Med13p Prevents Mitochondrial Fission and Programmed Cell Death in Yeast Through Nuclear Retention of Cyclin C

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Med13p prevents mitochondrial fission and programmed cell death in yeast through nuclear retention of cyclin C

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ABSTRACT The yeast cyclin C-Cdk8 kinase forms a complex with Med13p to repress the transcription of genes involved in the stress response and meiosis. In response to oxidative stress, cyclin C displays nuclear to cytoplasmic relocalization that triggers mitochondrial fission and promotes programmed cell death. In this report, we demonstrate that Med13p mediates cyclin C nuclear retention in unstressed cells. Deleting MED13 allows aberrant cytoplasmic cyclin C localization and extensive mitochondrial fragmentation. Loss of Med13p function resulted in mitochondrial dysfunction and hypersensitivity to oxidative stress–induced programmed cell death that were dependent on cyclin C. The regulatory system controlling cyclin C-Med13p interaction is complex. First, a previous study found that cyclin C phosphorylation by the stress-activated MAP kinase Slt2p is required for nuclear to cytoplasmic translocation. This study found that cyclin C-Med13p association is impaired when the Slt2p target residue is substituted with a phosphomimetic amino acid. The second step involves Med13p destruction mediated by the 26S proteasome and cyclin C-Cdk8p kinase activity. In conclusion, Med13p maintains mitochondrial structure, function, and normal oxidative stress sensitivity through cyclin C nuclear retention. Releasing cyclin C from the nucleus involves both its phosphorylation by Slt2p coupled with Med13p destruction.

INTRODUCTION

Elevated levels of reactive oxygen species (ROS) are commonly observed during aging or in response to environmental stress. High ROS levels cause lipid oxidation, protein aggregation, and DNA damage (Morano et al., 2012), leading to activation of stress-response pathways (Estruch, 2000). If the damage is too extensive, the cell will initiate the programmed cell death (PCD) pathway (Mazzoni and Falcone, 2008). An early step in the stress-response pathway is extensive mitochondrial fragmentation or fission (Chan, 2012). Several studies support a model that hyperfission helps facilitate mitochondrial outer membrane permeability, leading to release of proapoptotic factors and caspase activation (Youle and Karbowski, 2005).

In yeast and mammalian cells, cyclin C-Cdk8 regulates transcription through association with the RNA polymerase II holoenzyme (for a review, see Nemet et al., 2014). The cyclin C-Cdk8 kinase controls transcription though modification of the basal transcriptional machinery (Akoulitchev et al., 2000), chromatin (Meyer et al., 2008; Knuesel et al., 2009b), or transcription factors (Hirst et al., 1999; Nelson et al., 2003). In addition to Cdk8p, cyclin C also associates with two additional proteins (Med12p and Med13p) in a complex termed the Cdk8 module (Borggreve et al., 2002). This module is highly conserved and is found either free (Knuesel et al., 2009a) or associated (Conaway and Conaway, 2011) with the Mediator, a 25–30 protein complex that associates with the RNA polymerase II holoenzyme (Ansari and Morse, 2013). Expression profiling revealed that components of this module control the expression of a similar subset of genes (Zhu et al., 2006). However, the individual components have also been shown to have varying roles in transcriptional control during Drosophila development (Gobert et al., 2010). In addition, Med12p, but not the other components of the module, is required for induction of the...
multidrug transporter PDR5 in mitochondrial DNA–deficient (rho0) cells (Shahi et al., 2010), indicating an important exchange of information occurs between these two organelles.

Recent studies have revealed an important second role for cyclin C that is independent of Cdk8p. In response to oxidative or ethanol stress, cyclin C, but not Cdk8p, translocates from the nucleus to the cytoplasm (Cooper et al., 2012), where it interacts with the fission machinery to induce mitochondrial hyperfission (Cooper et al., 2014). Consistent with a role for mitochondrial fission and PCD execution, loss of cyclin C function restricts fission (Cooper et al., 2014) and enhances cell viability following stress (Krasley et al., 2006). Conversely, aberrant localization of cyclin C in the cytoplasm induces stress-independent hyperfission and sensitizes the cell to oxidative stress (Cooper et al., 2014). These results indicate that the decision to retain cyclin C in the nucleus or release it into the cytoplasm is an important regulator of PCD initiation. A previous study revealed that Cdk8p is required for normal cytoplasmic translocation of cyclin C (Cooper et al., 2012), but the mechanism was unknown. Here, we provide evidence that Med13p plays the opposite role to Cdk8p by retaining cyclin C in the nucleus in unstressed cells. In response to stress, cyclin C release from Med13p requires the stress-activated MAP kinase Slt2p and Cdk8p activity. Aberrant cyclin C relocalization to the cytoplasm results in continuous mitochondrial fragmentation and dysfunction. These results indicate that Med13p-cyclin C interaction is controlled by multiple signals to insure the proper subcellular localization of cyclin C in stressed and unstressed cells.

