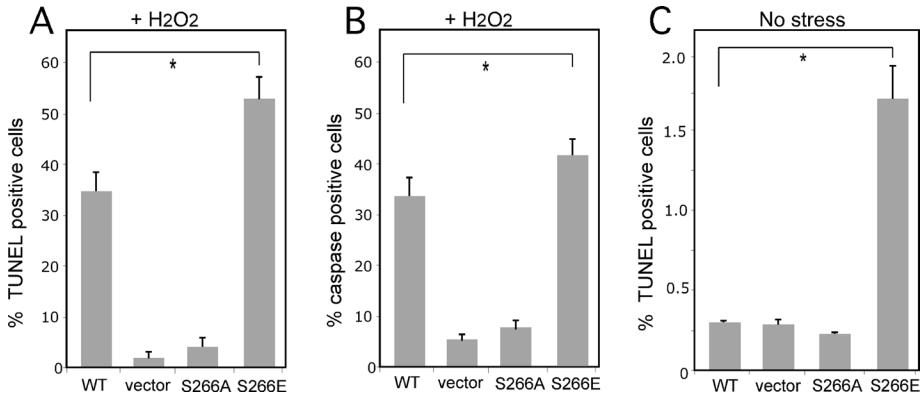

FIGURE 5: Phosphorylation of cyclin C by Slt2p regulates stress-induced programmed cell death. (A) Bar graph showing percentage TUNEL-positive cells in *cnc1Δ* strains expressing myc-cyclin C (WT), vector control, myc–cyclin C^{S266A}, or myc–cyclin C^{S266E} after 2-h treatment with 2 mM H_2O_2. (B) As in A, except that catalase activity was monitored. (C) As in A, except that mid-log unstressed cultures were subjected to TUNEL assays. For each assay, three independent isolates of each culture was analyzed. Graphs represent mean ± SEM. $*p < 0.05$.

Kdx1p associates with Ask10p and is required for its H_2O_2-induced phosphorylation

Ask10p associates with cyclin C and is required for its efficient destruction in response to H_2O_2-induced oxidative stress (Cohen *et al.*, 2003). In addition, Ask10p is hyperphosphorylated in response to H_2O_2-induced stress in an Mkk1p/Mkk2p-dependent, but, surprisingly, Slt2p-independent, manner (Cohen *et al.*, 2003). Therefore we first asked whether Kdx1p is required for H_2O_2-induced Ask10p phosphorylation. Wild-type and *kdx1Δ* strains were transformed with a plasmid expressing an Ask10p derivative fused to a single HA epitope (Ask10p-HA). As controls, *mkk1Δ/mkk2Δ* and *slt2Δ* strains were included in these experiments as well. These cultures were grown to mid log phase and treated with 0.4 mM H_2O_2 for 30 min. Total protein extracts were prepared and then subjected to Western blot analysis. These studies revealed a slower-migrating Ask10p-HA species in the wild-type strain (Figure 8A) that we previously demonstrated was due to phosphorylation (Cohen *et al.*, 2003). As expected, the phosphorylated Ask10p species was largely absent in the *mkk1Δ/mkk2Δ* double mutant but still present in the *slt2Δ* extract. Of interest, Ask10p remained unphosphorylated in the *kdx1Δ* strain, indicating that Kdx1p is required for this modification. To investigate whether Kdx1p was regulating Ask10p through a direct or indirect mechanism, we performed coimmunoprecipitation studies. No interaction was detected between these proteins in extracts prepared from mid log, unstressed cells (Figure 8B, lane 1). However, Kdx1p-13myc was detected in Ask10p-HA immunoprecipitates in extracts prepared from an H_2O_2-treated culture (Figure 8B, lane 2). This interaction was detected regardless of the protein being immunoprecipitated (Supplemental Figure S4B). These results indicate that Ask10p and Kdx1p associate in an oxidative stress–dependent manner, although it is unclear whether this interaction is direct or an intermediary is required.

