2-1-2018


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Stieg, David C; Willis, Stephen D; Ganesan, Vidyaramanan; Ong, Kai Li; Scuorzo, Joseph; Song, Mia; Grose, Julianne; Strich, Randy; and Cooper, Katrina F, "A Complex Molecular Switch Directs Stress-Induced Cyclin C Nuclear Release Through SCFGrr1-Mediated Degradation of Med13." (2018). School of Osteopathic Medicine Faculty Scholarship. 60.
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A complex molecular switch directs stress-induced cyclin C nuclear release through SCF\textsuperscript{Grr1}\-mediated degradation of Med13

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**ABSTRACT** In response to oxidative stress, cells decide whether to mount a survival or cell death response. The conserved cyclin C and its kinase partner Cdk8 play a key role in this decision. Both are members of the Cdk8 kinase module, which, with Med12 and Med13, associate with the core mediator complex of RNA polymerase II. In Saccharomyces cerevisiae, oxidative stress triggers Med13 destruction, which thereafter releases cyclin C into the cytoplasm. Cytoplasmic cyclin C associates with mitochondria, where it induces hyperfragmentation and regulated cell death. In this report, we show that residues 742–844 of Med13’s 600–amino acid intrinsic disordered region (IDR) both directs cyclin C-Cdk8 association and serves as the degron that mediates ubiquitin ligase SCF\textsuperscript{Grr1}\-dependent destruction of Med13 following oxidative stress. Here, cyclin C-Cdk8 phosphorylation of Med13 most likely primes the phosphodegron for destruction. Next, pro-oxidant stimulation of the cell wall integrity pathway MAP kinase Slt2 initially phosphorylates cyclin C to trigger its release from Med13. Thereafter, Med13 itself is modified by Slt2 to stimulate SCF\textsuperscript{Grr1}\-mediated destruction. Taken together, these results support a model in which this IDR of Med13 plays a key role in controlling a molecular switch that dictates cell fate following exposure to adverse environments.

**INtRODUCTION**

Protein–protein interactions are at the heart of nearly all facets of cell physiology, including transducing exogenous signals necessary for proper cell fate decisions to be adopted. For example, following exposure to cytotoxic compounds, the cell must assess the level of damage and decide whether to arrest cell division and repair the damage or execute programmed cell death. Recently, it has become apparent that intrinsic-disordered regions (IDRs), defined by a continuous stretch of disordered promoting residues, play key roles in directing protein–protein interaction networks, especially those involved in macromolecular decisions including signaling and control pathways (reviewed in Dyson and Wright, 2005; Fuxreiter et al., 2014). This hotspot for communication is achieved in part by the ability of IDRs to contribute to the formation of large, malleable interfaces that can interact with multiple partners. These protein interactions can occur via short segments termed molecular recognition features (MoRFs) that undergo disorder-to-order transition upon binding to their cognate ligands (Fuxreiter et al., 2007). Consistent with these domains being communication hubs, the binding of IDRs to their targets is often regulated by covalent modifications including phosphorylation, which can serve as simple biological switches (Vuzman et al., 2012).

In budding yeast, cyclin C and its kinase partner Cdk8 are predominantly negative regulators of a diverse set of stress response genes (Surosky et al., 1994; Cooper et al., 1997; Holstege et al., 1998; Chi et al., 2001; van de Peppel et al., 2005). Together with...
Med13 and Med12, they form the Cdk8 kinase module (CKM) of the multisubunit Mediator complex, which acts as an interface between DNA bound transcription factors and RNA polymerase (Bourbon, 2008; Yin and Wang, 2014; Allen and Taatjes, 2015). In addition to its transcriptional role, cyclin C possesses another function following oxidative stress that is found in both yeast and mammalian cells (Cooper et al., 2012, 2014; Strich and Cooper, 2014; Wang et al., 2015). Specifically, nuclear release of cyclin C, but not Cdk8, allows its relocalization to the mitochondrial outer membrane. At this new subcellular address, cyclin C is necessary and sufficient for stress-induced mitochondrial fission and is required for MOM-dependent normal regulated cell death (RCD) execution (Cooper et al., 2012, 2014; Wang et al., 2015). This type of cell death is the reclassification of programmed cell death (PCD) passed by the international Nomenclature Committee on Cell Death in 2015 (Galluzzi et al., 2015). In budding yeast, cyclin C release is dependent on its direct phosphorylation by Slt2 MAPK (Jin et al., 2014) and the ubiquitin-mediated degradation of Med13 (Khakhina et al., 2014, and see Figure 1) In the CKM, Med13 contains a large centrally located IDR, the extent and placement of which is conserved across metazoans, plants, and fungi (Nagulapalli et al., 2016). As Med13 also bridges the CKM to the Mediator complex (Knuesel et al., 2009a) this centrally placed IDR may provide structural plasticity, allowing the CKM to interface with different mediator components according to the environment. Consistent with Med13 IDR being a potential communication hub, computer predictions suggest the presence of several MoRFs that are biased toward an alpha helical confirmation (Toth-Petroczy et al., 2008).

In this report, we provide mechanistic details on how Med13 is targeted for destruction following oxidative stress in the budding yeast Saccharomyces cerevisiae. We show that the cell wall integrity (CWI) pathway MAPK Slt2 directly phosphorylates Med13, which is required for its recognition by the SCF<sup>Grr1</sup> E3 ligase complex. Consistent with other SCF targets in yeast and higher eukaryotes (reviewed in Ang and Wade Harper, 2005), degradation of Med13 by SCF<sup>Grr1</sup> also requires phosphorylation by cyclin C-Cdk8, which most likely primes this phosphodegron. Furthermore, this phosphodegron partly overlaps with the predicted Med13 IDR (Toth-Petroczy et al., 2008). Consistent with this region serving as an interaction hub (Uversky, 2013), this IDR directs both Cdk8 and Grr1 binding and is sufficient to retain cyclin C in the nucleus. Taken together, these results define a multistep switch required for the cellular decision to release cyclin C from the nucleus and identify a Med13 IDR as the communication hub that coordinates this prodeath decision.