RESULTS
Med13p is required for nuclear retention of cyclin C in unstressed cultures
In response to several types of stress, the transcription factor cyclin C translocates from the nucleus to the cytoplasm through a mechanism that requires Cdk8p (Cooper et al., 2012, 2014). Therefore we next determined whether the two remaining components of the Cdk8 module, Med12p and Med13p, also regulate cyclin C relocalization. Using fluorescence microscopy, we monitored the localization of a functional cyclin C–yellow fluorescent protein (cyclin C-YFP) reporter protein in med12Δ or med13Δ mutants before and following H2O2 stress application. In wild-type cells, H2O2 induces cyclin C translocation from the nucleus to the cytoplasm, where it interacts with the mitochondria to induce fission (see Cooper et al., 2014; and Figure 1A). Deleting MED12 did not affect cyclin C nuclear localization in unstressed cells or stress-induced cytoplasmic relocalization (Supplemental Figure S1A). However, cyclin C-YFP formed multiple cytoplasmic foci in the unstressed med13Δ strain (Figure 1B) similar to those observed in oxidatively stressed wild-type cells. In addition, these foci colocalized with fragmented mitochondria. These results indicate that Med13p is required for cyclin C retention in the nucleus of unstressed cells.

In addition to cyclin C mislocalization, we also observed that the mitochondria were highly fragmented in the med13Δ mutant similar to what is observed in stressed wild-type cells (compare mt-DsRed panels in Figure 1, A and B, quantified in C). We previously reported that the stress-induced cytoplasmic relocalization of cyclin C triggers extensive mitochondrial fission (Cooper et al., 2014). Therefore we next determined whether the fragmented mitochondrial phenotype observed in the med13Δ mutant was dependent on cyclin C. A med13Δ cnc1Δ double mutant was constructed, and mitochondrial morphology was monitored in unstressed cultures. These experiments indicated that cyclin C is required for the hyperfission phenotype associated with the med13Δ allele (Figure 1C). Finally, we determined whether this fragmentation was dependent on Dnm1p, the dynamin-like GTPase required for fission (Sesaki and Jensen, 1999). Similar to the med13Δ strain, cyclin C-YFP exhibited cytoplasmic localization in the unstressed dnm1Δ med13Δ double mutant (Figure 1D). Although cyclin C could be observed associated with the mitochondria in the double mutant, the mitochondria retained their aggregated or net-like phenotype similar to the dnm1Δ single
However, the overall Cdk8p-GFP signal also remained diffused over mutant in unstressed cells. Following H2O2 treatment, Cdk8p-GFP also exhibited nucleolar localization in 78% (SEM ± 8, n = 3) of the unstressed med13Δ cells (Figure 2B, top panels). These observations suggest that precocious cytoplasmic localization of cyclin C in itself is insufficient to induce its destruction. However, cytoplasmic cyclin C appears more rapidly targeted by a stress-activated destruction pathway.

**Med13p regulates Cdk8p nucleolar localization**

Our previous work revealed that stress-induced relocation to the cytoplasm triggers cyclin C proteolysis (Cooper et al., 2012). Consistent with these results, cyclin C was destroyed more rapidly in med13Δ mutants exposed to H2O2 stress (Figure S1B). This instability required the oxidative stress response, as glucose-repressible shutoff experiments revealed no significant difference in cyclin C stability in unstressed cells (Figure S1C). These results indicate that precocious cytoplasmic localization of cyclin C in itself is insufficient to induce its destruction. However, cytoplasmic cyclin C appears more rapidly targeted by a stress-activated destruction pathway.

**Med13p protects cells for H2O2-induced cell death**

Mitochondrial hyperfission is an early step in the stress response and is associated with PCD induction (Eisenberg et al., 2007). Supporting this model, cnc1Δ mutants fail to undergo stress-induced mitochondrial hyperfission (Cooper et al., 2014) and are resistant to H2O2-induced PCD (Krasley et al., 2006; Cooper et al., 2014). Therefore we next examined the role of Med13p in regulating the cellular response to oxidative stress. Although extensive mitochondrial fragmentation is observed in unstressed med13Δ cells, this event on its own did not induce cell death, as evidenced by the similar plating efficiency of unstressed wild-type and mutant cultures (Figure 3A, top rows). However, med13Δ cells demonstrated a hypersensitivity to H2O2 (second panel) compared with wild-type while cnc1Δ strains (third panel) were resistant to this pro-oxidant. To determine whether this hypersensitivity was due to aberrant cyclin C localization, we repeated the experiment with a med13Δ cnc1Δ double mutant. Loss of cyclin C suppressed the H2O2 hypersensitivity associated with the med13Δ allele (Figure 3A, right panel) similar to levels observed for the cnc1Δ allele alone. These results suggest that Med13p-dependent retention of cyclin C in the nucleus prevents hypersensitivity to H2O2. Finally, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays and flow-cytometry analyses indicated that the elevated cell death observed in stressed med13Δ mutants corresponded to an increase in cells exhibiting positive TUNEL signal (Figure 3B). As expected, TUNEL-positive cells were reduced in either the cnc1Δ or cnc1Δ med13Δ strains. Taken together, these results suggest that loss of Med13p function predisposes cells to programmed cell death through a cyclin C-dependent mechanism.