We previously showed that Ask10p is required for cyclin C–mediated cell death (Cohen *et al.*, 2003). One mechanism to explain this result is that Ask10p is required for nuclear-to-cytoplasmic translocation of cyclin C. To test this possibility, we monitored cyclin C–YFP localization by fluorescence microscopy in wild-type and *ask10Δ* cells before and after H_2O_2 application. No differences were observed in cyclin C–YFP localization between the wild type and *ask10Δ* mutant in unstressed cultures (Figure 8C). However, cyclin C–YFP remained mostly nuclear in the *ask10Δ* mutant after H_2O_2-induced stress, with the appearance of cytoplasmic foci occurring in <5% of the mutant population (compared with 88% for wild type). These results indicate that Ask10p is required for normal cyclin C relocalization to the cytoplasm. In addition, the requirement of Kdx1p for Ask10p phosphorylation and cyclin C destruction suggests that these two processes are linked.

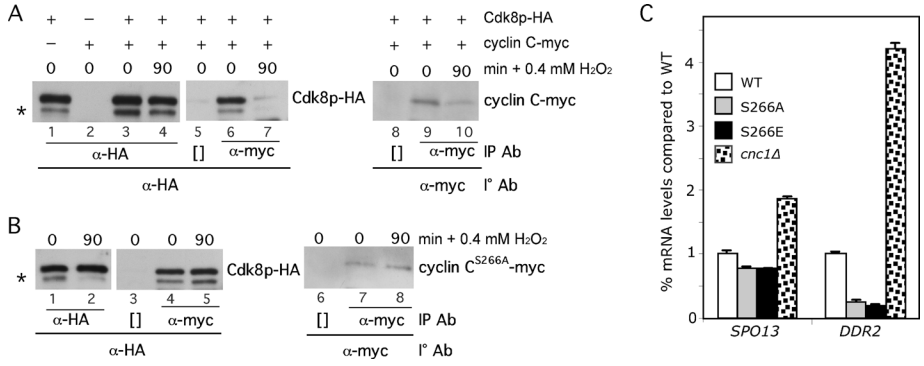

FIGURE 6: Cyclin C^{S266A} can coimmunoprecipitate with Cdk8p after H_2O_2 stress. (A) Cdk8p-HA and myc-cyclin C coimmunoprecipitation. Extracts prepared from unstressed and stressed *cnc1Δ cdk8Δ* (RSY1727) cells expressing Cdk8p-HA (pUM504) and myc-cyclin C (pKC337) as indicated were immunoprecipitated with the listed antibodies, and then the immunoprecipitates were probed for the presence of the indicated proteins. Brackets, [], represent the no-antibody control. Lanes 1–4 and 8–9 represent the inputs and lanes 5–7 the coimmunoprecipitations. (B) as in A, except that cyclin C^{S266A} was tested. Lanes 1, 2, and 6–8 represent the inputs and lanes 3–5 the coimmunoprecipitations. Asterisks indicate nonspecific cross-reactivity. (C) Cyclin C^{S266A} and cyclin C^{S266E} repress transcription. mRNA levels of *SPO13 and DDR2* were determined using reverse transcriptase (RT)-PCR. The graph represents the mean (±SEM) of three independent isolates for each locus analyzed.

DISCUSSION

One of the primary roles of MAPK pathways is to alter the transcription program to induce genes necessary for survival after exposure to exogenous stress. In most cases examined, the effect of MAPK pathways on transcriptional activation has focused on stimulation of transactivators (for a recent review, see Morano *et al.*, 2012). For example, activation of the CWI pathway stimulates the Slt2p MAPK and a closely related pseudokinase Kdx1p. Independently and in concert, these proteins activate several transcription factors (e.g., Rlm1p, SBF) through both catalytic and noncatalytic mechanisms (reviewed in Levin, 2005, 2011). However, little is known about how transcriptional repression activity is removed to allow normal induction of these genes. Under normal growth conditions, cyclin C–Cdk8p is part of the Mediator complex associated with the RNA polymerase II holoenzyme (Liao *et al.*, 1995; Cooper *et al.*, 1999) and functions to

