

The head module of Mediator directs activation of preloaded RNAPII *in vivo*

Sarah K. Lee*, Xu Chen, Liangqun Huang and Laurie A. Stargell

Department of Biochemistry and Molecular Biology, Colorado State University, CO 80523, USA

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ABSTRACT

The successful synthesis of a transcript by RNA polymerase II (RNAPII) is a multistage process with distinct rate-limiting steps that can vary depending on the particular gene. A growing number of genes in a variety of organisms are regulated at steps after the recruitment of RNAPII. The best-characterized *Saccharomyces cerevisiae* gene regulated in this manner is *CYC1*. This gene has high occupancy of RNAPII under non-inducing conditions, defining it as a poised gene. Here, we find that subunits of the head module of Mediator, Med18 and Med20, and Med19 are required for activation of transcription at the *CYC1* promoter in response to environmental cues. These subunits of Mediator are required at the preloaded promoter for normal levels of recruitment and activity of the general transcription factor TFIID. Strikingly, these Mediator components are dispensable for activation by the same activator at a different gene, which lacks a preloaded polymerase in the promoter region. Based on these results and other studies, we speculate that Mediator plays an essential role in triggering an inactive polymerase at *CYC1* into a productively elongating form.

INTRODUCTION

Control of gene expression via the regulation of events after the recruitment of RNA polymerase II (RNAPII) is now a well-recognized feature of transcriptional regulation (1–4). At genes regulated in this manner, events after the recruitment of polymerase are rate limiting for successful production of transcripts (5–7). Consequently, the promoter regions of these genes contain key members of the transcription machinery (including RNAPII) in the absence of transcript production and exhibit increased expression via the stimulation of inactive complexes. Here, we refer to genes with general transcription

factor and RNAPII promoter occupancy in the absence of transcription as poised, or preloaded (8). Poised promoters are found across the evolutionary spectrum; poising has been observed in bacteria, yeast, worms, flies and humans (5–7,9–13). Despite their prevalence, how poised genes are triggered in response to diverse biological signals remains unclear.

The yeast *CYC1* gene is preloaded and postrecruitment regulated: in the uninduced (non-activated) state, the *CYC1* promoter region contains RNAPII, TATA binding protein, Spt-Ada-Gcn5-acetyltransferase (SAGA) and Spn1 occupancy, as well as detectable Ser5 phosphorylation on the Rpb1 C-terminal domain (CTD) (7–9,14,15). We previously found that the Mediator coactivator complex is required for activating the preloaded *CYC1* gene during growth in non-fermentable carbon sources (8). Mediator is a large multi-subunit coactivator that is conserved from yeast to humans with diverse and complex roles in transcriptional processes (4,16–18). The core complex consists of three modules termed the head, middle and tail (19,20). Mediator integrates the transcription process by linking upstream signals from the activator with the general transcription machinery (8,21–26), participating in postrecruitment (27–33) and termination steps of transcription (34), as well as in silencing and chromatin structure (35–38). Here, we investigate the activation of the preloaded *CYC1* gene during the response to oxidative stress. We find that activation of the poised promoter by oxidative stress occurs via an independent activator from the response to non-fermentable carbon source (8) and displays vastly different activation kinetics. Despite the differences in these responses, both pathways are dependent on similar Mediator subunits to upregulate *CYC1* gene expression. We also show that a specific activator (Yap1) can stimulate expression of postrecruitment and recruitment regulated genes. Importantly, the investigation of these two gene classes, which are coordinately regulated in the response to oxidative stress, revealed that specific Mediator subunits are essential for activation of the poised promoter and dispensable for activation of

*To whom correspondence should be addressed. Tel: +1 325 674 2319; Fax: 1 325 674 6988; Email: sarah.lee@acu.edu
Present address:

Sarah K. Lee, Chemistry and Biochemistry Department, Abilene Christian University, Abilene, TX 79699, USA.

the recruitment regulated promoter. Taken together, our results provide important new insights on regulation of poised promoter gene expression and the functions of Mediator in transitioning genes to an activated state.

MATERIALS AND METHODS

Yeast strains and culturing conditions

Strains used in this study are listed in Table 1. The parent BY4741 (*MATa his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*) strain and deletion strains were purchased from Research Genetics, except the *med2Δ* strain, which was generated using common protocols (8). We generated all tagged strains from the BY4741 parent strain using established protocols (42). Media used for routine culture of yeast is as described (43).

For phenotypic analysis, yeast cultures were grown overnight in rich media. The next morning, cultures were diluted and allowed to undergo two cell doublings to an OD_{600} of 0.7–0.9. Cells were collected and diluted in water to an OD_{600} of 0.1. Serial dilutions (10-fold) were plated on the indicated plates and incubated at 30° for 2–5 days (depending on the media). Yeast extract-peptone-dextrose (YPD) plates with hydrogen peroxide were made by supplementing cooled YPD with hydrogen peroxide (Sigma) to a final concentration between 2.5 and 4.5 mM. Yeast extract-peptone-ethanol-glycerol (YPEG) plates were made by supplementing YP with ethanol (3%) and glycerol (3%).

For oxidative stress induction, cultures were grown overnight in rich media, diluted and allowed to undergo two cell doublings. When cultures reached an OD_{600} of 0.7–0.8, cells were treated with hydrogen peroxide to a final concentration of 0.3 mM. Samples were taken at various time points after induction. For oxidative stress induction of the *MED17* wild-type and temperature sensitive strains, cells were grown at 30°C for the permissive temperature. For the non-permissive temperature, cells were shifted to 37°C for 45 min. At this point, the uninduced sample was removed, and H_2O_2 was added to a final concentration of 0.3 mM. For inducing *CYCI* via

growth in non-fermentable carbon sources, yeast cultures were grown overnight in rich media, then diluted and allowed to undergo two cell doublings in rich media. Cells were washed with YP three times and diluted into YP medium containing 3% ethanol as the sole carbon source and were cultured at 30°C for various times (30 min to 6 h, as indicated). For uninduced samples, cells were grown in YPD media for 6 h at 30°C.