**RESULTS**

**SCF<sup>Grr1</sup> is necessary for Med13 H<sub>2</sub>O<sub>2</sub>-mediated degradation**

We have previously shown that Med13 degradation is dependent on a functional 26S proteasome, indicating that a ubiquitin ligase is required (Khakhina et al., 2014). In unstressed human cells, Med13 is turned over by the conserved SCF ubiquitin ligase utilizing the Fbw7 F-box recognition protein (Davis et al., 2013). Grr1, a yeast homologue of Fbw7, is a nonessential F-box protein that utilizes a leucine-rich region (LRR; Figure 2A) for substrate recognition (Flick and Johnston, 1991; Hsiung et al., 2001). SCF<sup>Grr1</sup> is necessary for degradation of the mediator component Med3 (Gonzalez et al., 2014), as well as proteins regulating the glycolytic–gluconeogenic switch (Benanti et al., 2007). More recently, it has also been shown to regulate Whi7, a repressor of Start transcriptional gene expression (Gomar-Alba et al., 2017). To determine whether SCF<sup>Grr1</sup> plays a role in H<sub>2</sub>O<sub>2</sub>-induced Med13 degradation, we examined Med13 protein levels following 0.4 mM H<sub>2</sub>O<sub>2</sub> treatment in wild-type and grr1Δ cells harboring functional Med13-HA on a single copy plasmid. This concentration of H<sub>2</sub>O<sub>2</sub> has long been established to induce a MOM-dependent RCD response in yeast (Madeo et al., 1999; Carmona-Gutierrez et al., 2010; Galluzzi et al., 2011), as well as triggering cyclin C nuclear release (Cooper et al., 2012, 2014; Jin et al., 2014; Khakhina et al., 2014). The results (Figure 2B) show that Med13-HA is significantly more stable in grr1Δ cells. We next repeated these experiments using endogenous MED13 tagged with the myc epitope in wild-type (RSY1798) and grr1Δ mutant strains (RSY1771) harboring a plasmid expressing either wild-type GRR1, a vector control, or a GRR1 derivative deleted for the LRR domain (grr1ΔL) (Hsiung et al., 2001). Following H<sub>2</sub>O<sub>2</sub> treatment, Med13-myc was again significantly more stable in grr1Δ cells harboring either the vector control or grr1ΔL than in wild-type cells (Figure 2C and Supplemental Figure S1A). These results suggest that SCF<sup>Grr1</sup> is the E3 ligase responsible for mediating Med13 degradation following H<sub>2</sub>O<sub>2</sub> stress.

If SCF<sup>Grr1</sup> is the ubiquitin ligase directing Med13 proteolysis, then Grr1 should interact with Med13. To test this possibility, Grr1 and Med13 association was assayed using a two-hybrid strategy. This approach has been used previously both to identify and confirm Grr1 substrates (Wang and Solomon, 2012; Gonzalez et al., 2014). We expressed either the wild-type or the grr1ΔL mutant GRR1 allele fused to the Gal4 DNA-binding domain bait with the full-length Med13 fused to the activator domain (AD) prey. These studies revealed that Grr1 interacts with Med13, and this interaction survives addition of the histidine analogue 3-amino-1,2,4-triazole (3-AT), suggesting that the His3 reporter gene induction is robust (Figure 2D).
FIGURE 2: SCFGrr1 mediates Med13 degradation following H2O2 stress. (A) Model of the SCFGrr1. (B) Wild-type (RSY10) and grr1Δ cells (RSY1770) harboring Med13-HA (pKC801) were treated with 0.4 mM H2O2 for the time points indicated and Med13 levels were analyzed by Western blot. (C) Top panel: RSY1798 (MED13-myc::KAN) was treated with 0.4 mM H2O2 for the time points indicated and Med13 levels were analyzed by Western blot. Tub1 levels were used as loading controls. Bottom panel: RSY1771 (grr1Δ::HIS3 MED13-myc::KAN) harboring ADH1Grr1 was analyzed as for RSY1798. Tub1 levels were used as loading controls. (D) Yeast two-hybrid analysis of Med13 and Grr1 derivatives. Y69a cells harboring Med13-activating domain plasmid (pKC800) and either pAS2, pAS-Grr1, or pAS2-Grr1Δ binding domain plasmids were grown on –LEU, –TRP, –HIS, –ADE dropout medium to select for both plasmids (left panel) and on –TRP, –LEU, –HIS –ADE2 (middle panel), and –TRP, –LEU, –HIS, –ADE + 3-AT (right panel) to test for Med13-Grr1 interaction.

With the grr1Δ mutant bait, an interaction is detected selecting for the dual HIS3 and ADE2 reporter genes, but colony formation is uneven (middle panel) or absent in the presence of 3-AT (right panel). Taken together with the increased stability of Med13 observed in grr1Δ cells, these results argue that Med13 is an SCFGrr1 substrate.

FIGURE 3: The unstructured domain of Med13 binds Grr1. (A) ProteinPredict (Yachdav et al., 2014) analysis of yeast Med13. Med13 contains amino- and carboxyl-terminal structured domains separated by an intrinsic disordered domain (IDR). Grr1 and Med13 interaction regions are indicated below the schematic. ++ = qualitatively reduced binding (B). Med13-Grr1 Y2H analysis. Y69a cells harboring pAS-Grr1 and the indicated Gal4AD-Med13 subclone were streaked on media selecting for plasmid maintenance (left) or induction of the ADE2 and HIS3 reporter genes (right) by Y2H interaction. (C) Wild-type (RSY10) and grr1Δ (RSY1770) cells harboring the minimal Med13 interaction domain expression plasmid (Gal4AD-Med13 571-906) were treated with 0.4 mM H2O2 for the time points indicated and Med13 571–906 levels were analyzed by Western blot. Pgk1 levels were used as loading controls.

The intrinsic disordered region of Med13 interacts with Grr1
Many SCF targets require phosphorylated substrates for F-box recognition (Skowyra et al., 1997). Consistent with this model, the LRR of Grr1 specifically recognizes phosphorylated serine/threonine–proline motifs within its substrate degron (Hsiung et al., 2001). In human cells, phosphorylation of T326 is required for Med13 destruction by SCF(Fbw7) (Davis et al., 2013). To address whether a similar mechanism directs H2O2-induced Med13 degradation in yeast, the requirement of the analogous threonine in yeast (T210; Supplemental Figure S1B) was tested. For these studies, we expressed Med13-HA from a single-copy plasmid in med13Δ cells. These studies revealed that both the wild type and the Med13ΔT210A derivative were still degraded following 0.4 mM H2O2 stress (Supplemental Figure S1C, D, and quantitated in E), indicating that T210 phosphorylation is not necessary for oxidative stress–induced destruction of Med13. Furthermore, consistent with our previously published results with endogenously tagged Med13-myc (Khakhina et al., 2014), Med13-HA degradation is not dependent on new protein synthesis, as it is still observed following cycloheximide treatment (Supplemental Figure S1C).