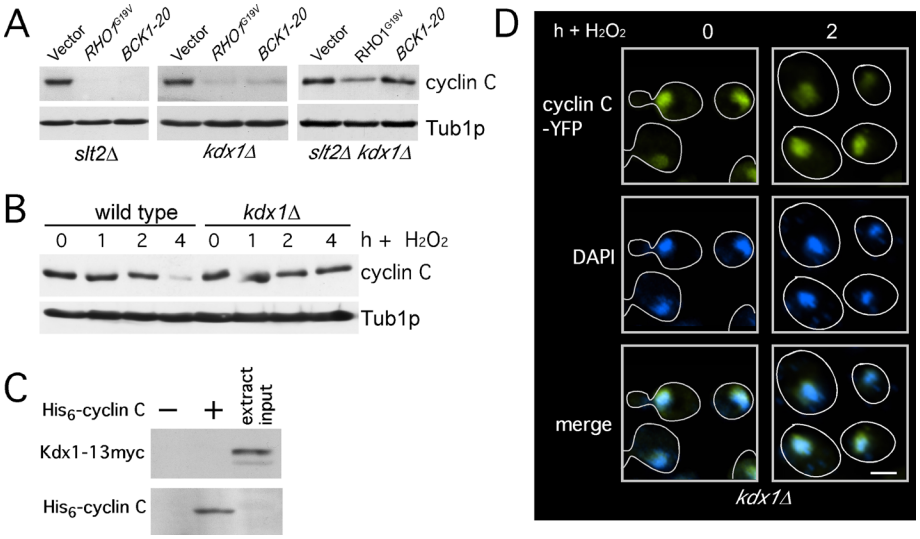

FIGURE 7: Kdx1p regulates cyclin C relocalization and destruction in response to H_2O_2 stress. (A) Westerns blot analysis of myc–cyclin C in unstressed cultures with the indicated genotypes expressing constitutively active alleles of *BCK1* (*BCK1-20*) and *RHO1* (*RHO1*^{G19V}). Tubl1p acts as a loading control. (B) Kdx1p is required for cyclin C destruction after H_2O_2 stress. Wild-type or *kdx1Δ* cells expressing myc–cyclin C were grown to mid log phase (0 h) and then treated with 0.4 mM H_2O_2 for the indicated times. Cyclin C levels were determined by Western blot analysis of immunoprecipitates. Tub1p levels were used as a loading control. (C) In vitro pull-down assays with Kdx1p-13myc and His_6–cyclin C as described in Figure 1B. (D) Kdx1p is required for YFP–cyclin C relocalization. Fluorescence microscopy was conducted on mid–log phase *kdx1Δ* yeast cells expressing cyclin C–YFP before (0 h) and after (2 h) 0.4 mM H_2O_2 treatment. Cells were fixed, stained with DAPI to visualize the nucleus, and then examined using fluorescence microscopy. The images are collapsed, deconvolved 0.2-μm slices. Bar, 5 μm.