RNA abundance

S1 nuclease assays were conducted as described (44). Yeast cells were harvested, and total RNA was extracted by the hot-phenol extraction method. Thirty micrograms of total RNA was hybridized with excess ^{32}P labeled probe in a 55°C water bath overnight. S1 nuclease digestion was performed on hybridized samples for 30 min at 37°C. Band intensity was normalized to the intensity of the tRNA^w band.

Chromatin immunoprecipitation experiments

Chromatin immunoprecipitation (ChIP) was performed as previously described (9) with few modifications. Cultures were induced as described earlier in the text. At the indicated time, cultures were cross-linked with 1% formaldehyde for 15 min, and then glycine (125 mM) was added to stop cross-linking. Sheared chromatin material (500 μl, corresponding to 25 ml of the initial culture) was incubated with 10 μl of anti-myc (Upstate), anti-HA (Santa Cruz), anti-RNAPII (Cat#: 8WG16, Covance) or anti-CTD Ser5 (Cat#: 39750, Active Motif) antibodies, rotating overnight at 4°C. Protein–DNA cross-links were reversed by incubation at 65°C, and the DNA was purified by phenol-chloroform extraction and used for quantitative PCR analysis. For all quantitative PCR samples, occupancy at the *GAL10* promoter region was subtracted from the occupancy of the promoter of interest from the identical replicate. Values equal to or below background levels after *GAL10* subtraction were set to 0. Primer locations are as follows: *CYCI* promoter (–149 to +36 relative to the ATG), *GTT2* promoter (–230 to +110 relative to the ATG), *GAL10* promoter (–139 to +142 relative to the

Table 1. *Saccharomyces cerevisiae* strains used in this study

Strain	Genotype	Source or reference
BY4741	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	Research genetics
<i>med2Δ</i>	BY4741 <i>med2Δ::URA3</i>	(8)
<i>yap1Δ^a</i>	BY4741 <i>yap1Δ::kanMX</i>	Research genetics
<i>YAP1-myc</i>	BY4741 <i>YAP1-myc₁₃::HIS3</i>	This work
<i>YAP1-myc, med20Δ</i>	BY4741 <i>YAP1-myc₁₃::HIS3, med20Δ::kanMX</i>	This work
<i>MED14-HA</i>	BY4741 <i>MED14-HA₃::HIS3</i>	This work
<i>MED15-myc</i>	BY4741 <i>MED15-myc₁₃::HIS3</i>	(39)
<i>MED15-myc, med20Δ</i>	BY4741 <i>MED15-myc₁₃::HIS3, med20Δ::kanMX</i>	(39)
<i>MED18-myc</i>	BY4741 <i>MED18-myc₁₃::HIS3</i>	(40)
<i>SRB4 wt</i>	<i>srb4Δ::HIS3 {RY2844 SRB4+}</i>	(41)
<i>srb4 ts</i>	<i>srb4Δ::HIS3 {RY2882 srb4-138}</i>	(41)
<i>RAD3-HA</i>	BY4741 <i>RAD3-HA₃::HIS3</i>	This work
<i>RAD3-HA, med20Δ</i>	BY4741 <i>RAD3-HA₃::HIS3, med20Δ::kanMX</i>	This work
<i>KIN28-myc</i>	BY4741 <i>KIN28-myc₁₃::HIS3</i>	This work
<i>KIN28-myc, med20Δ</i>	BY4741 <i>KIN28-myc₁₃::HIS3, med20Δ::kanMX</i>	This work

^aAll other deletion strains were purchased from Research Genetics and have the same markers.

ATG). ChIPs were performed in biological duplicate or triplicate, as indicated in the figure legend, RT-PCR of individual samples was performed in duplicate or triplicate. Unpaired *t*-tests were used to compare the means of two groups, when appropriate (www.graphpad.com). Two-tailed *P*-values are indicated in parentheses in the text.

Immunoblotting

Yeast cells were grown to log phase (OD₆₀₀ of 0.8) in rich media. Cells were harvested, washed and resuspended in 200 μl of lysis buffer [0.5 M phosphate buffer (pH 7.5)]. Whole-cell extracts were prepared by vigorous bead beating. Cellular debris was removed by spinning the extracts at 3000 rpm at 4°C for 15 min. An equal amount of total proteins from whole-cell extract was separated on a 10% SDS-PAGE gel and then transferred to a nitrocellulose membrane. The following antibodies were used at the given dilutions: anti-myc (Upstate, 1:500) and polyclonal anti-TATA binding protein (1:5000), Alexa Fluor goat anti-mouse (LI-COR Biosciences, 1:15000) and goat anti-rabbit (LI-COR Biosciences 1:15000). Proteins were detected with the Odyssey Infrared Imaging System (LI-COR).

RESULTS

Yap1 controls *CYC1* gene expression during oxidative stress

We previously discovered that the *CYC1* gene is poised with a preloaded RNAPII complex before activation, and that this complex is activated by growth in ethanol (8,9,45). Despite the constitutive presence of general transcription factors and RNAPII at the *CYC1* promoter, induction in response to ethanol takes several hours (4–5 h). This time scale seems inconsistent with the idea that poising potentiates genes for rapid activation (12,46–48). Given the connections between poising and speed of the transcriptional response, we were curious whether *CYC1* responds to other environmental changes with faster kinetics than the response to ethanol. Results from whole genome transcriptional profiling suggested that oxidative stress also induces *CYC1* expression (49). Indeed, we confirmed that sub-lethal amounts of hydrogen peroxide (H₂O₂) activate the *CYC1* gene quickly and transcript levels peak within 10 min after the addition of H₂O₂ (Figure 1A). This is in stark contrast to the 4 h required for growth in ethanol to elicit a similar transcriptional response at *CYC1*. After oxidative stress, transcript levels decline, reaching pre-induction levels 50 min after the treatment with H₂O₂. Yap1 is a transcriptional activator protein that is important during oxidative stress in yeast (50). Using a strain deleted for the Yap1 activator (*yap1Δ*), we found that the expression of *CYC1* during oxidative stress is dependent on this factor (Figure 1A). Yap1 is essential for cellular survival during growth in oxidative stress-inducing conditions, as cells lacking the *YAP1* gene fail to grow on plates containing hydrogen peroxide (51) (Figure 1B).

