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

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Running title: Med13p anchors cyclin C in the nucleus
Abbreviations:

Programmed cell death, PCD; Holoenzyme association domain, HAD; reactive oxygen species, ROS

Summary

The yeast cyclin C-Cdk8 kinase forms a complex with Med13p to represses the transcription of genes involved in the stress response and meiosis. In response to the 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 mitochondria fragmentation. Loss of Med13p function resulted in mitochondrial dysfunction and hypersensitivity to oxidative stress-induced programmed cell death that were dependent of cyclin C. The regulatory system regulating 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 phospho-mimic 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 hyper-fission helps facilitate mitochondrial outer membrane permeability (MOMP) leading to release of pro-apoptotic factors and caspase activation (Youle and Karbowski, 2005).

In yeast and mammalian cells, cyclin C-Cdk8 regulates transcription through association with the Mediator complex and the RNA polymerase II holoenzyme (see (Nemet et al., 2014) for review). 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 (Borggrefe 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 hyper-fission (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 hyper-fission 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 Med13-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; Cooper et al., 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, the localization of a functional cyclin C-YFP reporter protein was monitored 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 re-localization (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 co-localized 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 mis-localization, 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 Figures 1A and 1B, quantified in Figure 1C). We previously reported that the stress-induced cytoplasmic re-localization of cyclin C triggers extensive mitochondrial fission (Cooper et al., 2014). Therefore, we next determined if 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 hyper-fission 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 mutant. These results are consistent with a model that the extensive mitochondrial fragmentation observed in a med13Δ mutant requires cyclin C relocalization to the cytoplasm and Dnm1p activity.

Our previous work revealed that stress-induced re-localization 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 shut-off 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 does not mediate Cdk8p nuclear localization.**

Our previous studies (Cooper et al., 2012) found that Cdk8p does not relocate to the cytoplasm in H₂O₂ treated cells. Rather, it co-localizes with the nucleolar marker Nop1p-RFP in response to oxidative stress (Figure 2A). Therefore, we next determined whether Med13p is required for Cdk8p nuclear localization and/or its stress-induced concentration in the nucleolus. To address this question, we monitored Cdk8p-GFP localization in unstressed or stressed med13Δ mutant cultures. In the unstressed med13Δ culture, the Cdk8p-GFP signal exhibited a diffuse nuclear pattern similar to that observed in wild-type cells (Figure 2B). However, Cdk8p-GFP also exhibited nucleolar localization in 78% (s.e.m. ±8, n = 3) of the unstressed med13Δ cells (top panels, Figure 2B). These observations suggest that Med13p normally prevents Cdk8p entry into the nucleolar compartment in unstressed cells. Following H₂O₂ treatment, Cdk8p-GFP and Nop1p-RFP co-localization still occurred in the med13Δ cells. However, the overall Cdk8p-GFP signal also remained diffused over the nucleus rather than concentrating in the nucleolus as observed in wild-type cells. These results suggest a complicated role for Med13p in regulating Cdk8p sub-nuclear localization. In unstressed cells, Med13p prevents Cdk8p nucleolar localization. However, Med13p is also required for normal consolidation of Cdk8p in the nucleolus of stressed cells.
**Med13p protects cells for H$_2$O$_2$-induced cell death**

Mitochondrial hyper-fission is an early step in the stress response and associated with PCD induction (Eisenberg *et al.*, 2007). Supporting this model, *cnc1Δ* mutants fail to undergo stress-induced mitochondrial hyper-fission (Cooper *et al.*, 2014) and are resistant to H$_2$O$_2$-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 H$_2$O$_2$ (second panel) compared to 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, the experiment was repeated with a *med13Δ cnc1Δ* double mutant. Loss of cyclin C suppressed the H$_2$O$_2$ 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 H$_2$O$_2$. Finally, TUNEL assays and flow cytometry analyses indicated that the elevated cell death observed in stressed *med13Δ* mutants corresponded to an increase in cells exhibiting of 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. To further explore this possibility, the *med13Δ dnm1Δ* double mutant described earlier was assayed for mitochondrial function. Following approximately ninety generations in the presence of a fermentable carbon source, 97% (±3 n=4) of the double mutant cells were still respiration competent compared to >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 mtDNA, a condition termed Rho⁰. To determine if med13Δ mutants retained their mtDNA, DAPI staining was conducted followed by fluorescence microscopy. Wild-type cells exhibited small nucleoids throughout the mitochondrial continuum (arrow heads, Figure 4B). 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 arrow). 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 qPCR to test for the presence of COX1 and 21S rRNA alleles. Both alleles were quantitated using qPCR then compared to a nuclear gene control (ACT1). The primers were
chosen to generate relatively small amplicons (102 and 29 bp, respectively) to detect retention of discreet 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 destroyed in response to H$_2$O$_2$ stress**