**Med13p maintains mitochondrial DNA integrity**

Previous studies have implicated excessive mitochondrial fission with loss of mitochondrial function (Chen et al., 2005). These experiments were accomplished using knockdown or deletion of fusion machinery components. To ask this same question without altering the basic fission or fusion machinery, we monitored mitochondrial function in med13Δ mutants, using growth on a respiratory-necessary carbon source (acetate) as the readout. These studies revealed that the med13Δ mutant was not able to grow on acetate medium but displayed nearly normal growth rate in the presence of the fermentable carbon dextrose (Figure 4A). This result indicates that Med13p is required for mitochondrial function, confirming the results of an earlier study (Shahi et al., 2010). Next we examined whether loss of mitochondrial function was dependent on cyclin C. Double mutant experiments revealed that, similar to the stress
hypersensitivity phenotype, deleting CNC1 also suppressed the mitochondrial defect observed in med13Δ mutants. These results suggest that the extensive mitochondrial fragmentation induced by aberrant cyclin C cytoplasmic localization is deleterious to mitochondrial function. For further exploration of this possibility, the med13Δ dnm1Δ double mutant described earlier was assessed for mitochondrial function. Following approximately 90 generations in the presence of a fermentable carbon source, 97% (±3, n = 4) of the double mutant cells were still respiration competent compared with <5% for med13Δ mutants. These results suggest, as others have reported, that continuous mitochondrial fission is deleterious to long-term maintenance of mitochondrial function.

Loss of mitochondrial function can be the result of mutations in either the mitochondrial or nuclear genome. In yeast, respiration-deficient cells can exhibit total loss of mitochondrial DNA (mtDNA), a condition termed Rho0. To determine whether med13Δ mutants retained their mtDNA, we conducted 4′,6-diamidino-2-phenylindole (DAPI) staining followed by fluorescence microscopy. Wild-type cells exhibited small nucleoids throughout the mitochondrial continuum (Figure 4B, arrowheads). In med13Δ cells, three classes of mtDNA signals were observed. The predominant class 1 phenotype exhibited normal-appearing mtDNA signals associated with the mitochondria (white arrows). However, two additional classes were observed. Class 2 mutants displayed mtDNA-mitochondrial association but also exhibited abnormal nuclear morphology (green arrows). In addition, DAPI-staining signals were absent in a subset of the mitochondrial signals (red arrows). It is important to note that DAPI staining alone is not sufficient to conclude the absence of mtDNA, but rather only that the DNA signal is reduced. Finally, class 3 mutants display a more degraded nuclear DAPI signal with additional DAPI staining bodies not associated with the nucleus or the mitochondria (yellow arrows). Taken together, these results indicated that med13Δ mutants retain mtDNA, although the amount of the DNA may be reduced.

To further characterize mtDNA integrity in med13Δ mutants, we utilized quantitative real-time PCR (qPCR) to test for the presence of COX1 and 21S rRNA alleles. Both alleles were quantitated using qPCR and then compared with a nuclear gene control (ACT1). The primers were chosen to generate relatively small amplicons (102 and 29 base pairs, respectively) to detect retention of discrete regions of the mitochondrial genome. In addition, these loci are on opposite sides of the mitochondrial genome. This experiment produced a calculated wild-type copy number of COX1 and 21S rRNA at 28 and 29, respectively (Figure 4C). This copy number is in the normal range for mtDNA (Williamson and Fennell, 1979). However, less than one copy of either locus was measured in the med13Δ mutant. These results indicate that significant deletions of mtDNA occurred in the med13Δ strain and support the model that excessive fission is deleterious to overall mitochondrial genome maintenance.

Med13p is required for normal oxidative stress sensitivity (A) Mid-log wild-type (RSY10), med13Δ (RSY1701), cnc1Δ (RSY391), and cnc1Δ med13Δ (RSY1712) cultures were treated with the H2O2 concentrations as indicated for 2 h and then serially diluted (1:10) before being plated on rich growth medium. The plates were incubated 3 d before image collection. (B) TUNEL assays were performed on strains described in A that were treated with 2 mM H2O2 for 20 h. Typical histograms are shown depicting fluorescence-activated cell sorting analysis of untreated and treated samples as indicated. The numbers in the lower right of each panel indicate the percentage of the population exhibiting a TUNEL-positive signal (SEM < 5%, n = 3).

FIGURE 3: Med13p is required for normal oxidative stress sensitivity. (A) Mid-log wild-type (RSY10), med13Δ (RSY1701), cnc1Δ (RSY391), and cnc1Δ med13Δ (RSY1712) cultures were treated with the H2O2 concentrations as indicated for 2 h and then serially diluted (1:10) before being plated on rich growth medium. The plates were incubated 3 d before image collection. (B) TUNEL assays were performed on strains described in A that were treated with 2 mM H2O2 for 20 h. Typical histograms are shown depicting fluorescence-activated cell sorting analysis of untreated and treated samples as indicated. The numbers in the lower right of each panel indicate the percentage of the population exhibiting a TUNEL-positive signal (SEM < 5%, n = 3).
Med13p anchors cyclin C in the nucleus