We next determined whether the transcriptional dependence on Yap1 is a direct result of promoter

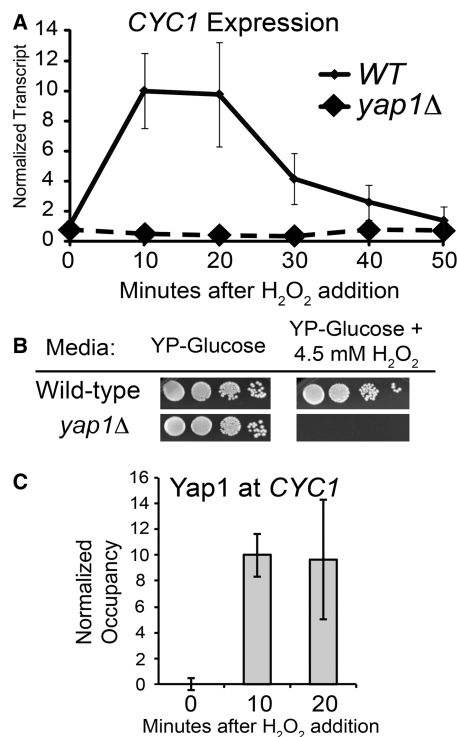


Figure 1. Yap1 directly regulates *CYC1* expression during oxidative stress. (A) *CYC1* transcript levels are rapidly induced in response to oxidative stress. An S1 nuclease protection assay was performed with RNA isolated from wild-type and *yap1Δ* cells before exposure to H₂O₂ and in 10-min intervals after adding H₂O₂ (0.3 mM). A probe for *tRNA^{Phe}* was used as a loading control, and the transcript level in the wild-type strain 10 min after H₂O₂ exposure was set to 10. Results represent the average of at least three biological replicates ±SD. (B) Serial spot dilutions of the wild-type and *yap1Δ* strains on YP-Glucose and on YP-Glucose containing H₂O₂ (4.5 mM). Plates were incubated at 30°C for 3 days before photographing. (C) Yap1-myc occupies the *CYC1* promoter during oxidative stress. Occupancy was determined with a ChIP using a Yap1-myc strain. Formaldehyde was added to cross-link proteins and DNA at the time point indicated on the *x*-axis. Occupancy at the *GAL10* promoter region was subtracted from the occupancy at *CYC1*, and the 10-min time point was set to 10. Bars represent the average ±SD of three biological replicates.

occupancy by this activator. A time course ChIP assay was performed using a strain containing a tagged version of Yap1. We found no significant occupancy of Yap1 at the *CYC1* promoter before induction with H₂O₂, whereas occupancy increases greatly after induction (Figure 1C). This occupancy pattern is consistent with the pattern of *CYC1* transcript production. Therefore, Yap1 is directly involved in the rapid activation of the poised *CYC1* promoter during the response to oxidative stress in yeast.

Yap1 is not required for preloading the *CYC1* promoter or for the response to changing carbon sources

During normal conditions, Yap1 is maintained primarily in the cytoplasm via interaction with the nuclear exporter Crm1 (52). During oxidative stress, this interaction is masked owing to oxidant-induced changes in the structure of Yap1, and Yap1 accumulates in the nucleus (53,54). As

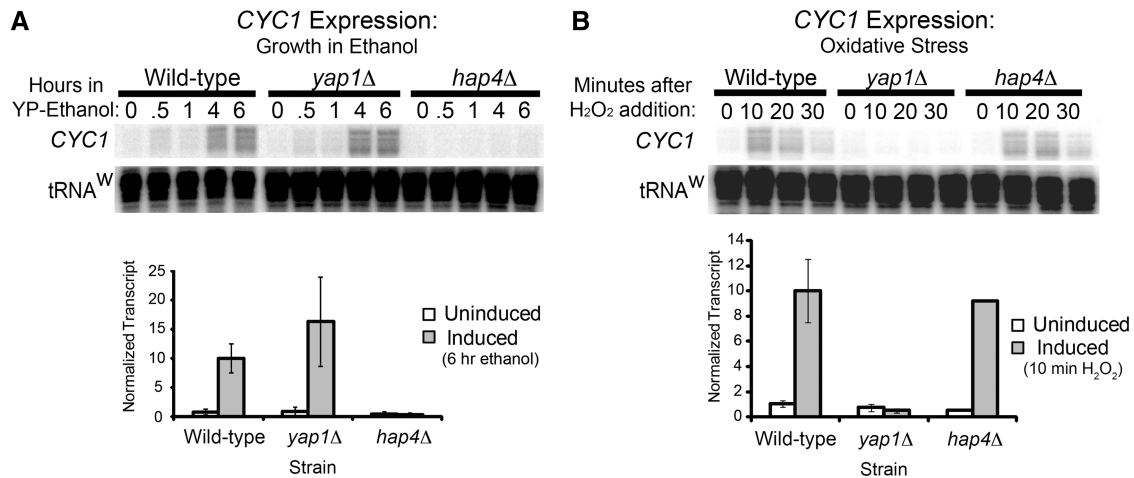


Figure 2. Activator dependence at *CYC1*. (A) *CYC1* activation during growth in non-fermentable carbon sources is Hap4-dependent. An S1 nuclease protection assay was used to analyze RNA prepared from the wild-type, *yap1*Δ and *hap4*Δ strains after cells were transferred to media containing 3% ethanol as the carbon source. A tRNA^W probe was used as a loading control. Representative gel is shown with quantification of transcript levels 6 h after transfer to a non-fermentable carbon source. Levels were analyzed in biological triplicate. (B) *CYC1* activation during oxidative stress is Yap1-dependent. Transcript levels were analyzed as described earlier in the text with RNA collected 10, 20 and 30 min after the addition of H₂O₂ to growth media. A tRNA^W probe was used as a loading control in the S1 assays. Representative gel is shown with quantification of *CYC1* transcript levels in each strain 10 min after the addition of H₂O₂. Levels were analyzed in biological triplicate.