We next employed Y2H assays to identify the Med13 region able to interact with Grr1. Med13 has structured head and tail domains flanking a large IDR (Toth-Petroczy et al., 2008; Figure 3A). A series of Gal4AD-Med13 constructs that span the length of Med13 were built and tested for their ability to interact with Gal4BD-Grr1. The results revealed that the Grr1 interaction domain lies within the unstructured domain, between amino acids 571 and 906 (Figure 3B, summarized in Figure 3A). To confirm that the Med13 degron lies within this region, we performed a classical degron assay. The levels of the prey Gal4AD-Med13 571–906 fusion protein were monitored following 0.4 mM H2O2 stress in wild-type and grr1Δ cells. The results indicated that Med13 571–906 was destroyed following oxidative stress in a Grr1-dependent manner (Figure 3C). Taken together, these results indicate that the SCFGrr1 degron lies within the IDR.

The Med13 intrinsic disordered region binds cyclin C
As cyclin C nuclear release represents an important step toward entering the cell death pathway, we next sought to identify the Med13 region that binds cyclin C, using two-hybrid strategies. However, the yeast cyclin C self-activates when tethered to a yeast two-hybrid (Y2H) bait protein (Cooper et al., 1997). Therefore, we used the human cyclin C, as it does not self-activate two-hybrid
The Med13 IDR binds cyclin C. (A) Y2H analysis of the human cyclin C DNA-binding domain plasmid (pSW108) and the indicated Gal4AD-Med13 subclone derivatives. The numbers indicate the amino acids remaining in the activation domain plasmids. Transformants were patched onto either –LEU, –TRP (L–T) or –ADE–LEU–TRP (H–A–L–T) to select for plasmid maintenance or Y2H interaction, respectively. (B) Western blot analysis of pull-down assays with His6-human cyclin C and GST-Med13\textsuperscript{742-844} (DS30). The load control contains 1/10 of the input. Single and double asterisks represent cleaved GST and cross-reaction between anti-GST antibody and human cyclin C, respectively. Molecular weight markers (kDa) are indicated. (C) As in B except that yeast cyclin C was used. (D) Western blot analysis of pull-down assays with GST yeast cyclin C and His\textsubscript{6}-Med13\textsuperscript{571-906} (DS22). The load control contains 1/50 of the input. Molecular weight markers (kDa) are indicated.

However, many substrates require two phosphorylation marks: one to “prime” the substrate and another that represents the trigger for ubiquitylation (reviewed in Ang and Wade Harper, 2005). Previous work revealed that protein kinase A (PKA) phosphorylates Med13 on Serine 608 and 1236 (Chang et al., 2004). However, a derivative mutated for both phosphor acceptor sites (Med13\textsuperscript{S608A,S1236A}-HA) was still destroyed with kinetics similar to wild type (Supplemental Figure S3A), indicating that PKA phosphorylation is not required for Med13 degradation following H\textsubscript{2}O\textsubscript{2} treatment. Consistent with this result, Med13\textsuperscript{S608A,S1236A}-HA complements the aberrant mitochondrial morphology exhibit by med13\textsuperscript{A} cells (Supplemental Figure S3B).

In addition to PKA, cyclin C-Cdk8 phosphorylates Med13 in human cells (Knuesel et al., 2009b; Poss et al., 2016) suggesting the possibility that Cdk8 represents the priming kinase. To test this model, Med13 destruction kinetics were monitored in a cnc1\textsuperscript{A} null strain transformed with either the wild-type CNC1 gene or the vector control. These experiments indicated that cyclin C-Cdk8 kinase is necessary for Med13 destruction (Figure 6A and quantified in Supplemental Figure S4A). Our previous studies showed that Slt2 phosphorylates cyclin C on Ser266 following H\textsubscript{2}O\textsubscript{2} and this modification is required for its release from Med13 and translocation to the cytoplasm (Jin et al., 2014). Therefore, one possibility is that
To test whether cyclin C-Cdk8 activity was required before or after oxidative stress, a Cdk8-N-end rule degron was constructed. This system takes advantage of the rapid turnover of proteins that contain arginine as the amino terminal residue (Bachmair et al., 1986; Varshavsky, 1992; Gnanasundram and Kos, 2015). The Cdk8-N-end rule construct is under the control of a doxycycline-repressible promoter, so that the administration of doxycycline coupled with the rapid turnover of the Cdk8-N-end rule fusion protein results in rapid depletion of Cdk8 from yeast cells (Figure 6C). To test the execution point for Cdk8 phosphorylation, a mid–log culture expressing Med13-HA and Cdk8-N-end degron was untreated or treated with doxycycline for 1 h prior to H$_2$O$_2$ addition to ensure Cdk8 depletion. A time course was conducted and samples were collected, extracts prepared, and Med13-HA levels monitored by Western blot analysis. These studies revealed that Med13-HA levels were reduced similarly in both cultures (Figure 6D), indicating that Cdk8 function was required before oxidative stress. Taken together, these results are consistent with a model in which Cdk8 phosphorylation primes Med13 for degradation.