The requirement of cyclin C for Med13p destruction suggested a role for Cdk8p in this process. Therefore, we next monitored Med13p levels in a cdk8Δ mutant expressing a kinase-dead cdk8 allele (cdk8KD; Surosky et al., 1994). Similar to the cnc1Δ mutant, Med13p was protected from destruction in the strain expressing the kinase-dead allele (Figure 6A). These results indicate that Cdk8p kinase activity is required for Med13p destruction. We next examined the impact that loss of Cdk8p kinase activity had on cyclin C localization. A cdk8Δ-null strain expressing either wild-type or the kinase-dead allele of CDK8, along with cyclin C-YFP, was subjected to H2O2 stress (0.8 mM) for 2 h; this was followed by fluorescence microscopy. As expected, the strain expressing wild-type CDK8 exhibited normal cyclin C-YFP relocation to the cytoplasm (Figure 6B, top panels). Conversely, cyclin C-YFP formed a single focus associated with the nuclear periphery (bottom panels). This observation is similar to our previous study, which found nucleolar targeting of cyclin C-YFP in stressed cdk8Δ cells (Cooper et al., 2014). These results are consistent with a model that Med13p destruction is required for cyclin C translocation from the nucleolus to the cytoplasm.

**Med13p destruction is mediated by Cdk8p activity**

Modification of cyclin C on Ser-266 by the cell wall integrity (CWI) MAP kinase Slt2p/Mpk1p is required for normal cyclin C translocation in response to H2O2 stress (Jin et al., 2014). This MAP kinase module is stimulated by Rho1p through protein kinase C (Pkc1p; for a review, see Levin, 2011). To determine whether Med13p stability is controlled by the CWI pathway, we monitored the levels of Med13p-myc in unstressed cultures harboring plasmids expressing wild-type RHO1 or one of two constitutively active alleles (Q68L or G19V; Sekiya-Kawasaki et al., 2002). Our previous studies found that the presence of activated Rho1p was sufficient to induce cyclin C relocation and destruction in the absence of stress (Jin et al., 2014). Western blot analysis revealed that Med13p-myc levels were not altered in the presence of the activated RHO1 alleles (Figure 6C). These results indicate that Slt2p activity is not sufficient to drive Med13p destruction. These results raised the question of the relationship between cyclin C phosphorylation and Med13p destruction with respect to cyclin C release from the nucleus. To address this question, we used a Ser-266 to alanine (S266A) mutant form of cyclin C that prevents Slp2p phosphorylation and its subsequent relocation to the cytoplasm under low-stress conditions (Jin et al., 2014). Localization of cyclin C5266A-YFP was monitored in unstressed cnc1Δ and cnc1Δ med13Δ mutants. Although nuclear in the wild-type strain, we found cyclin C5266A in the cytoplasm in the med13Δ mutant (Figure 6D). These results indicate that med13Δ is epistatic to cyclin C5266A and formally implies that Med13p function is either downstream or independent of Ser-266 phosphorylation.

To further test this model, we conducted commmunoprecipitation experiments between Med13p and either cyclin C or cyclin C5266A. This substitution mutation mimics cyclin C phosphorylation and allows partial release of cyclin C into the cytoplasm (Jin et al., 2014). Extracts were prepared from mid-log cultures (no stress) expressing Med13p-myc and either cyclin C-YFP or cyclin C5266A-YFP. These samples were immunoprecipitated with either α-GFP or α-myc antibodies, and then the immunoprecipitates were subjected to Western blot analysis probing for the presence of cyclin C-YFP or cyclin C5266A-YFP. This experiment revealed a reduction cyclin C5266A-YFP able to immunoprecipitate with Med13p-myc (compare lanes 6 and 7, Figure 6E). No significant differences were observed in cyclin C or cyclin C5266A levels (lanes 2 and 3). In addition, this interaction was

Med13p anchors cyclin C in the nucleus

expressing cyclin C with a small internal deletion of 10 amino acids in the HAD (HADΔ). In response to H2O2 exposure, Med13p destruction was also prevented in cells expressing cyclin C4HADΔ (Figure 5D). These results indicate that oxidative stress–induced Med13p destruction required the proteasome and cyclin C association.

**Med13p destruction and cyclin C translocation are controlled by separate signaling pathways**

Modification of cyclin C on Ser-266 by the cell wall integrity (CWI) MAP kinase Slt2p/Mpk1p is required for normal cyclin C translocation in response to H2O2 stress (Jin et al., 2014). This MAP kinase module is stimulated by Rho1p through protein kinase C (Pkc1p; for a review, see Levin, 2011). To determine whether Med13p stability is controlled by the CWI pathway, we monitored the levels of Med13p-myc in unstressed cultures harboring plasmids expressing wild-type RHO1 or one of two constitutively active alleles (Q68L or G19V; Sekiya-Kawasaki et al., 2002). Our previous studies found that the presence of activated Rho1p was sufficient to induce cyclin C relocation and destruction in the absence of stress (Jin et al., 2014). Western blot analysis revealed that Med13p-myc levels were not altered in the presence of the activated RHO1 alleles (Figure 6C). These results indicate that Slt2p activity is not sufficient to drive Med13p destruction. These results raised the question of the relationship between cyclin C phosphorylation and Med13p destruction with respect to cyclin C release from the nucleus. To address this question, we used a Ser-266 to alanine (S266A) mutant form of cyclin C that prevents Slp2p phosphorylation and its subsequent relocation to the cytoplasm under low-stress conditions (Jin et al., 2014). Localization of cyclin C5266A-YFP was monitored in unstressed cnc1Δ and cnc1Δ med13Δ mutants. Although nuclear in the wild-type strain, we found cyclin C5266A in the cytoplasm in the med13Δ mutant (Figure 6D). These results indicate that med13Δ is epistatic to cyclin C5266A and formally implies that Med13p function is either downstream or independent of Ser-266 phosphorylation.