previously shown, the *CYC1* promoter contains preloaded RNAPII (7,8,15). Thus, the RNAPII enzyme occupies the promoter region prior to gene activity. Given that the Yap1 protein shuttles into and out of the nucleus, it is possible that Yap1 molecules that escape the export machinery are responsible for recruiting the inactive RNAPII at the *CYC1* promoter during uninduced conditions. To test this, we analyzed RNAPII occupancy in the wild-type and *yap1*Δ strain under normal conditions and found that RNAPII still occupies this promoter in the *yap1*Δ strain (Supplementary Figure S1). This indicates that Yap1 is not responsible for loading the promoter with RNAPII before induction. To determine whether the response to oxidative stress is independent from the response to changing carbon source (the traditional way to induce *CYC1* expression), we tested the impact of deleting the gene for Yap1 on the activation profile for ethanol activation (Figure 2A). We also tested the converse, in that we assayed whether the response to oxidative stress was impacted in a strain defective for the response to ethanol induction. Carbon-source dependent activation requires the evolutionarily conserved Hap2, Hap3, Hap4 and Hap5 complex of proteins (55–57). The Hap4 protein provides the transcriptional activation function of the complex (58), and expression of the *HAP4* gene is induced during growth in non-fermentable carbon sources (58). Therefore, we tested this deletion strain for the response to oxidative stress (Figure 2B). We found that the Yap1 and Hap4 activators are specific to their respective inducing conditions, and there is no evidence for connectivity between these two pathways upstream of the poised RNAPII complex.

Yap1-dependent Mediator requirements at *CYC1*

Given our finding that two different activators stimulate the preloaded *CYC1* gene, we next wondered whether the

co-activator requirements overlap or are distinct. We previously found that *CYC1* is Mediator-dependent during growth in non-fermentable carbon sources like ethanol (8). Therefore, we explored the role of Mediator at *CYC1* during oxidative stress. We first tested a classical temperature-sensitive strain of *MED17* (*srb4-138*) and found that this strain is defective for *CYC1* expression under the non-permissive conditions in response to oxidative stress (Figure 3A). We next tested for specific Mediator subunit requirements by assaying deletion strains of the non-essential Mediator subunits (Figure 3B). We found that *CYC1* transcript levels during oxidative stress are statistically significantly diminished in strains containing deletions of the *MED18*, *MED19* and *MED20* genes ($P < 0.015$ using a *t*-test). Transcript levels were not statistically significantly changed in the uninduced condition in any of the strains. *MED18* and *MED20* encode proteins that form a heterodimer in the head module of the complex (59). They are the only non-essential proteins within this module. The precise location of the Med19 protein within Mediator is currently unknown, as it is not included in any high-resolution models of the complex (23,60). Although Med19 was previously assigned to the head module (61), more recent studies suggest this subunit is better viewed as a connector between the head and middle modules (23,62–64). The transcriptional defect of *CYC1* is largely specific for these head-related non-essential subunits, as transcript levels are not statistically significantly affected in strains containing deletions of the middle or tail module subunits of Mediator. Thus, activation of the poised complex during oxidative stress requires specific subunits of Mediator, which are similar to the requirements observed for activation during ethanol induction (8).

We also performed a ChIP assay to determine whether Mediator occupies the *CYC1* promoter during oxidative stress. We tested occupancy of three tagged subunits of Mediator, representing the head module (Med18), the

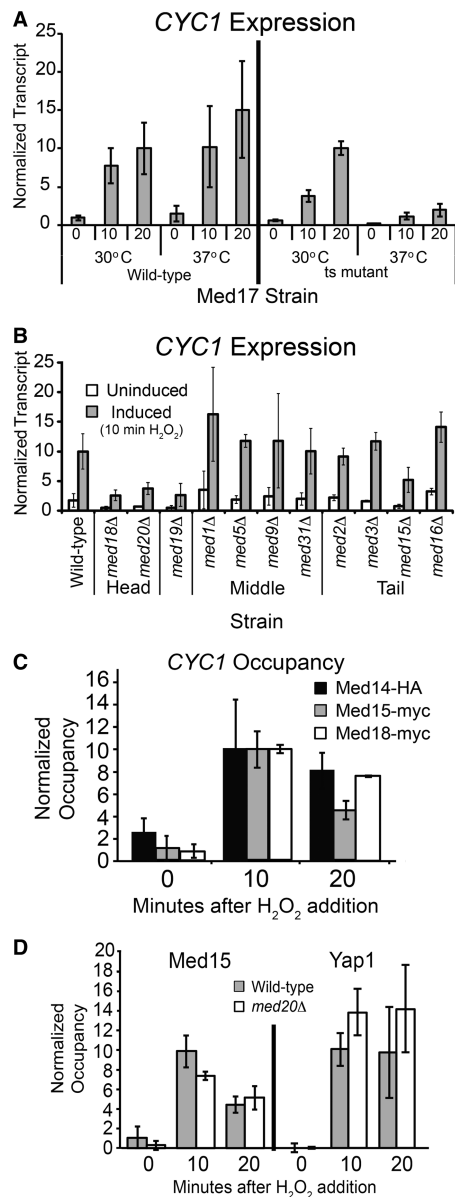


Figure 3. *CYC1* expression is Mediator dependent during oxidative stress. **(A)** *CYC1* expression in strains with wild-type Med17 and a temperature sensitive mutant of Med17 (*srb4-138*). Transcript levels are shown at the permissive (30°C) and non-permissive temperature (37°C) in the uninduced condition, and 10 and 20 min after the addition of H₂O₂, as indicated. *CYC1* transcript levels were normalized to a tRNA^w probe, expression level in the wild-type strain 20 min after activation was set to 10. The average ±SD of three biological replicates is shown. **(B)** Certain non-essential subunits of the Mediator complex are required for *CYC1* activation during oxidative stress. Transcript levels were analyzed with RNA prepared from the wild-type strain and strains containing deletions of the indicated Mediator subunit in both uninduced (YP-Glucose, unfilled bars) and induced (10 min H₂O₂ treatment, gray bars) conditions. *CYC1* transcript level was normalized as described earlier in the text. Expression in the wild-type strain at 10 min after activation was set to 10. The average ±SD of three biological replicates is shown for all strains except the tail module deletions, which is shown in biological duplicate. **(C)** Mediator subunits are recruited to *CYC1* during oxidative stress. Occupancy of Med14-HA, Med15-myc and Med18-myc was determined with a time course ChIP. Occupancy at the *GAL10* promoter region was subtracted from the occupancy at *CYC1*, and the 10-min time point was set to 10. Bars represent the average ±SD of two or three biological replicates. **(D)** Mediator and Yap1 occupancy at the *CYC1* promoter is