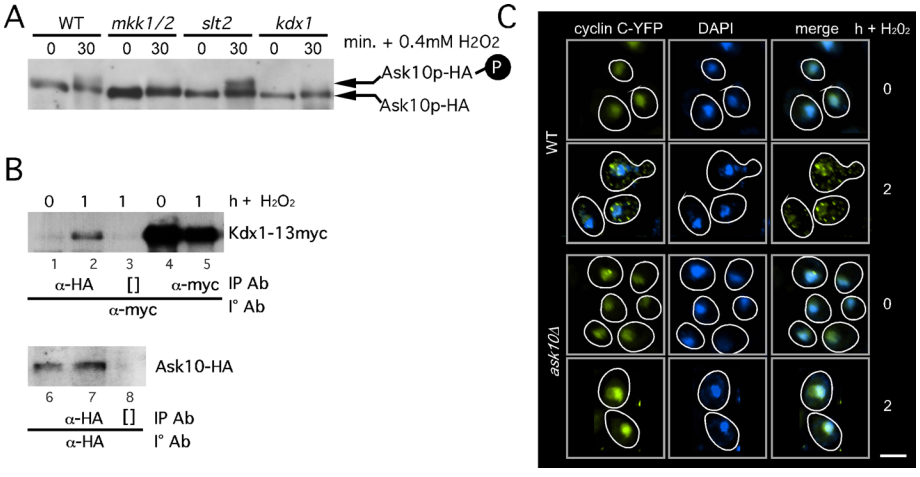

FIGURE 8: Kdx1p is required for H_2O_2 stress–induced Ask10p hyperphosphorylation. (A) Kdx1p-dependent hyperphosphorylation of Ask10p. Wild-type, *mkk1/2*, *slt2Δ*, and *kdx1Δ* cells harboring Ask10p-HA (pAK3) were treated with 0.4 mM H_2O_2 and then analyzed for Ask10p hyperphosphorylation as described (Cohen *et al.*, 2003). The hyperphosphorylated Ask10p species is indicated. (B) Ask10p and Kdx1p can interact in a stress-dependent manner. Extracts prepared from RSY1840 (Kdx1p-13myc) harboring Ask10p-HA at the time points indicated were immunoprecipitated as indicated and then probed for the presence of Kdx1p-13myc. Brackets, [], represent the no-antibody control. Lanes 1 and 2 represent the coimmunoprecipitation, lanes 4–7 the input. (C) Ask10p is required for cyclin C–YFP relocalization in response to H_2O_2. Fluorescence microscopy was conducted on mid–log phase wild-type or *ask10Δ* yeast cells expressing cyclin C–YFP (pBK37) before (0 h) and after (2 h) 0.4 mM H_2O_2 treatment. The cells were fixed, stained with DAPI, and then examined by fluorescence microscopy. The images are collapsed, deconvolved 0.2-μm slices. Bar, 5 μm.

repress many stress response genes (Strich *et al.*, 1989; Cooper *et al.*, 1997; Holstege *et al.*, 1998). This repression is relieved when cyclin C translocates from the nucleus to the cytoplasm, where it is destroyed (Cooper *et al.*, 1997, 2012). In this study, we found that Slt2p and Kdx1p mediate the oxidative-induced relocalization of cyclin C. Slt2p directly phosphorylates cyclin C, and this modification is required for translocation from the nucleus to the cytoplasm (see model in Figure 9). Kdx1p is also required for cytoplasmic relocalization but appears to function indirectly through the cyclin C–associating factor Ask10p. Kdx1p associates with Ask10p and is required for H_2O_2-induced phosphorylation of this factor (see model in Figure 9). In addition to removing transcriptional repression, cyclin C relocalization also promotes H_2O_2-induced mitochondrial fission and programmed cell death (Cooper *et al.*, 2014). These results describe a bifurcated CWI MAPK pathway that tightly controls cyclin C subcellular localization, which coordinates stress gene induction with mitochondrial fragmentation and cell death.

One surprising result from our work is that both Slt2p and Kdx1p, the two effectors of the CWI pathway, contribute to cyclin C relocalization and destruction through different mechanisms. Our results show that after H_2O_2-induced stress, Slt2p triggers cyclin C relocalization by phosphorylation. These results represent one of the few examples of a transcriptional repressor being a direct target of a MAPK cascade. It has been well demonstrated that, once activated by the CWI pathway, albeit by stressors other than H_2O_2, Slt2p translocates to the nucleus (Baetz *et al.*, 2001; Hahn and Thiele, 2002; Mao *et al.*, 2011). Indeed, Slt2p interacts with transcriptional activators at gene promoters (Jung *et al.*, 2002). Two pieces of data suggest that Slt2p phosphorylates cyclin C in the nucleus. First, Slt2p is phosphorylated in response to H_2O_2 stress in a CWI pathway–dependent manner (Vilella *et al.*, 2005; Jin *et al.*, 2013). Second, activated Slt2p can coimmunoprecipitate with the cyclin C A110V mutant (Figure 1F), which remains nuclear after H_2O_2 stress (Cooper *et al.*, 2012). Different from its other nuclear targets, Slt2p-mediated modification of cyclin C is required for its cytoplasmic relocalization, which results in mitochondrial fission and cell death execution. How does Ser-266 phosphorylation control this critical step of relocalization? Serine 266 is at the carboxyl end of the protein and therefore not within the cyclin box domain required for Cdk8p binding (Cooper *et al.*, 1999). This observation is consistent with our finding