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Mediator to control gene transcription. In response to stress, cyclin C translocates from the nucleus to the cytoplasm, where it promotes both mitochondrial fragmentation and PCD. Therefore, the switch governing cyclin C retention or release from the nucleus is an important cell fate discriminator. In this paper, we demonstrate that Med13p is responsible for retaining cyclin C in the nucleus in unstressed cells. Deleting MED13 releases cyclin C into the cytoplasm, inducing extensive mitochondrial fission, oxidative stress hypersensitivity, and loss of mtDNA integrity. Our previous studies revealed that cyclin C relocalization requires Cdk8p and activation of the CWI pathway. We now provide mechanisms to explain the requirement of each factor. We found that Med13p is destroyed in response to oxidative stress in a manner dependent on Cdk8p activity. In addition, our data indicate that Slt2p-dependent phosphorylation of cyclin C helps destabilize its interaction with Med13p. Taken together, these data reveal that Med13p maintains mitochondrial function and protects the cells from aberrant PCD execution through retention of cyclin C in the nucleus.

We find that loss of Med13p activity results in extensive mitochondrial fragmentation in unstressed cells. In light of our previous report (Cooper et al., 2014) and results from the present study, release of cyclin C into the cytoplasm is responsible for this dramatic fission phenotype. Two additional phenotypes associated with the med13Δ allele include H2O2 hypersensitivity and loss of mitochondrial function. The respiration dysfunction could be caused by mutation within mitochondrial genome or misexpression of a nuclear gene caused by the med13Δ allele. Several results indicate that the respiration deficiency in med13Δ mutant is due to mtDNA defect and not the transcriptional role of Med13p. First, we quantified a significant loss in two mtDNA loci in med13Δ strains. Second, dissection of MED13/med13 diploids resulted in spores exhibiting both active and inactive mitochondria. However, the mitochondrial defective phenotype did not cosegregate with the med13Δ mutant allele. Finally, studies in mice (Chen et al., 2003, 2005; An et al., 2013) and yeast (Hermann et al., 1998; Sesaki and Jensen, 2001) have reported that the inability to undergo fusion causes elevated mtDNA damage. These data argue that aberrant mitochondrial fission induced by constitutive cytoplasmic cyclin C localization accelerates loss of active mitochondria. An alternative explanation is that we cannot rule out is that a transcriptional defect associated with loss of Med13p function reduces the efficiency of mitochondrial maintenance. Therefore, unlike nuclear petite mutants that display instant loss of mitochondrial activity, med13Δ mutants may undergo an overall decrease in mtDNA copy number, which is due to mtDNA defect and not the transcriptional role of Med13p.

**DISCUSSION**

In unstressed cells, cyclin C and Cdk8p form a complex with two additional proteins (Med12p and Med13p) that associates with the mitochondrial activity, which is responsible for retaining cyclin C in the nucleus. However, our finding that Med13p activity results in extensive mitochondrial fragmentation in unstressed cells. In light of our previous report (Cooper et al., 2014) and results from the present study, release of cyclin C into the cytoplasm is responsible for this dramatic fission phenotype. Two additional phenotypes associated with the med13Δ allele include H2O2 hypersensitivity and loss of mitochondrial function. The respiration dysfunction could be caused by mutation within mitochondrial genome or misexpression of a nuclear gene caused by the med13Δ allele. Several results indicate that the respiration deficiency in med13Δ mutant is due to mtDNA defect and not the transcriptional role of Med13p. First, we quantified a significant loss in two mtDNA loci in med13Δ strains. Second, dissection of MED13/med13 diploids resulted in spores exhibiting both active and inactive mitochondria. However, the mitochondrial defective phenotype did not cosegregate with the med13Δ mutant allele. Finally, studies in mice (Chen et al., 2003, 2005; An et al., 2013) and yeast (Hermann et al., 1998; Sesaki and Jensen, 2001) have reported that the inability to undergo fusion causes elevated mtDNA damage. These data argue that aberrant mitochondrial fission induced by constitutive cytoplasmic cyclin C localization accelerates loss of active mitochondria. An alternative explanation is that we cannot rule out is that a transcriptional defect associated with loss of Med13p function reduces the efficiency of mitochondrial maintenance. Therefore, unlike nuclear petite mutants that display instant loss of mitochondrial activity, med13Δ mutants may undergo an overall degradation of mitochondrial function that is manifested only after many generations. However, our finding that dnm1Δ alleles are able
by which BH-3 proteins can induce PCD. In yeast, a BH-3 protein (Ybh3p) has been identified (Buttner et al., 2011). Currently, studies are underway to determine the relationship between the stress hypersensitivity associated with med13∆ alleles and Ybh3p activity.