middle module (Med14) and the tail module (Med15). We observed that occupancy of each subunit is not detectable before activation and increases significantly on gene induction with oxidative stress (Figure 3C). This demonstrates that Mediator does not occupy the promoter in the uninduced condition but is recruited to *CYC1* during activation.

Since activator proteins interact with Mediator, activator occupancy could be affected by a change in Mediator composition (65). To test this, we performed a ChIP for Yap1 in the *med20Δ* strain. We found that Yap1 occupancy is not compromised on deletion of Med20 but is recruited normally in the *med20Δ* background (Figure 3D and Supplementary Figure S2). Therefore, lack of Med20 does not affect activator occupancy at *CYC1*. We also tested whether Mediator recruitment is changed in the absence of Med20, by examining occupancy of the Med15 subunit in the *med20Δ* strain (Figure 3D and Supplementary Figure S2). There is no statistically significant difference in Med15 occupancy when Med20 is missing; therefore, Yap1 and Mediator are present even when transcription is compromised by deletion of this head module subunit.

Mediator subunits are necessary during oxidative stress and growth on non-fermentable carbon sources

We found that Med18, Med19 and Med20 are critical for controlling transcription of the preloaded *CYC1* gene; yet, these subunits are non-essential for cell viability under normal conditions. To test the physiological importance of these proteins, we analyzed phenotypes of 11 Mediator deletion strains through serial spot analysis on media supplemented with H₂O₂ or on media containing a non-fermentable carbon source (ethanol). We found that strains lacking *MED18*, *MED19* and *MED20* show diminished cell growth on both types of plates compared with growth on glucose (Figure 4). This indicates the head-related proteins are only non-essential during optimal conditions and are important for expression of genes that help the cell survive during growth on both oxidative stress inducing conditions and non-fermentable carbon sources. Thus, *MED18*, *MED19* and *MED20* are essential for induction of the preloaded *CYC1* gene and for cell viability under the activating conditions.

GTT2 is a Yap1-dependent gene induced by oxidative stress but is not poised

We next wanted to test whether other genes induced by H₂O₂ were preloaded with RNAPII and poised for activation. Using genomic transcriptional profiling data (49,66), we selected a group of H₂O₂-responsive genes: *YSR3*, *FAD1*, *AIM13*, *TRX2*, *GLR1* and *GTT2*. We found that

Figure 3. Continued independent of Med20, as occupancy of Med15-myc and Yap1-myc increases on gene activation in both the wild-type (gray bars) and *med20Δ* strain (white bars). Occupancy of Med15-myc and Yap1-myc was determined with a time course ChIP. Occupancy at the *GAL10* promoter region was subtracted from the occupancy at *CYC1*, and the 10-min time point was set to 10. Bars represent the average ±SD of three biological replicates.

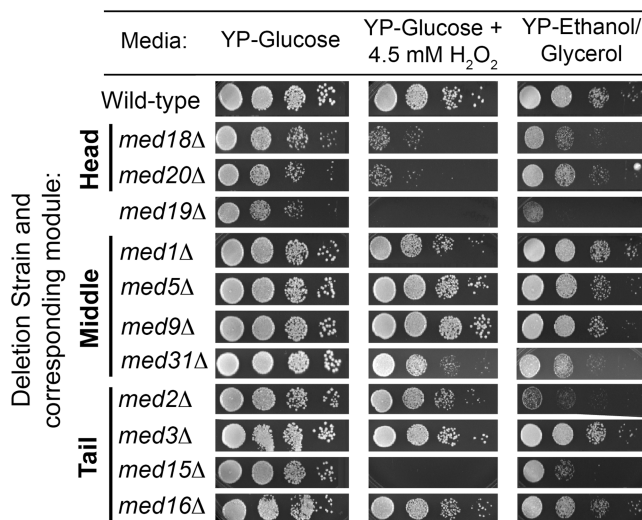


Figure 4. *MED18*, *MED19* and *MED20* are required during oxidative stress and growth on non-fermentable carbon sources. Serial spot dilutions of the wild-type strain and strains containing deletions of 11 Mediator subunits on YP-Glucose, YP-Glucose supplemented with H₂O₂ and YP-Ethanol/Glycerol. Plates were incubated at 30°C for 2–5 days before photographing.

YSR3, *FAD1* and *AIM13* were not H₂O₂-responsive in our strain background, and although *TRX2* and *GLR1* were induced in response to H₂O₂, these genes have high levels of constitutive transcription before induction (data not shown). This basal transcription confounds our ability to clearly distinguish between poised and non-poised promoters; therefore, we did not follow-up with these genes. However, we did find that *GTT2* is H₂O₂-responsive, Yap1-dependent and has low transcript levels in the uninduced condition (Figure 5A). Yap1 binds to the promoter only during activation (Figure 5B), and RNAPII does not occupy this promoter before activation, but is recruited shortly after (Figure 5C). Therefore, unlike *CYCI*, *GTT2* is a bona fide recruitment-regulated gene, as RNAPII is only detectable at the promoter after gene activation.