The CWI pathway is required for reactive oxygen species–mediated destruction of Med13
The CWI signal transduction pathway is the major MAPK pathway in yeast that transmits the reactive oxygen species (ROS) stress signal...
Slt2 and Cdk8 phosphorylation of the intrinsic disordered region is required for Med13 destruction

IDRs confer conformational flexibility on proteins that are often targets of posttranslational modifications (Diella et al., 2008). Notably, IDRs are enriched in phosphorylation sites, raising the possibility that they are substrates for multiple kinases (Collins et al., 2008). As our data indicate that Cdk8 and Slt2 are required for Med13 degradation, we next asked if the sites they phosphorylate lie within the same IDR of Med13 that associates with Grn1 and cyclin C (amino acids 651–906). Slt2 and Cdk8 are both proline-directed kinases that preferentially phosphorylate the consensus sequences PX(S/T)P and S/T-P-X-K/R (where X is any amino acid), respectively. They also can phosphorylate the minimal consensus sequence S/T-P (Nigg, 1993). Med13571–906 contains a conserved Slt2 consensus sequence at position 748 that has been identified as potential phosphorylation site in two genomie screens (Albuquerque et al., 2008; Holt et al., 2009) as well as four additional TP sites (Figure 8A). Thus, to address whether these kinases phosphorylate the five S/T-P sites in Gal4AD-Med13571–906 fusion protein, its degradation was monitored following H2O2 stress in wild-type, slt2Δ and cdk8Δ cells. The results show that Gal4AD-Med13571–906 fusion protein is stable in either slt2Δ or cdk8Δ cells (Figure 8B). These results are consistent with a model where the Med13 phosphodegron is targeted by both Cdk8 and Slt2. Consistent with the Y2H data presented in Figure 3B, the phosphodegron is stable in grn1Δ cells (Figure 8C). If the Med13 phosphodegron is regulated by two kinases, then two separate S/T-P sites within the degron should stabilize the protein. To test this, wild-type cells harboring either a wild-type Gal4AD-Med13571–906 fusion protein or mutant derivatives were subjected to 0.4 mM H2O2 stress and analyzed as described above. As anticipated, the smaller Gal4AD-Med13571–844 fusion protein that contains all five S/T-P sites was still degraded in wild-type cells (Figure 8C). Interestingly, the S748A, T781A, and T803A mutants were also degraded, with the same kinetics as wild type (Supplemental Figure S4C). In addition, the double mutants (T781A, T803A and S748A, T781A) were also degraded with wild-type kinetics (Figure 8, D and E). However, the T835A, T837A double mutant was significantly more stable than the wild type (Figure 8, C and E), suggesting that these sites are needed for Med13 degradation. Taken together, these results suggest that both kinases have to be active for SCF<sup>GRN1</sup> to recognize this 100–amino acid phosphodegron at positions T835 and T837.
FIGURE 7: Slt2 directly stimulates Med13 destruction. (A) Wild-type (RSY10), kdx1Δ (RSY1736) and slt2Δ (RSY1006) cultures expressing pKC801 (CEN, Med13-HA) were grown to mid–log phase (0 h) and then treated with 0.4 mM H2O2 for the indicated times. Med13-HA levels were determined by Western blot analysis. (B) As in A except that the slt2Δ cells expressed a kinase dead SLT2 mutant (slt2ΔK54R) plasmid. (C) Top panel: Slt2-HA or Slt2Δ-HA (kinase dead) was immunoprecipitated from extracts prepared from cells treated for 60 min with 5 mM sodium orthovanadate was mixed with Med13Δ906 and radioactive ATP as indicated. All reactions were conducted in duplicate and then analyzed by SDS-PAGE and subjected to autoradiography. Med13Δ906 is indicated by the arrowhead. Bottom panel: Coomassie stained gel showing the Med13Δ906 input used in the kinase assays. (D) Wild-type (RSY10) or cdk8Δ (RSY1796) mutant cells expressing either an empty vector control or the hyperactive allele of BCK1 (BCK1-20) and MED13-HA (pKC803) were grown to mid-log and Med13-HA levels determined by Western blot analysis. Tub1 served as a loading control.

DISCUSSION

Communication between organelles is critical to coordinate the cellular response to a variety of external or internal signals, including oxidative stress. The data presented here and in previous publications (Cooper et al., 1997, 2014; Krasley et al., 2006; Jin et al., 2013, 2014, 2015; Khakhina et al., 2014; Wang et al., 2015) have revealed that the CKM plays a key role in both yeast and mammalian cells. Previously, we reported that cyclin C, but not Cdk8, translocates from the nucleus to the mitochondria following oxidative stress in both yeast and mammalian cells. At the mitochondria, cyclin C associates with the fission machinery to induce fragmentation and direct programmed cell death (Cooper et al., 2012, 2014; Wang et al., 2015). Therefore, releasing cyclin C from the nucleus represents an important step determining whether the cell initiates the RCD pathway or not. Importantly, we found that the nuclear anchor for cyclin C, Med13, is destroyed in response to oxidative stress, providing a mechanism for orchestration of cyclin C nuclear release (Khakhina et al., 2014). Interestingly, Med12 is neither required for this response (Khakhina et al., 2014) nor destroyed following H2O2 stress (Supplemental Figure S4D). In this report, we provide the molecular details on how Med13 destruction is triggered to operate this important molecular switch in yeast. First, Med13 proteolysis is mediated by the ubiquitin ligase SCF and the specificity factor Grn1. Several studies in yeast and higher eukaryotes found that SCF substrates require a complex interplay between multiple kinases to generate the requisite phosphodegron on the substrate (reviewed in Ang and Wade Harper, 2005). For example, Cdk1 acts as a priming kinase for Cdc5 (Asano et al., 2005; Yoshida et al., 2006), whereas Cdc5 acts as a priming kinase for Wee1 (Watanabe et al., 2004). In all cases, the priming kinase first phosphorylates the substrate, while the second kinase completes the formation of the phosphodegron by modifying residues that are recognized by the SCF. This has led to the current model, in which priming phosphorylations create docking sites for downstream kinases. Moreover, local structural disorder most likely facilitates multiple kinases regulating a protein within a small region. We identified a small region (amino acids 742–844) within the IDR of Med13 that acts as a communication hub. This region is sufficient to retain cyclin C in the nucleus, associates with Grn1, and contains the Med13 phosphodegron that is primed by Cdk8 and activated by Slt2 following oxidative stress exposure. This dual kinase requirement provides the cell with a mechanism to integrate multiple input signals into a single readout.