Our results are consistent with a model that disrupting cyclin C-Med13p association is important to release the cyclin into the cytoplasm in stressed cells. Therefore, we next investigated whether Med13p regulation itself provided insight into how this interaction is dissolved. Initially, we monitored Med13p localization in the cell expressing an endogenously tagged functional *MED13*-YFP allele. As expected, fluorescence microscopy revealed that Med13p-YFP displayed diffuse nuclear staining in unstressed cells (Figure S2). Following one hour H$_2$O$_2$ treatment, no difference in Med13p-YFP localization was noted indicating that relocalization of this factor is not a primary regulatory mechanism. Next, we examined Med13p levels following in an H$_2$O$_2$ stressed culture expressing an endogenously tagged *MED13* allele (13-myc). Western blot analysis revealed a dramatic reduction in Med13p levels following stress application
Med13p-myc reduction was due to enhanced degradation, and not translation inhibition, as Med13p is relatively stable as determined by translation inhibition experiments in unstressed cells (right panel). Thus, Med13p is actively targeted for degradation during oxidative stress. To determine if Med13p destruction required the 26S proteasome, this experiment was repeated in a strain deleted for UMP1, a gene whose product is required for 20S proteasome maturation (Ramos et al., 1998). In response to H$_2$O$_2$ stress, Med13p-myc was protected from destruction in the ump1Δ mutant compared to the wild-type control (Figure 5B). These results indicate that Med13p destruction is most likely directed by a ubiquitin-mediated mechanism.

To determine if cyclin C is involved in H$_2$O$_2$-induced Med13p destruction, Med13p-myc levels were monitored in stressed cnc1Δ mid-log cultures. Similar to ump1Δ strains, Med13p-myc levels remained elevated in the cnc1Δ mutant following H$_2$O$_2$ addition. These results indicated that cyclin C is required for Med13p turnover in stressed cells. We have previously demonstrated that a domain at the amino terminal end of cyclin C, the holoenzyme association domain or HAD (Cooper and Strich, 1999), is required for Med13p association (Cooper et al., 2014). Therefore, we next examined whether cyclin C association per se was necessary for Med13p destruction. A cnc1Δ strain was transformed with a plasmid expressing cyclin C with a small internal deletion of 10 amino acids in the HAD (HADΔ). In response to H$_2$O$_2$ exposure, Med13p destruction was also prevented in cells expressing cyclin C$^{\text{HADΔ}}$ (Figure 5D). These results indicate that oxidative-stress induced Med13p destruction required the proteasome and cyclin C association.
**Med13p destruction is mediated by Cdk8p activity.**

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 H\textsubscript{2}O\textsubscript{2} stress (0.8 mM) for two hours followed by fluorescence microscopy. As expected, the strain expressing wild type *CDK8* exhibited normal cyclin C-YFP relocalization 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 that 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 and cyclin C translocation are controlled by separate signaling pathways.**
Modification of cyclin C on Ser266 by the cell wall integrity (CWI) MAP kinase Slt2p/Mpk1p is required for normal cyclin C translocation in response to H$_2$O$_2$ stress (Jin et al., 2014). This MAP kinase module is stimulated by Rho1p through Protein Kinase C (Pkc1p) (see (Levin, 2011) for review). To determine if Med13p stability is controlled by the CWI pathway, the levels of Med13p-myc were monitored 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 relocalization 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 Serine 266 to alanine (S266A) mutant form of cyclin C that prevents Slt2p phosphorylation and its subsequent re-localization to the cytoplasm under low stress conditions (Jin et al., 2014). Localization of cyclin C$^{S266A}$-YFP was monitored in an unstressed cnc1Δ and cnc1Δ med13Δ mutants. Although nuclear in the wild-type strain, we found cyclin C$^{S266A}$ in the cytoplasm in the med13Δ mutant (Figure 6D). These results indicate that med13Δ is epistatic to cyclin C$^{S266A}$ and formally implies that Med13p function is either downstream or independent of Ser266 phosphorylation.