Mediator requirements at *GTT2*

To determine whether the Mediator-dependency profile of *CYCI* is shared with *GTT2*, we next tested the requirement for this co-activator during activation of *GTT2*. Similar to *CYCI*, we found the temperature-sensitive strain of *MED17* (*srb4-138*) was defective in its ability to activate the *GTT2* gene in response to oxidative stress at the non-permissive temperature (Figure 6A). This indicates that Mediator is required for normal *GTT2* activation. Given the finding that Med18, Med19 and Med20 are required for stimulating the preloaded *CYCI* gene, we next tested the effect of removing these proteins on *GTT2* expression. Although there is a slight reduction in *GTT2* transcript levels in the *med18Δ* and *med20Δ* strains (Figure 6B), the difference from wild-type is not statistically significant, whereas the reduction is significant and severe for *CYCI* expression (Figure 3B). To confirm Mediator occupancy at the *GTT2* promoter, we performed ChIP assays for head, middle and tail subunits before and

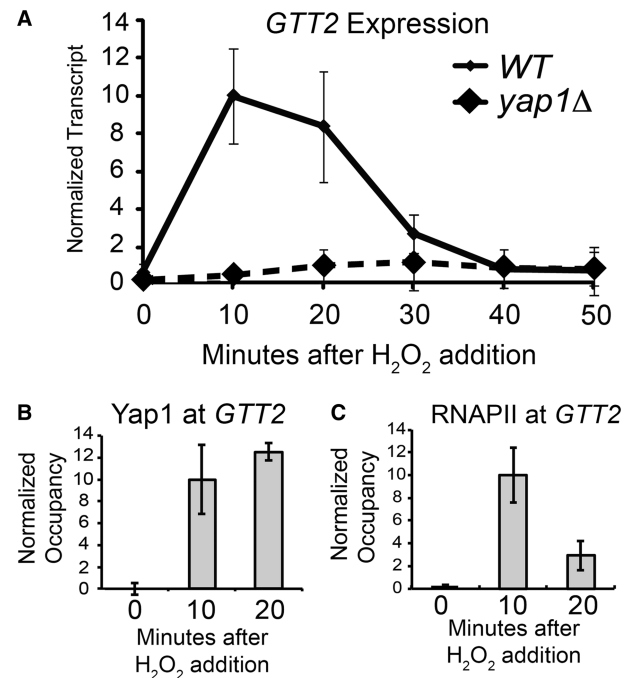


Figure 5. *GTT2* is a Yap1-dependent gene without preloaded RNAPII. (A) *GTT2* transcript levels are rapidly induced in response to oxidative stress. An S1 nuclease protection assay was performed with RNA isolated from wild-type and *yap1Δ* cells before exposure to H₂O₂ and in 10-min intervals after adding H₂O₂ (0.3 mM). A probe for *tRNA^{Phe}* was used as a loading control, and the transcript level in the wild-type strain 10 min after H₂O₂ exposure was set to 10. Points represent the average ±SD of at least three biological replicates for the wild-type, and in biological duplicate for the *yap1Δ* strain. (B) Yap1 directly stimulates *GTT2* transcription, as Yap1-myc occupies this promoter during oxidative stress. Occupancy was determined with a ChIP of a Yap1-myc fusion protein. Occupancy at the *GAL10* promoter region was subtracted from the occupancy at *GTT2*, and the 10-min time point was set to 10. Bars represent the average ±SD of three biological replicates. (C) RNAPII does not occupy the *GTT2* promoter before gene activation but is recruited on activation. Occupancy at the *GAL10* promoter region was subtracted from the occupancy at *GTT2*, and the 10-min time point was set to 10. Bars represent the average ±SD of three biological replicates.

during treatment with H₂O₂. We found Mediator does not occupy this promoter in the uninduced condition, but occupancy of each module increases significantly on activation (Figure 6C). Furthermore, absence of Med20 does not disrupt recruitment of Med15 or Yap1 (Figure 6D). Taken together, these results indicate that Mediator is required for *GTT2* activation, but the Med18, Med19 and Med20 subunits are dispensable.

Mediator promotes postrecruitment events at *CYCI*

Mediator is well-characterized for its functional role in recruiting RNAPII to promoters upon activation (25,67). However, this function is unnecessary for *CYCI* expression, as RNAPII is present at the poised promoter prior to gene activation (7–9,14,15), and this occupancy does not depend on Med20, as it is maintained in the *med20Δ* strain (Figure 7A). While this promoter is classified as poised (i.e. possesses RNAPII occupancy before induction), we do observe an ~3-fold increase in