This study and our previous work have identified two domains, the HAD and Ser-266 region, as sites controlling cyclin C nuclear localization. Structural analysis of the Saccharomyces pombe cyclin provides a clue as to how these domains act together. Cyclins contain a repeat of the cyclin box fold, a five alpha-helix bundle (Hoeppner et al., 2005). The amino cyclin box universally binds its cognate Cdk, while a role for the second cyclin box remains elusive. In addition, all cyclins possess an amino terminal helix of varying length that appears to have different functions. For cyclin A, the amino terminal helix folds back on itself to make contact with Cdk2 (Jeffrey et al., 1995). Conversely, this region in cyclin C contains the HAD (see Figure 7A) and has been described as flexible (Hoeppner et al., 2005) or more rigid (Schneider et al., 2011). Previously, we to suppress the loss of mitochondrial function in a med13∆ strain argues against this possibility. Additional studies into the exact role of cyclin C in promoting mitochondrial fission may help distinguish between these possibilities.

The second phenotype we observed is oxidative stress hypersensitivity. This observation may also be related to the impact of constant mitochondrial fragmentation. Many studies have observed that mitochondrial fragmentation is an early step in the stress-response pathway (for reviews, see Scott et al., 2003; Youle and Karbowski, 2005). Consistent with this connection, we have previously demonstrated that cells lacking cyclin C fail to undergo fission and are resistant to oxidative stress (Cooper et al., 2014). Studies in mammalian cells have found that the proapoptotic BH-3 family member Bax is recruited to sites of fission (Karbowsk et al., 2002; Yuan et al., 2007; Cassidy-Stone et al., 2008; Brooks et al., 2011).

Therefore it is possible that the constitutive recruitment of the fission machinery to the mitochondria by cyclin C may elevate the efficiency

FIGURE 6: Med13p destruction requires Cdk8p activity. (A) Med13p-13myc levels were monitored in an H2O2 (0.8 mM) stressed mid-log cdk8∆ strain (RSY1954) expressing the wild-type CDK8 (pPL144-21) or a kinase-dead derivative (pPL144-23). Pgk1p levels were used as a loading control. (B) Localization of cyclin C-YFP in wild-type or Cdk8pKD-expressing cells following 2 h treatment with H2O2 (0.8 mM). Subnuclear localization of cyclin C-YFP in the cdk8∆ expressing cells is indicated by the arrowheads. The Nomoski (Nom.) and nuclear (DAPI) images are indicated. (C) Endogenously tagged Med13p-3 hemagglutinin (HA) levels were monitored in an unstressed wild-type strain (RSY1788) harboring constitutively active RHO1 (RHO1G19V or RHO1Q68L) expression plasmids. Protein extracts were immunoprecipitated with HA monoclonal antibodies, and the immunoprecipitates were probed for the presence of Med13p-HA. The parental strain (con) and no antibody controls are shown. Tub1p levels were monitored as a loading control. (D) Cyclin C5266A-YFP localization was monitored in unstressed cnc1∆ (RSY391) or cnc1∆ med13∆ (RSY1712) strains. The Nomoski (Nom.) and nuclear (DAPI) images are indicated. (E) Immunoprecipitation studies were conducted in extracts prepared from a wild-type strain expressing Med13p-myc (RSY1786) and either cyclin C-YFP (pBK38) or cyclin C5266A-YFP (pBK53). α-my or α-GFP immunoprecipitates were probed for the presence of cyclin C-YFP (E) or Med13p-myc (F) as indicated. Open brackets indicate the no immunoprecipitation antibody controls. GFP lanes contain extracts prepared from cells expressing only GFP (pUG36) to control for interaction of GFP alone with Med13p-myc. Scale bar: 5 μM; h = hours.
complete release of cyclin C into the cytoplasm. Med13p susceptible to ubiquitin-mediated proteolysis, resulting in disruption of this interaction. Phosphorylation by Cdk8p makes Ser-266 region as indicated. Activation of the CWI MAP kinase Slt2p regulation of cyclin C release from Med13p. Proposed interaction of loop region in which it resides was not modeled. (B) Model for the space-filling model. The Ser-266 region is approximate, as the model diagram is rotated 90° with respect to the space-filling model. The cyclin box region that binds Cdk8p is on the other side of the space-filling model. The Ser-266 region is approximate, as the loop region in which it resides was not modeled. (B) Model for regulation of cyclin C release from Med13p. Proposed interaction of Med13p with the cyclin box II region is depicted with the HAD and Ser-266 region as indicated. Activation of the CWI MAP kinase Slt2p results in phosphorylation of cyclin C on Ser-266, causing partial disruption of this interaction. Phosphorylation by Cdk8p makes Med13p susceptible to ubiquitin-mediated proteolysis, resulting in complete release of cyclin C into the cytoplasm.