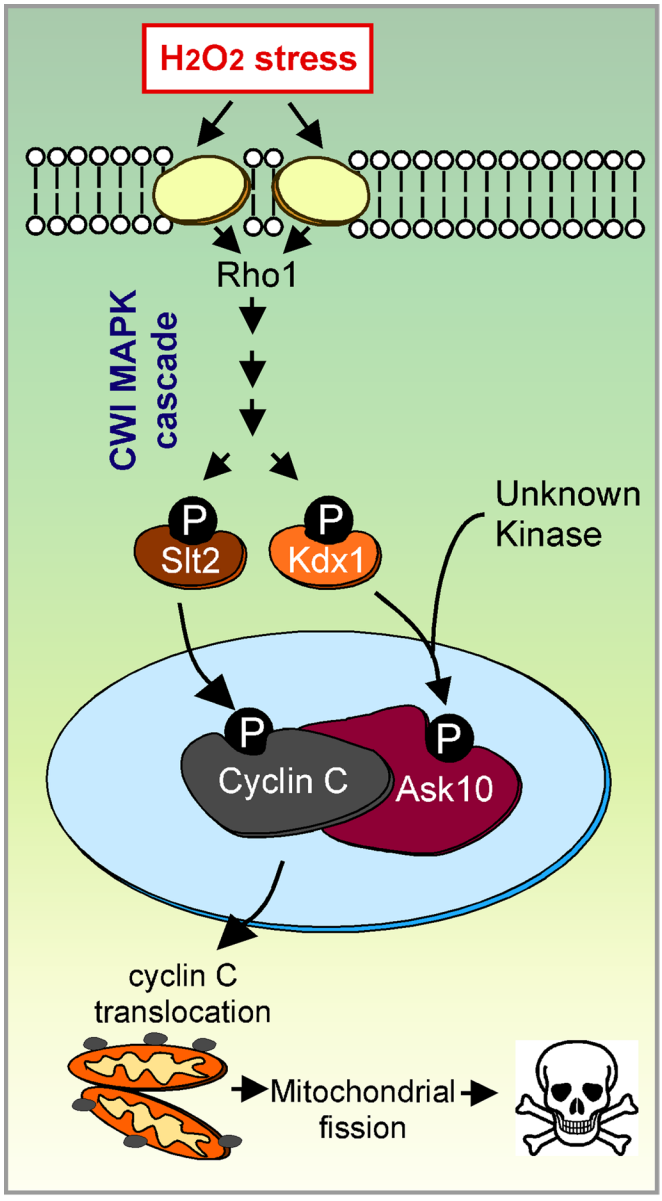

FIGURE 9: Regulation of cyclin C relocalization by the CWI pathway after H_2O_2 stress. H_2O_2-induced damage is recognized by the cell wall sensors (yellow), which transmit the stress signal via Rho1 to the CWI MAPK, pathway resulting in phosphorylation of Slt2p and Kdx1 (P). Activated Slt2p translocates to the nucleus and phosphorylates cyclin C at serine 266. Activated Kdx1p is also imported into the nucleus, where it binds to Ask10p. This binding permits Ask10p to be phosphorylated by an unknown kinase. Thereafter, cyclin C translocates to the cytoplasm, where it is required for mitochondrial fission and cell death.

that preventing phosphorylation at Ser-266 or introducing a phosphomimic mutation does not alter cyclin C repressor function (Figure 6C). In addition, a region of cyclin C has been identified, termed the holoenzyme association domain (HAD), that is required for association to the Mediator complex (Cooper *et al.*, 1999). This region is located within the first 50 amino acids of cyclin C, suggesting that Slt2p-dependent phosphorylation does not regulate HAD function. Another possibility is that phosphorylation may mark cyclin C for recognition by exportin proteins. Changes in the phosphorylation status of substrates have been shown to trigger nuclear export mediated by the exportin Msn5p (Kaffman *et al.*, 1998; DeVit and Johnston, 1999). However, we found no evidence that Msn5p mediates cyclin C relocalization (unpublished data), suggesting that other exportins may be involved. Alternatively, phosphorylation may serve to sever the association between cyclin C and its nuclear anchor. Such a function would likely reside in the Mediator complex or, more specifically, in the Cdk8p module that also contains Med12p and Med13p (Bourbon, 2008). Analyzing the role of these proteins may help to define the role of phosphorylation in controlling cyclin C localization.