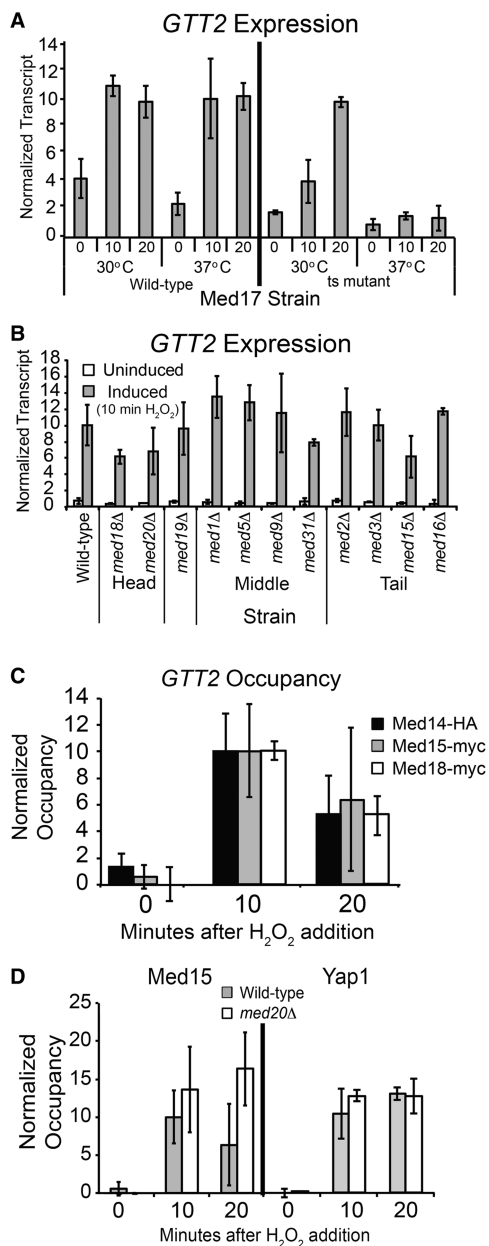


Figure 6. *GTT2* activation is Mediator dependent, but does not rely on the Med18, Med20 and Med20 subunits for expression. (A) *GTT2* expression in strains with wild-type Med17 and a temperature sensitive mutant of Med17 (*srb4-138*). Transcript levels are shown at the permissive (30°C) and non-permissive temperature (37°C) in the uninduced condition, and 10 and 20 min after the addition of H₂O₂, as indicated. Transcript levels were analyzed as described in Figure 3. The average \pm SD of three biological replicates is shown. (B) *GTT2* activation is largely independent of the Med18, Med19 and Med20 proteins. RNA levels from indicated strains were analyzed as described in Figure 3. The average \pm SD of three biological replicates is shown for all strains except the tail module deletions, which is shown in biological duplicate. (C) Mediator subunits are recruited to *GTT2* during oxidative stress. Occupancy of Med14-HA, Med15-myc and Med18-myc were determined with a time course ChIP and analyzed as described in Figure 3. (D). Mediator and Yap1 occupancy at the *GTT2* promoter is independent of Med20, as occupancy of Med15-myc and Yap1-myc increases on gene activation in both the wild-type (gray bars) and *med20Δ* strain (white bars). Occupancy of Med15-myc and Yap1-myc was determined with a time course ChIP and analyzed as described in Figure 3.

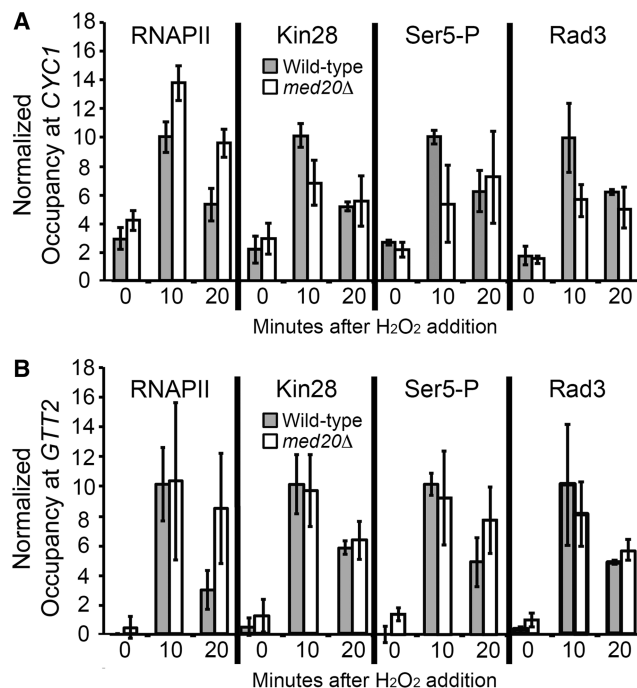


Figure 7. Med20 is required for normal TFIIH recruitment and Ser5 phosphorylation at the promoter of *CYC1* but not at *GTT2*. (A) Occupancy of RNAPII, Kin28-myc, Ser5-P and Rad3-HA in the wild-type (gray bars) and *med20Δ* (white bars) strains was determined at the *CYC1* promoter region with a time course ChIP. Occupancy at the *GAL10* promoter region was subtracted from the occupancy at *CYC1*, and the 10-min time point in the wild-type strain was set to 10. Bars represent the average \pm SD of three biological replicates. (B) Occupancy of RNAPII, Kin28-myc, Ser5-P and Rad3-HA in the wild-type (gray bars) and *med20Δ* (white bars) strains was determined at the *GTT2* promoter as described in (A).

RNAPII occupancy after induction with H₂O₂ (Figure 7A). This is not observed when *CYC1* is induced by ethanol (8), which may be due to the much more rapid induction observed during oxidative stress. This increase in occupancy is still observed in the *med20Δ* strain, although it is not correlated with a corresponding increase in productive transcripts (to the contrary; Figure 3B) and may depend on other subunits of Mediator or other unknown components. Like *CYC1*, the *Drosophila* Hsp70 gene, which is the best-characterized postrecruitment regulated gene, also shows an increase in RNAPII occupancy during induction (68).

Mediator is also known to stimulate TFIIH-dependent phosphorylation of the CTD (25,69). Kin28, the cyclin-dependent kinase activity of TFIIH, phosphorylates the CTD of RNAPII (41,70–72). Therefore, we tested whether Kin28 occupancy at the *CYC1* and *GTT2* promoters is dependent on the Med20 subunit of the Mediator complex. We tagged Kin28 in a wild-type and *med20Δ* strain (Supplementary Figure S2) and performed a ChIP assay. In wild-type cells, we found that Kin28 is preloaded at the *CYC1* promoter, but the occupancy increases upon activation by oxidative stress (Figure 7A). At the *GTT2* promoter Kin28 is recruited only during activation (Figure 7B). H₂O₂-induced Kin28 recruitment is significantly diminished at the *CYC1*

promoter region upon deletion of the Med20 protein ($P < 0.05$ at the 10-min time point). In contrast, recruitment of Kin28 is unaffected at the *GTT2* promoter. Consistent with the kinase occupancy observation, we also observe statistically significantly diminished Ser5 phosphorylation of the CTD of RNAPII at *CYC1* upon induction in the *med20Δ* strain ($P < 0.05$ at the 10-min time point) but found no impact at the *GTT2* gene (Figure 7A and B). Mediator also interacts with one of the helicase subunits of TFIIF, Rad3 (25). Recent structural studies of TFIIF and new models of the preinitiation complex reveal Rad3, along with the kinase submodule, is positioned near the proposed location of the Mediator head (64,73,74). Therefore, we also tagged Rad3 (Supplementary Figure S2) and tested Rad3 occupancy in the presence and absence of Med20. We found that H₂O₂-induced Rad3 recruitment is statistically significantly diminished at *CYC1* in the absence of Med20 ($P < 0.05$ at the 10-min time point), but the deletion does not affect recruitment at *GTT2* (Figure 7A and B). These findings reveal that the head module of Mediator, specifically the Med20 protein, is required for normal levels and activity of the Kin28 kinase subunit of TFIIF, and for normal recruitment of the Rad3 helicase at the preloaded *CYC1* gene. Although Med20 is required for wild-type levels and activity of TFIIF, there is still a considerable increase in recruitment of these factors in the *med20Δ* strain (at the 20-min time point, the defect seen at 10 min is abolished). At the *GTT2* gene, which does not contain a preloaded polymerase, Kin28 recruitment and activity and Rad3 recruitment are completely independent of the Med20 subunit (Figure 7B).