demonstrated that the hydrophobic residues in this domain (indicated by yellow coloring), as well as its alpha-helical nature, are required for HAD function (Cooper and Strich, 1999). In addition, the cyclin C HAD mutant is less able to copurify with Med13p, causing partial release from the nucleus and an intermediate, mixed fusion–fission phenotype, in unstressed cells (Cooper et al., 2014). Similarly, another study demonstrated that a phosphomimetic substitution mutation at Ser-266 (S266E) also displays a reduced ability to associate with Med13p and causes a partial release of cyclin C from the nucleus (Jin et al., 2014). These results suggest a common role for these domains. The solved cyclin C structure (Hoeppner et al., 2005) allowed us to model Ser-266 to the loop region between the third and fourth helix of the second cyclin box (Figure 7A). Interestingly, both the HAD and Ser-266 regions are on the same side of cyclin C, raising the possibility that they represent a docking site for Med13p, as previously suggested (Hoeppner et al., 2005). Therefore combining our genetic results with the crystal data suggests that the HAD and Ser-266 regions form a protein-binding domain on cyclin C away from Cdk8p interaction. Consistent with this model, the crystal structure also predicts a hydrophobic pocket (Figure 7A, light blue region) that may facilitate protein:protein interaction.

Our finding that cyclin C is cytoplasmic in unstressed med13Δ mutants indicates that the system controlling cyclin C translocation does not require a stress signal. Therefore Med13p release appears to be the critical decision point in controlling cyclin C localization. We have recently demonstrated that the CWI MAP kinase Slt2p phosphorylates Ser-266 (Jin et al., 2014) and that this modification is required for efficient cyclin C cytoplasmic translocation. Therefore cyclin C phosphorylation reduces its ability to bind Med13p (Figure 7B). To relieve HAD binding, Med13p is destroyed, which commits the cell to cyclin C release. Interestingly, the CWI signal transduction pathway that mediates cyclin C phosphorylation is not involved in Med13p proteolysis, indicating the existence of another pathway triggering this process. Indeed, Med13p is a known substrate of the protein kinase A signal transduction pathway (Chang et al., 2004). We found that cyclin C and Cdk8p kinase activities are required for Med13p destruction. As a previous study found that mammalian Med13 is a substrate of Cdk8 (Knuesel et al., 2009b), the interaction we observe may be direct. In addition, a recent study found that Cdk8 phosphorylation induced the destruction of the Mediator component Med3 (Gonzalez et al., 2014). Finally, similar to our results, steady-state turnover of mammalian Med13 is mediated by a ubiquitin-mediated process (Davis et al., 2013). Taken together, our findings are consistent with a model that stress-activated destruction of Med13p requires phosphorylation by Cdk8p. This possibility implies that activation of two separate pathways is required for full release of cyclin C. It is reasonable to expect that cyclin C translocation to the cytoplasm is tightly controlled to prevent aberrant mitotic progression.

FIGURE 7: Models for Med13p control of cyclin C subcellular localization. (A) RasMol-generated images of the cyclin C crystal structure solved by Hoeppner et al. (2005). A space-filling (left) and ribbon (right) diagram with the HAD (yellow), Ser-266 (dark red), and the hydrophobic pocket (light blue) imposed on the cyclin C structure. The ribbon diagram is rotated 90° with respect to the space-filling model. The cyclin box region that binds Cdk8p is on the other side of the space-filling model. The Ser-266 region is approximate, as the loop region in which it resides was not modeled. (B) Model for regulation of cyclin C release from Med13p. Proposed interaction of Med13p with the cyclin box II region is depicted with the HAD and Ser-266 region as indicated. Activation of the CWI MAP kinase Slt2p results in phosphorylation of cyclin C on Ser-266, causing partial disruption of this interaction. Phosphorylation by Cdk8p makes Med13p susceptible to ubiquitin-mediated proteolysis, resulting in complete release of cyclin C into the cytoplasm.