Unlike Slt2p, Kdx1p may regulate cyclin C destruction via an indirect mechanism though Ask10p. Previous expression profiling (Jung and Levin, 1999) and unpublished studies (Levin, 2005) suggested that Kdx1p is dramatically (100-fold) up-regulated in response to cell wall damage in an Slt2p-Rlm1p–dependent manner. These observations led to the proposal of a positive feedback loop that could enhance the role of Kdx1p in mediating the stress response (Levin, 2005). However, our analysis of endogenously tagged Kdx1p did not detect any change in protein levels after oxidative stress (Supplemental Figure S4A). Therefore our results do not support a model in which Kdx1p activation is dependent on Slt2p. Instead, our results indicate that Kdx1p associates with Ask10p and is required for its oxidative stress-induced phosphorylation. These findings explain our previous results that although Mkk1p and Mkk2p are required for Ask10p phosphorylation, Slt2p is not (Cohen *et al.*, 2003). Mkk1p and Mkk2p phosphorylation on the activator T-loop is required for Kdx1p activity (Kim *et al.*, 2008). Pseudokinases represent ~10% of the human "kinome," with ~50 members identified based on their homology to protein kinases but their lack of one or more of the three domains required for catalysis (Manning *et al.*, 2002). In general, it is believed that these pseudokinases, which are found in all protein kinase subfamilies, play a scaffolding role in transducing signals. For example, the CASK pseudokinase contains an inactive guanylate kinase domain that directs protein:protein interactions (Li *et al.*, 2002). However, other proteins originally designated as pseudokinases based on lack of canonical kinase motifs have been found to possess catalytic activity. *KSR1* lacks the critical lysine required for ATP binding in subdomain II but is still able to phosphorylate *MEK1* (Goettel *et al.*, 2011). Kdx1p contains an identical arginine substitution in this motif. However, Kdx1p also lacks a critical aspartate in subdomain VIb that is present in *KSR1*. Therefore, although Kdx1p is required for oxidative stress–induced Ask10p phosphorylation, it seems more likely that Kdx1p plays a scaffolding role in this modification.

The results presented here demonstrate that cells use both arms of the bifurcated MAPK pathway via different mechanisms to release cyclin C from the nucleus. Why is this signaling pathway so complex? Our findings (Cooper *et al.*, 1997, 2012, 2014; Cohen *et al.*, 2003; Krasley *et al.*, 2006; Jin *et al.*, 2013) suggest that cyclin C sits in an important regulatory seat, driving the decision of whether to initiate PCD or not. Thus the decision to relocate cyclin C to the cytoplasm must be carefully considered. Maintaining independent effectors for this process that require both the Slt2p and Kdx1p pathways to be engaged ensures that the cell is making the appropriate molecular decision. Furthermore, Kdx1p's requirement for Ask10p phosphorylation by a kinase most likely independent of the CWI pathway implicates another signaling system in this decision. Consistent with this model of interactive signaling pathways, the interplay between the CWI pathway and other MAPK cascades has been reported in yeast (for review see Jendretzki *et al.*, 2011). Furthermore, we recently demonstrated that the level of H_2O_2-induced stress exposure affects the plasma membrane sensor configuration

involved in signaling cyclin C destruction (Jin *et al.*, 2013). These findings suggest that, similar to higher systems, yeast use an interactive system composed of multiple sensing proteins, signaling pathways, and molecular readouts to ensure that the decision to execute the PCD pathway is made correctly.

MATERIALS AND METHODS

Yeast strains and plasmids

Experiments were performed in *Saccharomyces cerevisiae* W303–related strain RSY10 (Strich *et al.*, 1989). The yeast strains used in this study are listed in Supplemental Table S1. In accordance with the Mediator nomenclature unification effort (Bourbon *et al.*, 2004), the cyclin C (*SSN8*/*UME3*/*SRB11*) and Cdk8p (*SSN3*/*UME5*/*SRB10*) will use *CNC1* and *CDK8* gene designations, respectively. The *cnc1Δ*, *ask10Δ*, *slt2Δ*, and *CNC1*-TAP strains have been previously described (Cooper *et al.*, 1997, 2014; Cohen *et al.*, 2003; Krasley *et al.*, 2006). The *KDX1*-13Myc, *kdx1*::*KANMX6*, *cdk8Δ* and *SLT2*-3HA strains were constructed using gene replacement methodology as described (Longtine *et al.*, 1998). All primers and plasmids used are listed in Supplemental Tables S2 and S3, respectively. Plasmids pKC337 (*ADH1*-myc-cyclin C), pKC354 (*ADH1*-myc-cyclin C^{A110V}), and pBK37 (*ADH1*-cyclin C–YFP) are functional and have been previously described (Cooper *et al.*, 1997, 2012; Jin *et al.*, 2013). The S266A and S266E mutations were introduced into these constructs by using site-directed mutagenesis according to manufacturer's instructions (Stratagene, Santa Clara, CA). pMS3 was made by cloning PCR-amplified (primers KCO1089 and KCO1090) *CNC1* open reading frame into pQE30 (Qiagen, Valencia, CA). The remaining MAPK plasmids (Slt2-HA, $Slt2^{K54R}$-HA, $Slt2^{TAYF}$-HA, Kdx1-HA, $Kdx1^{YF}$-HA) and the initial *BCK1-20* construct (used to create pLR106) were gifts from D. Levin, Boston University. The RHO^{19V} construct was a gift from Y. Ohya, University of Tokyo. The plasmid used to visualize mitochondria (mt-DsRed) was a gift from J. Nunnari, University of California, Davis.