To further test this model, co-immunoprecipitation experiments were conducted between Med13p and either cyclin C or cyclin C$^{S266E}$. 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 C$_{S266E}$-YFP. These samples were immunoprecipitated with either α-GFP or α-myc antibodies then the immunoprecipitates were subjected to Western blot analysis probing for the presence of cyclin C-YFP or cyclin C$_{S266E}$-YFP. This experiment revealed a reduction cyclin C$_{S266E}$-YFP able to immunoprecipitate with Med13p-myc (compare lanes 6 and 7, Figure 6E). No significant differences were observed in cyclin C or cyclin C$_{S266E}$ levels (lanes 2 and 3). In addition, this interaction was independent of the YFP tag (lane 5) and required the myc antibody (lane 8). If the co-immunoprecipitation is examined probing for the presence of Med13p-myc, a similar result was obtained. The substitution mutation was again less able to interact with Med13p-myc (compare lanes 2 and 3, Figure 6F). These results indicate that cyclin C$_{S266E}$ reduces, but does not eliminate, Med13p association. In addition, these results provide a mechanism for the requirement of Slt2p in mediating cyclin C relocalization to the cytoplasm. Taken together, our results support a two-step system controlling cyclin C-Med13p association and ultimately cyclin C nuclear retention or release.

Discussion

In unstressed cells, cyclin C and Cdk8p form a complex with two additional proteins (Med12p and Med13p) that associates with the 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 report, 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 cell wall integrity (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, this report reveals 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
mis-expression of a nuclear gene caused by the med13Δ allele. Several results indicate
that the respiration deficiency in med13Δ mutant is due to mitochondrial DNA 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 that did not demonstrate a 2:2 segregation pattern (data not shown) or always segregate with med13Δ. Finally, studies in mice (Chen et al., 2003; Chen et al., 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 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 to suppress the loss of mitochondrial function in a med13Δ 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 (see (Scott et al., 2003; Youle and Karbowski, 2005) for reviews). 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 pro-apoptotic BH-3 family member Bax is recruited to sites of fission (Karbowski 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 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 med13Δ alleles and Ybh3p activity.

This study and our previous work have identified two domains, the HAD and Ser266 region, as sites controlling cyclin C nuclear localization. Structural analysis of the S. pombe cyclin C 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 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 co-immunoprecipitate 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 phospho-mimetic substitution mutation at Ser266 (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 Ser266 to the loop region between the third and forth helix of the second cyclin box (Figure 7A). Interestingly, both the HAD and S266 regions are on the same side of cyclin C raising the possibility that they represent a docking site to Med13p as previously suggested (Hoeppner et al., 2005). Therefore, combining our genetic results with the crystal data suggests that the HAD and Ser266 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 (light blue region, Figure 7A) 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 Ser266 (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 (PKA) 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. Taken together, 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 control to prevent aberrant mitochondrial fission and/or elevated sensitivity to stress, two outcomes that are deleterious to cell fitness.

Materials and methods.

Yeast strains and plasmids.

All S. cerevisiae strains used in this study are derivatives of a W303-1A variant (Strich et al., 1989) and 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; Cooper et al., 2012). pBK217 containing the CNC1^{HADΔ} allele was previously described (Cooper et al., 2014). Mitochondria visualization was achieved using pMt-DsRed (a gift from J. Nunnari (Naylor et al., 2006)). Plasmids pUG36 (MET25-GFP control), pBK38 (cyclin C-YFP) and pBK53 (cyclin C^{S266E}-YFP) have been previously described (Niedenthal et al., 1996; Jin et al., 2014). The CDK8/SSN8/SRB10 (pPL144-21) and cdk8^{KD} (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.