Med13 phosphodegron activation requires a three-step process

Our results indicate that cyclin C-Cdk8 provides the priming signal for Med13 destruction (Figure 9, Step 1). Although technical reasons prevented us from addressing whether this activity is direct or indirect, four independent experiments support a model of this as a direct phosphorylation event: 1) The observation that Med13Δ906 was not destroyed following H2O2 stress in cdk8Δ cells (Figure 8B). 2) The observation of a slower migrating band seen in Phos-Tag SDS–PAGE analysis of unstressed wild-type extracts that was missing in the cdk8Δ sample (Figure 6B). 3) The end-N rule experiments conducted in Figure 6, C and D, also revealed that Med13-HA levels were reduced regardless of the presence of Cdk8 after stress. 4) Last, in vitro evidence revealed that cyclin C interacts directly with this region of Med13 (Figure 4, B and C), which would position the kinase with direct access to the Med13 degron. Thus, taken together, these results suggest that Cdk8 phosphorylation primes Med13 for degradation, most likely by a direct event. Consistent with this, human Cdk8 directly phosphorylates human Med13 on S749, which lies in the intrinsic disorder domain in what appears to be a nonconserved residue compared with yeast (Poss et al., 2016). This requirement may be necessary to inform the cell that Med13 is in the Cdk8 module and its destruction will lead to cyclin C release and Cdk8 inactivation. Slt2 activation by oxidative stress provides the trigger mechanism that commits the cell to destroying Med13 and releasing cyclin C from the nucleus (Step 3). This event accomplishes two goals. First, removing cyclin C and destroying Med13 inhibits the repressor activity of Cdk8 for several stress-responsive genes, including chaperones (e.g., SSA1), anti-oxidants such as catalase (CTT1), and the multi-stress-responsive gene DDR2 (Cooper et al., 1997, 2012; Holstege et al., 1998). In addition, Slt2 activation stimulates several transcription factors, such as Rlm1, which is required for the transcriptional arm of the stress response (Dodou and Teisman, 1997; Watanabe et al., 1997; Jung et al., 2002). Therefore, Slt2 activation both stimulates transcriptional activators and inhibits repressors. By releasing cyclin C, the cell is able to transmit the stress signal to the mitochondria. We have previously demonstrated that cyclin C–induced fission alone is not sufficient to induce cell death, but does make cells hypersensitive to oxidative stress (Khakhina et al., 2014). Therefore, cyclin C release poises the cell to initiate RCD, but additional regulatory layers exist. Unlike other priming/trigger two-kinase degron phosphorylation switches, we have identified an additional step in the system regulating Med13. Specifically, Slt2 also modifies cyclin C on S266, which promotes its RCD pathway or not. Importantly, we found that the nuclear anchor for cyclin C, Med13, is destroyed in response to oxidative stress, providing a mechanism for orchestration of cyclin C nuclear release (Khakhina et al., 2014). Interestingly, Med12 is neither required for this response (Khakhina et al., 2014) nor destroyed following H2O2 stress (Supplemental Figure S4D). In this report, we provide the molecular details on how Med13 destruction is triggered to operate this important molecular switch in yeast. First, Med13 proteolysis is mediated by the ubiquitin ligase SCF and the specificity factor Grn1. Several studies in yeast and higher eukaryotes found that SCF substrates require a complex interplay between multiple kinases to generate the requisite phosphodegron on the substrate (reviewed in Ang and Wade Harper, 2005). For example, Cdk1 acts as a priming kinase for Cdc5 (Asano et al., 2005; Yoshida et al., 2006), whereas Cdc5 acts as a priming kinase for Wee1 (Watanabe et al., 2004). In all cases, the priming kinase first phosphorylates the substrate, while the second kinase completes the formation of the phosphodegron by modifying residues that are recognized by the SCF. This has led to the current model, in which priming phosphorylations create docking sites for downstream kinases. Moreover, local structural disorder most likely facilitates multiple kinases regulating a protein within a small region. We identified a small region (amino acids 742–844) within the IDR of Med13 that acts as a communication hub. This region is sufficient to retain cyclin C in the nucleus, associates with Grn1, and contains the Med13 phosphodegron that is primed by Cdk8 and activated by Slt2 following oxidative stress exposure. This dual kinase requirement provides the cell with a mechanism to integrate multiple input signals into a single readout.

Med13 phosphodegron activation requires a three-step process

Our results indicate that cyclin C-Cdk8 provides the priming signal for Med13 destruction (Figure 9, Step 1). Although technical reasons prevented us from addressing whether this activity is direct or indirect, four independent experiments support a model of this as a direct phosphorylation event: 1) The observation that Med13Δ906 was not destroyed following H2O2 stress in cdk8Δ cells (Figure 8B). 2) The observation of a slower migrating band seen in Phos-Tag SDS–PAGE analysis of unstressed wild-type extracts that was missing in the cdk8Δ sample (Figure 6B). 3) The end-N rule experiments conducted in Figure 6, C and D, also revealed that Med13-HA levels were reduced regardless of the presence of Cdk8 after stress. 4) Last, in vitro evidence revealed that cyclin C interacts directly with this region of Med13 (Figure 4, B and C), which would position the kinase with direct access to the Med13 degron. Thus, taken together, these results suggest that Cdk8 phosphorylation primes Med13 for degradation, most likely by a direct event. Consistent with this, human Cdk8 directly phosphorylates human Med13 on S749, which lies in the intrinsic disorder domain in what appears to be a nonconserved residue compared with yeast (Poss et al., 2016). This requirement may be necessary to inform the cell that Med13 is in the Cdk8 module and its destruction will lead to cyclin C release and Cdk8 inactivation. Slt2 activation by oxidative stress provides the trigger mechanism that commits the cell to destroying Med13 and releasing cyclin C from the nucleus (Step 3). This event accomplishes two goals. First, removing cyclin C and destroying Med13 inhibits the repressor activity of Cdk8 for several stress-responsive genes, including chaperones (e.g., SSA1), anti-oxidants such as catalase (CTT1), and the multi-stress-responsive gene DDR2 (Cooper et al., 1997, 2012; Holstege et al., 1998). In addition, Slt2 activation stimulates several transcription factors, such as Rlm1, which is required for the transcriptional arm of the stress response (Dodou and Teisman, 1997; Watanabe et al., 1997; Jung et al., 2002). Therefore, Slt2 activation both stimulates transcriptional activators and inhibits repressors. By releasing cyclin C, the cell is able to transmit the stress signal to the mitochondria. We have previously demonstrated that cyclin C–induced fission alone is not sufficient to induce cell death, but does make cells hypersensitive to oxidative stress (Khakhina et al., 2014). Therefore, cyclin C release poises the cell to initiate RCD, but additional regulatory layers exist. Unlike other priming/trigger two-kinase degron phosphorylation switches, we have identified an additional step in the system regulating Med13. Specifically, Slt2 also modifies cyclin C on S266, which promotes its
The Med13 intrinsic disordered region is a communication hub