Cell growth

Yeast cells were grown in either rich, nonselective medium (YPDA) or synthetic minimal medium (SC), allowing plasmid selection as previously described (Cooper *et al.*, 1997). For all experiments, the cells were grown to mid log phase (~6×10^6 cells/ml) before treatment with low concentrations of 0.4 mM H_2O_2 as previously described (Jin *et al.*, 2013). In Figures 2E and 3B, the cells were treated with 50 µg/ml cycloheximide for 1 h before analysis or addition of H_2O_2, respectively. *E. coli* cells were grown in Luria–Bertani broth medium with selective antibiotics.

Survival assays

TUNEL assays were conducted essentially as previously described (Madeo *et al.*, 1997; Krasley *et al.*, 2006; Cooper *et al.*, 2014) using the cell death kit (Roche) and flow cytometry (counting 30,000 cells/time point). Caspase assays were conducted as described (Vachova and Palkova, 2005; Jin *et al.*, 2013), except that the cells were incubated with the caspase substrate (CaspSCREEN; BioVision, Milpitas, CA) at 37°C for 24 h in the dark. At least 30,000 cells were counted per sample, with three independent cultures analyzed. Statistical analysis was performed using the unpaired Student's *t* test, with $p < 0.05$ being considered significant.

Western blot analysis

Western blot and coimmunoprecipitation analyses were performed essentially as described (Cooper *et al.*, 1997), except that RIPA 5 buffer (150 mM NaCl, 1% NP-40, 0.15% deoxycholate, 50 mM Tris-HCl, pH 7.5) was used to prepare the protein extracts using glass bead lysis. Ask10p-HA phosphorylation assays were also performed exactly as described (Cohen *et al.*, 2003). To detect myc–cyclin C, Slt2p-HA, Kdx1p-HA, and Ask10p–HA, 500 µg of soluble protein was immunoprecipitated with anti-myc antibodies (Roche) or anti-HA antibodies (Clontech, Mountain View, CA), respectively, collected on agarose A beads (Sigma-Aldrich, St. Louis, MO), and analyzed by Western blot as previously described (Tan *et al.*, 2011). The anti-His antibodies were used as recommended by the manufacturer (Roche). Tub1p was visualized as previously described (Tan *et al.*, 2011), except that anti–α-tubulin antibodies (12G10) were obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA). Western blot signals were detected using either goat anti-mouse or goat anti-rabbit secondary antibodies conjugated to alkaline phosphatase (Sigma-Aldrich) and the Tropix chemiluminescence CDP-star kit (Life Technologies, Grand Island, NY). Signals were quantitated by phosphorimaging (Kodak, Rochester, NY). Cyclin C levels were standardized to Tub1p levels before comparing to other values. Half-life determinations were calculated by linear regression analysis with curves possessing $r > 0.9$. All degradation assays were performed more than once.

His_6–cyclin C expression and pull-down assays

His_6-tagged cyclin C was purified from *E. coli* using nickel affinity chromatography as suggested by the manufacturer (Qiagen, Valencia, CA) after an overnight induction with 0.1 mM isopropyl-thiogalactopyranoside (US Biological, Salem, MA) at 16°C. The pooled elutes were analyzed by SDS–PAGE to confirm purity. For the pull-down assays, 25 µg of purified His_6-tagged cyclin C was incubated with 200 µl of Ni-agarose bead suspension for 1 h at 4°C. Protein–bead complexes were centrifuged at 10,000 × *g* for 30 s, the supernatant removed, and the pellet washed three times with wash buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 35 mM imidazole, 5% glycerol). Next, 500 µg of yeast extract, prepared as described from mid log cultures (5×10^6 cells/ml in minimal media) treated with 0.4 mM H_2O_2 (1 h) was incubated with the washed pellets on a rocking platform at 4°C overnight. Protein–bead–extract complexes were isolated by centrifugation at 10,000 × *g* for 30 s, followed by three washes with holoenzyme buffer 3 (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.5, 0.25 M potassium acetate, 5 mM EDTA, 0.1% NP-40), and were eluted off the beads by adding 3× SDS sample buffer. The presence of His_6–cyclin C was determined by Western blot analysis probing for His_6 as described, using anti-histidine antibodies.