**Growth and stress assays.**

Cells were grown in either rich, non-selective medium (YPDA) or synthetic minimal medium (SC) allowing plasmid selection as previously described (Cooper et al., 1999). Clonogenic viability studies were conducted with mid-log phase (6 x 10^6 cells/ml) treated with 1 or 2 mM H_2O_2 for 2 h then serially diluted (1:10) and plated on the non-selective 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 timepoint. Quantitative PCR analysis of mtDNA loci was accomplished using Taqman™ cybergreen method (Applied Biosystems). The threshold cycle number (C>T) values were normalized to the nuclear ACT1 locus. COX1-F-5’-CTACAGATACAGCATTCCAAGA; COX1-R-5’-GTGCCTG-AATAGATGATAATGGT;
21S-F-5’-AATTGACCGAAAGCAAAACG’ 21S-R-5’-TTGCAACAT-CAACCTGTTCGA;
ACT1-F-5’-GTATGTGAAAGCCGGTTTTG; ACT1-R-5’ CATGATACCT-TGGTGTTCTTGG

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 4’, 6-diamidino-2-phenylindole (DAPI) as described (Cooper and Strich, 2002). For all experiments, the cells were grown to mid-log (5 x 10^6 cells/ml), treated with 1 mM H_2O_2 for time indicated in text then analyzed by fluorescence microscopy. Images were obtained using a Nikon microscope (model E800) with a 60X 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. In all images, the bar indicates 5 µM.
Western blot analysis

Straight Western blot analysis was performed as described (Kushnirov, 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% doxycycline, 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 x 10^6 cell/ml) cultures treated with cycloheximide (10 mg/L). Co-immunoprecipitation analyses were performed using 500 µg of whole cell extract. Immunoprecipitations were conducted overnight at 4°C with agitation. Than protein complexes were bound to protein G-agarose beads (Roche) and processed as according to manufacturer’s manual. GFP polyclonal antibody (Living Colors®, Clontech) or anti-c-myc monoclonal (9E10) antibody (Roche) was used for immunoprecipitations and Western blot analysis. The 12G10 mouse monoclonal anti-Tub1p antibody (Developmental Studies Hybridoma Bank, University of Iowa) and 3-phosphoglycerate K (Pgtk1p) mouse IgG1 monoclonal (22C5D8) antibody (Invitrogen) were used to detect Tub1p and Pgtk1p 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) and the CDP-Star chemiluminescence reagent (Tropix).
Acknowledgements.

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References:


economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. Yeast 14, 953-961.