DISCUSSION

Here, we investigated the response of the preloaded *CYC1* gene to different biological stimuli. We find that this preloaded promoter responds to two environmental conditions via two different activators: the Hap complex and Yap1. The Hap complex controls *CYC1* transcription during growth in non-fermentable carbon sources such as ethanol. Yap1 controls *CYC1* transcription during oxidative stress. Both activators stimulate this gene via Mediator. Interestingly, our investigation of *CYC1* expression under both conditions reveals that three non-essential subunits (Med18, Med19 and Med20) are required for gene activation. Therefore, two different activator proteins stimulate the poised *CYC1* promoter via Mediator, and the same subunits of this large complex are required for each activation scheme.

Our study of the regulation of *GTT2* transcription revealed differences at this promoter compared with *CYC1*. In the uninduced condition, the *GTT2* promoter does not contain polymerase, whereas polymerase is present at *CYC1* (Figure 7). On induction with H₂O₂, Yap1 is rapidly recruited to both genes (Figures 1 and 5), followed by activation of gene expression of both *CYC1* and *GTT2*. These findings highlight the versatility of activator proteins, as Yap1 can operate at two fundamentally different promoter settings *in vivo* (one preloaded and one lacking polymerase before stimulation). Further,

Med18, Med19 and Med20 appear to play a more important role at *CYC1* than at *GTT2*. These proteins are not generally required for transcriptional processes, as these subunits are non-essential under rich growth conditions. In addition, genome-wide studies show the expression of only a small set of genes is impacted by the deletion of *MED18* or *MED20* when yeast are grown under standard conditions (59). However, we demonstrate that these proteins are critical for cell growth in ethanol or during oxidative stress (Figure 4). These conditions induce the *CYC1* gene, indicating they form an accessory domain that is essential in certain contexts. A study of the genes induced by the Met4 activator demonstrated that Med18 and Med20 are not required for activation of recruitment-regulated Met4 target genes (75). Our study of *CYC1* and *GTT2* transcription also suggests that this accessory domain is more important at poised genes than recruitment-regulated genes.

At *CYC1*, we found that Kin28 occupancy is diminished 10 min after activation when Med20 is deleted. Although the defect was slight (albeit statistically significant), we observed a similar pattern for two uniquely tagged subunits of TFIIF (Kin28-myc and Rad3-HA), and for the phospho-Ser5 mark on the CTD of RNAPII, providing support for the trend. The diminished occupancy and activity we observed could result from a loss of direct interactions between Mediator and TFIIF, as TFIIF interacts with several subunits of the Mediator complex, including the head region (24,25,76). Further, genetic interaction with the CTD was used in the original SRB screen that discovered many Mediator components, including head module subunits (77). Although we observed a statistically significant decrease in Kin28, Ser5-phosphorylation and Rad3 occupancy at *CYC1* in the *med20Δ* strain, there is still some Kin28 and Rad3 present and active in the absence of this Mediator subunit (Figure 7A). Therefore, TFIIF does not exclusively rely on Med20 for normal occupancy at *CYC1*. Furthermore, it seems unlikely that failure to fully recruit or activate Kin28 and Rad3 is the *only* defect in the *med20Δ* strain, given the significant decrease in transcript level observed in the absence of this protein.

What other function could these Mediator subunits play at the preloaded *CYC1* promoter? We propose two (not mutually exclusive) possibilities. First, it is possible that these subunits are required for recruitment or stability of an additional factor(s) at the *CYC1* promoter. As we found that *MED18*, *MED19* and *MED20* are not required for *GTT2* transcription, these subunits do not perform this proposed function at *GTT2*. A second possibility is that these Mediator subunits are critical for altering the conformation of the preloaded RNAPII at *CYC1* into an actively transcribing state. Activation of stalled human RNAPII is correlated with conformational changes induced by activator contact with the essential Med17 (Srb4) subunit in the Head (21). Biophysical evidence supports the idea that structural shifts occur in both Mediator and RNAPII on interaction in human and yeast systems (23,64,79–82). Recent structural studies of Mediator reveal that Med18 and Med20 are in a prime location to affect conformational changes in RNAPII.

This heterodimer is at the distal end of a movable jaw in the head module (23,63). The Rpb4/7 heterodimer of RNAPII is between the fixed and movable jaw of the head module (63). Rpb4/7 can dissociate from the polymerase and appears to control the conformation of the clamp domain of the enzyme (64,83). Given our findings at *CYC1* and *GTT2*, together with the biophysical evidence, we favor the second possibility. Here, Mediator, containing Med18, Med19 and Med20, is required for affecting a conformational change in RNAPII at the poised promoter, shifting it to a more active conformation that is compatible with full Kin28 and Rad3 binding and activity (Supplementary Figure S3). In this model, the impact we observed on TFIID at *CYC1* is a marker for the essential Med20-dependent conformational change that occurs in the poised complex to transition into a TFIID-stably associated and actively transcribing state.

Identification and characterization of additional poised genes will allow further study into the specific role of Mediator at genes with preloaded RNAPII. With these tools, we can test our model more rigorously. Additionally, biophysical studies can probe the Mediator-polymerase interaction more fully to determine the role of the accessory domain. Furthermore, we found that a single gene (*CYC1*) requires the same Mediator subunits for activation in response to growth in non-fermentable carbon sources or oxidative stress. Activation in response to these stimuli occurs via two distinct activators: the Hap complex and Yap1. As poising, Mediator and TFIID are conserved features of transcriptional regulation, these findings provide important new insight on the regulation of gene expression at preloaded promoters.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online, including [64,73,74].

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