It has recently been suggested that IDRs play important roles in protein:protein communication, as they provide a flexible interaction surface to multiple partners (Wright and Dyson, 2015). Using two different protein structure prediction algorithms, IUPred/ANCHOR and Phyre² (Dosztányi et al., 2005, 2009; Kelley et al., 2015), Med13 has a large IDR spanning the middle of the protein (amino acid residues 307–906 and Supplemental Figure S5, A and B). Although not conserved in amino acid sequence, it is notable that the position and presence of Med13’s IDR is conserved throughout three kingdoms (Nagulapalli et al., 2016). Also conserved is the observation that Med13 is the CKM member that brings this kinase module in contact with the Mediator complex (Knuesel et al., 2009a). This suggests a model in which the IDR of Med13 provides conformational plasticity to the CKM, which is important for bringing the kinase and other proteins into contact with different mediator members on a pro re nata basis. This idea is consistent with the results presented here, in which we show that the IDR of Med13 can bind to cyclin C before stress and Grr1 after. Although speculative, taken together, this suggests a model in which Med13 IDR is conserved functionally and may contain signature motifs that are important for protein binding. Consistent with this idea is the observation that two other conserved proteins that regulate transcription (CCR4-NOT4 and TFIIS) also bind to the IDR of yeast Med13, but in a different location (Supplemental Figure S5; Liu et al., 2001; Wery et al., 2004). Such a signature could be a MoRF, which is defined as a short binding region located within a longer intrinsically disordered region that binds to protein partners via disorder-to-order transitions (Kotta-Loizou et al., 2013). ANCHOR analysis (Dosztányi et al., 2009) predicts that the IDR of Med13 has many such domains, with two lying within the cyclin C binding region (residues 742–844, Supplemental Figure S5A). As additional factors are identified that bind the Med13 IDR, the rules that govern these interactions may become better understood.

FIGURE 8: The Med13 IDR contains the phosphodegron. (A) Map of Med13 showing the location of potential Cdk and MAPK sites within the IDR. (B) Wild-type (RSY10), slt2Δ (RSY1737), and cdk8Δ (RSY1796) cultures expressing pDS16 (Gal4AD-Med13745–906) were grown to mid–log phase (0 h) and then treated with 0.4 mM H2O2 for the indicated times. Med13651–906-HA levels were determined by Western blot analysis. Pgk1 levels were used as a loading control. (C, D) Wild-type (RSY10) cultures harboring wild-type Gal4AD-Med13742–844 (pDS32) or various point mutations as indicated were grown to mid–log phase (0 h) then treated with 0.4 mM H2O2 for the indicated times. Med13-HA levels were determined by Western blot analysis. Pgk1 levels were used as a loading control. The Gal4AD-Med13742–844 levels were also monitored in the grr1Δ strain. (E) Degradation kinetics of the Gal4AD-Med13742–844 constructs shown in C and D.

dissociation from Med13 (Jin et al., 2014). Our finding that the S266A mutation protects Med13 from destruction is consistent with a model in which S266 modification disrupts cyclin C–Med13 interaction (Step 2), allowing Slt2 access to the phosphodegron. Taken together, our model predicts a three-step system that controls Med13 stability and cyclin C nuclear release.

If cyclin C phosphorylation is sufficient to initiate cyclin C nuclear release, then why would it be important to completely destroy Med13? First, our results indicate that S266 phosphorylation is capable of stimulating only partial release of cyclin C in unstressed cells (Jin et al., 2014). Therefore, Med13 destruction may be necessary for complete cyclin C release. Not exclusive of this model, another possibility is that Med13 destruction prevents the reaccumulation of cyclin C in the nucleus after its cytoplasmic translocation. In this model, Med13 proteolysis fixes the decision of cyclin C release thus directing a complete transcriptional and mitochondrial response to oxidative stress.
paralogue of MED13 (Muncke et al., 2003), results in MED13L haploinsufficiency syndrome. This syndrome is complex and presents with a range of symptoms including congenital heart and neurodevelopmental defects, severe neurocognitive deficiencies, and facial dysmorphism (Adgebola et al., 2015; van Haelst et al., 2015; Asadollahi et al., 2017; Yamamoto et al., 2017). Interestingly many of these mutations lie in the IDR of MED13L (Asadollahi et al., 2017). In yeast, a hallmark of med13A cells is that they are respiratory deficient, which is dependent on cyclin C nuclear release (Khakhina et al., 2014). Thus, it would be anticipated that a strain deleted for Med13 residues 742–844 would have a similar phenotype. Taken together, these studies emphasize the need for further analysis of both yeast and human Med13 IDRs to understand the molecular details of how this region contributes to Med13 function.

Role of SCF<sup>Fbw7</sup> and Med13 in higher eukaryotes following stress

Our previous studies described the conserved role of cyclin C in stressed and unstressed cells (Cooper et al., 2014; Wang et al., 2015). However, differences are observed between yeast and humans in cyclin C regulation. In yeast, all of cyclin C is released into the cytoplasm, followed by its destruction by the Not1 ubiquitin ligase. Conversely, only ~10% of total cyclin C is released from the nucleus in mammalian cells following oxidative stress, and we do not detect any significant changes in its overall protein levels (Wang et al., 2015). Steady state turnover of the human Med13 is employed in these two systems or that stress-induced degradation is regulated differently than that observed under normal growth conditions. Consistent with this is the observation that Cdk substrates are often clustered in regions of intrinsic disorder (reviewed in Enserink and Kolodner, 2010). Similar to other IDRs, their exact position in the protein is often poorly conserved in evolution, indicating that precise positioning of phosphorylation may not be required to permit a shared function (reviewed in Enserink and Kolodner, 2010). Thus, it is possible that following oxidative stress in human cells, a similar two-kinase mechanism could regulate Med13 degradation. In support of this model, phosphorylation by two kinases has been observed for other SCF<sup>Fbw7</sup> substrates (Akhoondi et al., 2007) including oncogenic transcription factors. This has led to Fbw7 being classified as a tumor suppressor (reviewed in Welcker and Clurman, 2008). Fbw7 degrades numerous oncogenic transcription factors (e.g., Myc, Notch, and Jun), as well as other proteins that contribute to carcinogenesis. As cyclin C has also recently been given this tumor suppressor designation (Li et al., 2014), it is imperative to understand the relationship between these proteins following stress in higher eukaryotes.