In vitro phosphorylation assay

Phosphorylation assay were conducted as described (Kamada *et al.*, 1995), with the following modifications. Cells were grown to 8×10^6 cells/ml and harvested, and protein extracts were prepared using RIPA 5 buffer. To remove nonspecific association of other protein kinases, the cell lysates were precleared by incubation with 30 µl of protein A–Sepharose beads (Protein A-Sepharose 4B; Sigma-Aldrich) for 1 h at 4°C. The beads were removed by centrifugation (12,000 × *g*) for 10 min at 4°C, and the supernatants were incubated with anti–influenza HA monoclonal antibodies for 1 h to immunoprecipitate HA-tagged Slt2p for phosphorylation assays. The immunoprecipitates were washed four times in holoenzyme buffer 3 (Cooper and Strich, 1999) and once in kinase assay buffer (25 mM Tris, pH 7.5, 5 mM β-glycerolphosphate, 2 mM dithiothreitol, 0.1 mM Na_3VO_4, 10 mM $MgCl_2$). They were then resuspended in 18 µl of kinase buffer with 10 µg of His_6-tagged cyclin C or 1 µg of myelin basic protein (from bovine brain; Sigma-Aldrich) as a

positive control. Immunoprecipitates of the HA-tagged kinase-dead mutant Slt2p^{K54R} served as a negative control. The mixture was preincubated for 3 min at 30°C before adding 2 µl of [γ-^{32}P]ATP (6000 Ci/mmol; Perkin-Elmer, Waltham, MA). After incubation for 1 h at 30°C, the reaction was terminated by addition of 10 µl of 3× SDS sample buffer. The samples were subjected to SDS–PAGE on 12% acrylamide gel. After electrophoresis, the gel was soaked in 40% methanol, 10% acetic acid for 30 min, and Amplify for 30 min (Amplify NAMP100V; Amersham), followed by 5 min of soaking in 7% methanol, 7% acetic acid, and 1% glycerol. The gel was vacuum dried and subjected to autoradiography.

Fluorescence microscopy

YFP–cyclin C subcellular localization was monitored as described previously (Cooper *et al.*, 2012). For all experiments, the cells were grown to mid log phase (5×10^6 cells/ml), treated with 0.4 mM H_2O_2 for the time points indicated, and then analyzed by fluorescence microscopy. Briefly, the cells expressing YFP–cyclin C were grown to mid log phase and fixed with 4% paraformaldehyde/3.4% sucrose for 1 h at room temperature. The cells were washed three times in water and prepared for fluorescence microscopy as described previously (Guacci *et al.*, 1997) using mounting medium (10 mg/ml *p*-phenylenediamine, 50 ng/ml 4′,6-diamidino-2-phenylindole [DAPI]) to visualize nuclei and prevent photobleaching. Images were obtained using a Nikon (Cedar Knolls, NJ) microscope (model E800) with a 60× objective (Plan Fluor Oil, numerical aperture 1.3) and a charge-coupled device camera (model C4742; Hamamatsu). Data were collected using NIS software and processed using Image Pro software. All images of individual cells were optically sectioned (0.2-µm slices at 0.6-µm spacing) and deconvolved, and the slices were collapsed to visualize the entire fluorescence signal within the cell. Cyclin C–YFP foci were scored as being cytoplasmic when three or more foci were observed outside of the nucleus. Mitochondrial fission assays were performed on live cells as described (Cooper *et al.*, 2014). In brief, mitochondrial fission was scored positive if no reticular mitochondria were observed that traversed half the cell diameter. Fusion was scored when cells exhibited one or more reticular mitochondria the diameter of the cell. Fission and fusion was scored for 200 cells from three independent isolates. Statistical analysis was performed using the Student's *t* test, with $p < 0.05$ used to indicate significant differences.

RT-PCR assays

RT-PCR assays were conducted essentially as described (Cooper *et al.*, 2012). In brief, quantitative PCR was conducted using Taqman2 protocols (Applied Biosystems, Foster City, CA). Three independent cultures were subjected to H_2O_2 stress (0.4 mM) for the times indicated in the text. Total RNA prepared from each time point was assayed in triplicate, and all values are expressed relative to the internal *ACT1* control. The SD from three replicate reactions is indicated in the figures. The mRNA level of each gene was standardized compared with its level in the wild-type strain.

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