MATERIALS AND METHODS

Yeast strains and plasmids

All Saccharomyces cerevisiae strains used in this study are derivatives of a W303-1A variant (Strich et al., 1989) and are listed in Table 1. In accordance with the gene nomenclature standardization efforts (Bourbon et al., 2004), CNC1 (a.k.a. SSN3/SRB11/UME3), MED12 (a.k.a. SRB8/SSN5), MED13 (a.k.a. SSN2/SRB9/UME2), and CDK8 (SSN8/SRB10/UME5) gene designations will be used. Gene deletions were constructed as described previously (Longtine et al., 1998). The med13Δ cnc1Δ strain (RSY1712) was generated by deleting MED13 in the cnc1Δ mutant RSY391. The endogenous MED13-yECitrine::KanMX6 construct was made using pKT140 (Sheff and Thorn, 2004). The strain containing the integrated CNC1-TAP allele (RSY1010) was a gift from Nynke L. van Berkum. Plasmids pKC337, pKC333, and pBK37 were described previously (Cooper et al., 1999, 2012). pBK217 containing the CNC1ΔCA strain was described (Cooper et al., 2014). Mitochondria visualization was achieved using mPt-DsRed (a gift from J. Nunnari; Naylor et al., 2006). Plasmids pUG36 (MET25-GFP control), pBK38 (cyclin C-YFP), and pBK53 (cyclin CΔ2566-YFP) have been previously described (Niedenthal et al., 1996; Jin et al., 2014). The CDK8/SSN8/SRB10 (pPL144-21) and cdk8ΔΔ (pPL144-23) expression plasmids have been previously described (Surosky et al., 1994). The G19V (pYO964) and Q68L (pYO965) constitutively active RHO1 expression plasmids (Sekiya-Kawasaki et al., 2002) were a gift from Y. Ohya. A ump1 mutant strain (Ramos et al., 1998) used to generate RSY1961 was a gift from R. J. Dohmen.
Cells were grown in either rich, nonselective medium (YPDA) or synthetic minimal medium (SC) allowing plasmid selection as previously described (Cooper et al., 1999). Cells were grown to mid–log (5 × 10^6 cells/ml), treated with 1 mM H_2O_2 for 2 h and then serially diluted (1:10) and plated on the nonselective medium (YPDA). TUNEL assays were conducted essentially as previously described (Madeo et al., 1997; Krasley et al., 2006). TUNEL-positive cells were measured by fluorescence-activated cell analysis using the Accuri C6 cell analyzer. All statistical analysis was performed using the Student’s t test with p < 0.05 considered significant. All analyses were conducted with at least three independent cultures with 300 or more cells counted per time point. Quantitative PCR analysis of mtDNA loci was accomplished using Taqman cybergreen method (Applied Biosystems, Grand Island, NY). The threshold cycle number (Ct) values were normalized to the nuclear ACT1 locus. COX1-F-5′-CTACAGATACGCGATTCTCAAGA; COX1-R-5′-GTGCCG-TGAATA GATGATAATGGT; 21S-F-5′-GTGCC G-CTTGGTGT CTTGG; ACT1-F-5′-CTACAGATACGCGATTCTCAAGA; ACT1-R-5′-CTACAGATACGCGATTCTCAAGA; COX1-R-5′-GTGCCG-TGAATA GATGATAATGGT; 21S-F-5′-GTGCC G-CTTGGTGT CTTGG; ACT1-F-5′-CTACAGATACGCGATTCTCAAGA; ACT1-R-5′-CTACAGATACGCGATTCTCAAGA; COX1-R-5′-GTGCCG-TGAAT A GATGATAATGGT.

**TABLE 1:** S. cerevisiae strains.

<table>
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<th>Strain</th>
<th>Genotype</th>
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<tr>
<td>RSY10</td>
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<td>Strich et al., 1989</td>
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<tr>
<td>RSY391</td>
<td>cnc1::LEU2</td>
<td>Cooper et al., 1999</td>
</tr>
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<td>Cooper et al., 1999</td>
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Microscopy and cell analysis

Intracellular localization studies of chimeric fusion proteins were performed in fixed or living cells as indicated in the figure legends. Cells were fixed in 3.7% paraformaldehyde and stained with DAPI, as previously described (Cooper and Strich, 2002). For all experiments, the cells were grown to mid–log (5 × 10^5 cells/ml), treated with 1 mM H_2O_2 for the time indicated in the text, and then analyzed by fluorescence microscopy. Images were obtained using a Nikon microscope (model E800) with a 60× objective (Plan Fluor Oil, NA 1.3) and a CCD camera (RETIGA Exi). Data were collected using Autoquant and processed using Image Pro software. All images were obtained using the same exposures for the course of the experiment.

Western blot analysis

Straight Western blot analysis was performed as previously described (Kushnirev, 2000) using 20 ml of mid–log phase cells per sample. For immunoprecipitations, protein extracts were prepared from mid–log phase cultures using mild RIPA buffer (150 mM NaCl, 1% NP-40, 0.15% deoxychline, 50 mM Tris-HCl, pH 8) and glass bead lysis, as described previously (Cooper et al., 1999). Protein turnover rates were determined in mid–log phase (5 × 10^6 cells/ml) cultures treated with cycloheximide (10 mg/l). Coimmunoprecipitation analyses were performed using 500 μg whole-cell extracts. Immunoprecipitations were conducted overnight at 4°C with agitation. Then protein complexes were bound to protein G-agarose beads (Roche, Indianapolis, IN) and processed according to the manufacturer’s manual. GFP polyclonal antibody (Living Colors; Clontech, Mountain View, CA) or anti-c-myc monoclonal (9E10) antibody (Roche) was used for immunoprecipitations and Western blot analyses. The 12G10 mouse monoclonal anti-Tub1p antibody (Developmental Studies Hybridoma Bank, University of Iowa) and 3-phosphoglycerate K (Pgk1p) mouse immunoglobulin G1 (IgG1) monoclonal (22C5D8) antibody (Invitrogen) were used to detect Tub1p and Pgk1p, respectively, as loading controls in this study. Western blot signal was detected using alkaline phosphatase–conjugated goat anti-mouse IgG (H+L) or anti-rabbit IgG (H+L) (Jackson Laboratories, Bar Harbor, ME) and the CDP-Star chemiluminescence reagent (Tropix, Life Technologies, Grand Island, NY).

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REFERENCES