MATERIALS AND METHODS

Yeast strains and plasmids

All experiments, except the Y2H assays, were performed on the S. cerevisiae W303 strain RSY10 (Strich et al., 1989) and are listed in Supplemental Table S1. The Y2H assays were performed on PJK69-4a (James et al., 1996). In accordance with the Mediator nomenclature unification effort (Bourbon et al., 2004), cyclin C (SSN8/UME3/SRB11) and Cdk8 (SSN2/UME5/SRB10) will use the CNC1 and CDK8 gene designations, respectively. For reference, the human cyclin C gene name is CCNC. The cnc1Δ, cdk8Δ, med13Δ, sfl2Δ, kdx1-1Δ, and kdx1 sfl2Δ strains have been previously described (Cooper et al., 1997, 2014; Krasley et al., 2006; Jin et al., 2014). The grl1Δ strain RSY1770 and the myc-, tagged Med12 strain (RSY1787) were constructed using gene replacement methodology as described (Longtine et al., 1998). The MED13-1myc allele was generated in RSY1770 to create RSY1771 using the same methodology. RSY2066 was made by integrating pMK634 (Gnanasundram and Kos, 2015) into the CDK8 locus of RSY10. The strain was then transformed with pCM188 (Gari et al., 1997), which contains the TET activator on a plasmid. The yeast hybrid strain PJK69-4a was obtained from the Yeast Resource center, courtesy of a gift from S. Fields (University of Washington). All cells were grown at 30°C, apart from sfl2A and sfl2A kdx1Δ, which were grown at 23°C.

All plasmids used in this study are listed in Supplemental Table S2. The wild-type epitope tagged plasmids pHY1066 (MED13-HA, 2 µ, pKCC37 (ADH1p-cyclin C-myc), pUM511 (GPD1p-CDK8-HA), and pBKB3 (ADH1p-CNC1-YFP) are functional and have been described previously (Cooper et al., 1997, 2012; Cooper and Strich, 1999; Chang et al., 2004; Jin et al., 2013). pLR141 was made by cloning the ADH1p-CNC1-myc, CYC1<sub>Term</sub> from pKCC337 into pRS426. The 2 µ MED13-HA plasmids (PHY1066 and PHY1089) and were a gift from P. Herman (Ohio State University) (Chang et al., 2004). The wild-type MED13 centromere–based plasmids pKC801 and pKCB03 were made by PCR cloning the ADH1 promoter MED13-3HA ORF and the MED13 terminator from PHY1022 into pRS316 and pRS315, respectively. Site-directed mutagenesis (New England Bio-Ladigo Q5) was used to create plasmids harboring amino acid mutations, except for DS36 which was made using the ChangeIT kit by Affymetrix. The CNC1<sup>SL2<sub>SA</sub></sup> and the SLT2<sup>SAE2</sup> (a gift from D. Levin, Boston University) have been described previously (Kim et al., 2008; Jin et al., 2014). The MED13 Y2H plasmids were constructed by PCR cloning from pHY1022 into the XhoI site of the Gal4 activating domain plasmid pACT2. Likewise, the human cyclin Y2H Gal4 binding domain plasmid pSW108 was made by PCR cloning of cyclin C DNA amplified from pEGFP-cyclin C (unpublished data) into Nco1-digested pAS2. The GST-MED13 fusion plasmid was made by PCR cloning from PHY1022 into pGEX4T-1. Oligonucleotide
sequences were used to make plasmids, and strains are available upon request. In short, all constructs were amplified from plasmid DNA using Phusion Taq (Thermo) digested using NEB fast digest restriction enzymes and ligated using Thermo fast ligase into their respective vectors. All protein fusion constructs were sequenced (Eurofins Genomics). Other plasmids that were used in this study that have been previously described are listed in Supplemental Table S2.

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) before treatment with low concentrations of 0.4 mM H_2O_2 as previously described (Jin et al., 2013). Samples of 25 ml of cells were collected per time point and washed in water; then the pellets were flash frozen in liquid nitrogen. Y2H experiments were executed as described (Wang and Solomon, 2012). A sample of 10 mM 3-amino-1,2,4-triazole (3-AT) was added to the selective plates to increase the stringency of the interactions. E. coli cells were grown in LB medium with selective antibiotics. The degron experiments described in Figure 5, D and E, were executed as follows. In Figure 5D, cells (RSY2066) were grown to midlog and samples removed at the time points shown for analysis of Ubi-isoleucine::3HA-Cdk8 degradation after the addition of 2 µg/ml doxycycline. In Figure 5E, RSY2066 was transformed with Med13-3HA plasmid (pKC803), grown to midlog, and split and into one flask 2 µg/ml doxycycline was added. After 60 min, 0.8 mM H_2O_2 was added to both sets of cells and samples taken for NaOH lysis and Western analysis as described below.

Western blot analysis

Tagged Med13 constructs were detected using NaOH lysis of cell pellets as described by Zhang et al. (2011). In short, the frozen cell pellets were defrosted on ice, resuspended in 2 M LiOAc on ice for 5 min, centrifuged, and then resuspended in 0.4 M NaOH for a further 5 min on ice. Thereafter, the pellets were resuspended in 100 µl 2X SDS loading dye (Cooper et al., 1997) and boiled for 5 min, and 15 µl was loaded onto a 6% SDS–PAGE gel (Novagen). For the phos-tag gel (Figure 9B), proteins were prepared as just described and analyzed on a 7.5% polyacrylamide gel containing 7.5 µM phos-tag (Wako Laboratory Chemicals) and 15 µM MnCl_2. The proteins were transferred to a nylon membrane (Millipore) using 10% methanol, 0.02% SDS, and 10X running buffer (Cooper et al., 1997). To detect Med13-myc, Med12-myc, and Med13-3HA, 1-in-5000 dilutions of either anti-myc (Roche) or anti-HA antibodies (Clontech) were used. Tub1 and Pgk1 were visualized as previously described (Tan et al., 2011), except that anti–alpha tubulin antibodies (12G10) obtained from the Developmental Studies Hybridoma Bank, University of Iowa. Western blot signals were detected using either goat anti-mouse or goat anti-rabbit secondary antibodies conjugated to alkaline phosphatase (Sigma) and the CDP-Star chemiluminescence kit (Tropix). Signals were quantitated by CCD camera imaging (Kodak). All degradation assays were performed more than once. SEM were generated for each point and error bars are indicated on the graphs, which were generated using the GraphPad Prism 7 program.