Figure 1. Med13p retains cyclin C in the nucleus. (A) Subcellular localization of cyclin C (cyclin C-YFP), mitochondria (mt-DsRed) and nuclei (DAPI) were monitored by fluorescence microscopy in a wild-type cell (RSY10) before and following H$_2$O$_2$ treatment (1 mM) as indicated. (B) An unstressed med13Δ culture (RSY1701) was examined as in (A). (C) Representative image of mitochondrial morphology in med13Δ cnc1Δ mutant (RSY1712, left panel) and quantitation of mitochondrial fission in cells with the indicated genotype (mean ± s.e.m., n ≥ 3). (D) Experiment described in (B) was repeated in unstressed dnm1Δ (RSY1750) or dnm1Δ med13Δ (RSY1894) mutant strains. DAPI staining was omitted in these experiments. Bar = 5 µM in all panels, h = hours in all figures.
Figure 2. Regulation of GFP-Cdk8p localization by Med13p. GFP-Cdk8p localization was monitored by fluorescence microscopy in the wild-type RSY10 (A) or med13Δ mutant RSY1701 (B) strains before and 2 h after H₂O₂ addition as indicated. Nucleolar and nuclear locations were followed by Nop1p-RFP and DAPI signals, respectively. Arrows indicate cells that are enlarged in the zoom panels.
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 H_2O_2 concentrations as indicated for two hours, then serially diluted (1:10) before plating on rich growth medium. The plates were incubated three days prior to image collection. (B) TUNEL assays were performed on strains described in (A) were treated with 2 mM H_2O_2 for twenty hours. Typical histograms are shown depicting FACS analysis of untreated and treated samples and indicated. The numbers in the lower right of each panel indicate the % of the population exhibiting a TUNEL positive signal (s.e.m. <5%, n = 3).
Figure 4. Med13p is required to maintain mitochondrial DNA integrity. (A) Mid-log cultures as described in Figure 3 legend with the indicated genotype were grown in dextrose medium then serially diluted (1:10) before plating on medium containing either dextrose or acetate as the sole carbon source. The plates were incubated three days prior to image collection. (B) Mitochondrial morphology and mtDNA abundance was
monitored by mt-DsRed and DAPI staining, respectively in wild type (RSY10) and med13Δ mutant (RSY1701) cells. Arrowheads indicate typical mtDNA nucleoids within the mitochondria in wild-type cells. Representative images of the three general phenotypic classes observed in the med13Δ strain are shown with quantification. The white arrows indicate normal overlapping mtDNA-mitochondrial signals. Green arrows indicate abnormal nuclear morphology; red arrows indicate examples of fragmented mitochondria with out a visual mtDNA signal; yellow arrows indicate DAPI staining signals that may represent nuclear fragmentation or aberrant mtDNA signals. (C) qPCR analysis of two mtDNA loci COX1 and 21S rRNA in the strains indicated. Values are depicted relative to the single-copy ACT1 locus. Results shown are the means (±s.e.m.) from three biological replicates.
Figure 5. Med13p is destroyed in response to oxidative stress. (A) Western blot analysis of endogenously tagged Med13p-13myc (RSY17896) during an H₂O₂ timecourse experiment (left panel). Med13p-13myc turnover rate was monitored in an unstressed log-phase culture following the addition of cycloheximide (CHX, right panel). For all panels, “con” indicates the untagged parental strain (RSY10) controlling for non-specific α-myc cross reactivity. Pgk1p levels were used as loading controls. (B) Med13p-13myc levels were monitored by Western blot analysis during H₂O₂ stress
timecourse in wild type (RSY1786) and *ump1Δ* (RSY1961) strains as described in (A). (C) Med13p-myc levels were monitored by Western blot in extracts prepared from a wild type (RSY1786) or *cnc1Δ* (RSY1930) strains before and following H$_2$O$_2$ treatment. (D) Med13p-13myc levels were monitored by Western blot analysis during H$_2$O$_2$ stress timecourse in *cnc1Δ* strain (RSY1930) harboring the cyclin C$^{\text{HADΔ}}$ expression plasmid pBK217.
Figure 6. Med13p destruction requires Cdk8p activity. (A) Med13p-13myc levels were monitored in an H$_2$O$_2$ (0.8 mM) stressed mid-log cdk8Δ 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 Cdk8p$^{KD}$ expressing cells following 2 h treatment with H$_2$O$_2$ (0.8 mM). Subnuclear localization of cyclin C-YFP in the cdk8$^{KD}$ expressing cells is indicated by the arrowheads. The Normoski (Nom.) and nuclear (DAPI) images are indicated. (C) Endogenously tagged Med13p-3HA levels were monitored in an unstressed wild-type strain (RSY1788) harboring constitutively active RHO1 (RHO1$^{G19V}$ or RHO1$^{Q68L}$) expression plasmids. Protein extracts were immunoprecipitated with HA monoclonal antibodies and the
immunoprecipitates 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 C^{S266A}-YFP localization was monitored in unstressed cnc1Δ (RSY391) or cnc1Δ med13Δ (RSY1712) strains. The Normoski (Nom.) and nuclear (DAPI) images are indicated. (E) Co-immunoprecipitation studies were conducted in extracts prepared from a wild-type strain expressing Med13p-myc (RSY1786) and either cyclin C-YFP (pBK38) or cyclin C^{S266E}-YFP (pBK53). α-myc 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.
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), Ser266 (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 S266 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 S266 region as indicated. Activation of the CWI MAP kinase Slt2p results in phosphorylation of cyclin C on S266 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.
Table 1. List of *S. cerevisiae* strains.

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Genotype</th>
<th>Source</th>
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<tbody>
<tr>
<td>RSY10</td>
<td></td>
<td>(Strich <em>et al.</em>, 1989)</td>
</tr>
<tr>
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<td>cnc1::LEU2</td>
<td>(Cooper <em>et al.</em>, 1999)</td>
</tr>
<tr>
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<td>cnc1::TRP1</td>
<td>(Cooper <em>et al.</em>, 1999)</td>
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<td>This study</td>
</tr>
<tr>
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<td>cnc1::LEU2 med13::HIS3</td>
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</tr>
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<tr>
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<td>This study</td>
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</table>
*All strains are derived from the W303 background and contain the genotype $MAT^{a}$

$ade2$ $ade6$ $can1-100$ $his3-11,15$ $leu2-3,112$ $trp1-1$ $ura3-1$ except RSY1961.