Fluorescence microscopy

YFP-cyclin C subcellular localization and mitochondrial morphology were monitored as described previously (Cooper et al., 2012, 2014). For all experiments, the cells were grown to midlog (6 × 10^6 cells/ml), treated with 0.4 mM H_2O_2 for the time points indicated, and then analyzed by fluorescence microscopy. Cyclin C-YFP export analysis was performed on cells 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 (Gucci 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 photo bleaching. Images were obtained using a Nikon microscope (Model E800) with a 100x objective with 1.2x camera magnification (Plan Fluor Oil, NA 1.3) and a CCD camera (Hamamatsu Model C4742). 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 0.3 µM spacing) and deconvolved, and the slices were collapsed to visualize the entire fluorescent signal within the cell. Cyclin C-YFP foci were scored as being cytoplasmic when three or more foci were observed outside 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 transversed 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 Student’s t test, with p < 0.05 used to indicate significant differences.

Pull-down assays and kinase assays

Purified proteins that were used in the pull-down assay (Figure 4B) were made as follows. The GST constructs (DS30, GST hCCNC, and GST alone) were transformed into the BL21 DE3 E. coli strain and expression was induced with 0.5 M IPTG at 37°C for 3 h. Cell pellets were resuspended in 50 ml GST lysis buffer (150 mM KCl, 50 mM HEPES, pH 7.5, 1 mM βME) and sonicated (25 × 10 s bursts), and the supernatant was recovered by centrifugation at 40,000 × g for 30 min. The supernatant was then added to 1 ml of washed glutathione beads (LifeTech) rotated at 4°C for 1 h. The unbound fraction was removed from the beads by gravity flow. The beads were washed twice with lysis buffer and the bound protein was eluted in five 1 ml fractions with the elution buffer (lysis buffer supplemented with 10 mM reduced glutathione). The protein-containing fractions were pooled and then desalted using 4 ml Zeba7K desalting columns that had been preequilibrated with the lysis buffer. The protein fraction was supplemented with glycerol to a final concentration of 10% and stored at −80°C in small aliquots. His-tagged human or yeast cyclin C was purified the same way with the following exceptions. The lysis buffer (buffer A) was 500 mM KCl, 50 mM HEPES, pH 7.5, 1 mM βME, and 20 mM imidazole. Talon beads (Clontech) were used and washed with buffer B (150 mM KCl, 50 mM HEPES, pH 7.5, 1 mM βME, and 20 mM imidazole) and the bound protein was eluted in elution buffer (150 mM KCl, 50 mM HEPES, pH 7.5, 1 mM βME, and 500 mM imidazole). The desalting columns were preequilibrated with the B buffer without imidazole. The concentrations of all the recombinant proteins were determined by Bradford assay. The pull-down assays in Figure 4, B and C, were performed using His₅₉-tagged human or yeast cyclin C, respectively, as bait and GST-tagged Med13',12',24' (DS30) as target. A quantity of 500 mM cyclin C was mixed with 1 µM of Med13',12',24' in a final reaction volume of 250 µl with B buffer and incubated for 1 h at room temperature. Bound protein was precipitated with 100 µl of washed Talon beads and eluted using 500 mM imidazole. The protein mixture was resolved using SDS–PAGE and Western blotting with either anti-GST (Abcam), anti-His (Bethyl), or anti-human cyclin
C antibodies (Bethal). Controls with only the target protein and no bait protein were included in all the experiments. The pull-down assays in Figure 4D were performed using 500 nM of either GST or GST-tagged yeast cyclin C and 1 μM His6-Med13537-606 (DS22). The proteins were incubated together in GST lysis buffer and incubated at RT for 1 h. Then 100 μl of GSH beads (preequilibrated with GST wash/lysis buffer) was added to the protein mixture and incubated with rotation for 1 h. The beads were centrifuged for 30 s washed twice with 500 μl of GST wash/lysis buffer. The protein mixture was resolved using SDS–PAGE and Western blotting with either anti-GST or anti-His antibodies.

The in vitro kinase assays were executed basically as described by DeMille et al. (2015). Slt2-HA and Slt25354–560-HA kinases were immunoprecipitated from cells treated with 5 mM sodium orthovanadate for 60 min, which activates Slt2 (Martin et al., 2000). In short, yeast extracts harboring the respective plasmids were made by resuspending cells in lysis buffer (20 mM HEPES, 10 mM KCl, 1 mM EDTA, 1 mM ethylene glycol tetracetic acid (EGTA), 50 mM NaCl, 10% glycerol, 1 mM β-mercaptoethanol, and complete Protease Inhibitor Cocktail Tablet, pH 7.4, with phosphatase inhibitors for Cdk8–HA purification) and protein immunoprecipitation was conducted as previously described (DeMille et al., 2015), with anti-HA magnetic beads (PierceTM)/anti-myc magnetic beads (Cell Signal-ing). Med13537-606 was purified from an E. coli BL21 DE3 strain induced with 0.5 mM IPTG for 5 h at 37°C using the 6X His-tagged purification method previously described (DeMille et al., 2015). The Slt2 in vitro kinase assay was conducted as previously described (Carmody et al., 2010) by resuspending Slt2 beads in kinase buffer (20 mM HEPES, 10 mM MgCl2, 100 μM Na2VO3, pH 7.5), with 20 μM ATP, 5 μCi of 32P-ATP, and purified Med13537-606. Reactions were incubated at 30°C for 30 min.

ACKNOWLEDGMENTS

We thank C. Wittenburg, S. Fields, P. Herman, D. Levin, and M. Solomon for strains and plasmids. We also thank A. Sardallah and S. Khakhina for help in making Med13 plasmids and the Med12-myc strain, respectively. We thank members of the Cooper, Strich, and Grose laboratories for critical reading of the manuscript. This work was supported by grants from the National Institutes of Health (NIH) awarded to K.F.C. (GM113196), R.S. (GM113052), and J.G. (GM100376) and by the New Jersey Cancer Commission (DHF awarded to K.F.C. (GM113196), R.S. (GM113052), and J.G.

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