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Rrd1p, an RNA polymerase II-specific prolyl isomerase and activator of phosphoprotein phosphatase, promotes transcription independently of rapamycin response

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ABSTRACT

Rrd1p (resistance to rapamycin deletion 1) has been previously implicated in controlling transcription of rapamycin-regulated genes in response to rapamycin treatment. Intriguingly, we show here that Rrd1p associates with the coding sequence of a galactose-inducible and rapamycin non-responsive GAL1 gene, and promotes the association of RNA polymerase II with GAL1 in the absence of rapamycin treatment following transcriptional induction. Consistently, nucleosomal disassembly at GAL1 is impaired in the absence of Rrd1p, and GAL1 transcription is reduced in the Δrrd1 strain. Likewise, Rrd1p associates with the coding sequences of other rapamycin non-responsive and inducible GAL genes to promote their transcription in the absence of rapamycin treatment. Similarly, inducible, but rapamycin-responsive, non-GAL genes such as CTT1, STL1 and CUP1 are also regulated by Rrd1p. However, transcription of these inducible GAL and non-GAL genes is not altered in the absence of Rrd1p when the steady-state is reached after long transcriptional induction. Consistently, transcription of the constitutively active genes is not changed in the Δrrd1 strain. Taken together, our results demonstrate a new function of Rrd1p in stimulation of initial rounds of transcription, but not steady-state/constitutive transcription, of both rapamycin-responsive and non-responsive genes independently of rapamycin treatment.

INTRODUCTION

Rrd1p (resistance to rapamycin deletion 1) is an evolutionarily conserved protein. It shares 35% identity with human PTPA (phosphotyrosyl phosphatase activator) that is an activator of phosphotyrosyl activity of PP2A phosphatase (1–3). Like PTPA, Rrd1p is required for activation of inactive PP2A (4,5). Further, both PTPA and Rrd1p have peptidyl prolyl isomerase activity on a specific PP2A peptide (1). In yeast, Rrd1p interacts with PP2A-like phosphatase, Sit4p, and forms a ternary complex with Tap42p, a mediator of the TOR (target of rapamycin) signaling pathway (6). When TOR signaling pathway is inhibited by rapamycin or nutrient starvation, Tap42p dissociates from the Sit4p–Rrd1p–Tap42p ternary complex to dephosphorylate and translocate Gln3p to the nucleus for triggering transcription of the target genes (7,8). However, the Gln3p target gene MEP2 can also be activated independently of Rrd1p (8). These results suggest that Rrd1p has a role in transcription in response to rapamycin (8). Indeed, Douville et al. (8) have demonstrated the function of Rrd1p in modulation of transcription in the presence of rapamycin treatment.

In addition to interacting with Sit4p, Rrd1p also associates with CTD (carboxy-terminal domain) of RNA polymerase II (9). Through this interaction, Rrd1p associates with chromatin (9,10). Further, Rrd1p has peptidyl prolyl isomerase activity on the CTD of RNA polymerase II (9), similar to human peptidyl prolyl isomerase, Pin1, and its yeast homologue, Ess1p. Both Pin1 and Ess1p regulate transcription (11–15). Like Pin1 and Ess1p, Rrd1p controls transcription of the genes that are regulated by rapamycin or TOR pathway (8,10). Especially, Rrd1p has been implicated in promoting transcriptional elongation of rapamycin-responsive genes following rapamycin treat-

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ment (10). However, it remained unknown whether Rrd1p can also promote transcription independently of rapamycin treatment. To address this, we analyzed the role of Rrd1p in promoting transcription of several inducible genes such as GAL1, GAL7, GAL10, STL1, CTT1 and CUP1 (both rapamycin-responsive and non-responsive genes) following transcriptional induction. Our results reveal that Rrd1p promotes transcription of both rapamycin-responsive and non-responsive genes independently of rapamycin treatment. However, when the steady-state is reached after long transcriptional induction, altered transcription of these genes is not observed in the absence of Rrd1p. Consistently, transcription of the constitutively active genes is not changed in the Δrrd1 strain in comparison to the wild-type equivalent. Thus, our results demonstrate that Rrd1p promotes initial rounds of transcription of both rapamycin-responsive and non-responsive genes in the absence of rapamycin treatment (or TOR pathway), hence providing a new function of Rrd1p in regulation of gene expression as presented below.

**MATERIALS AND METHODS**

**Plasmids**

The plasmid, pFA6a-13Myc-KanMX6 (16), was used for genomic tagging of Rad3p and Rrd1p by Myc epitope. The plasmid, pRS403 (17), was used in the polymerase chain reaction (PCR)-based disruption of RRD1.

**Strains**

The endogenous **RRD1** gene of W303a was disrupted using the PCR-based gene knock-out method to generate SMY16 (Δrrd1::HIS3). Likewise, the RSY50 strain (Δrrd1::HIS3) was generated by knocking out RRD1 in the YKH045 strain that expresses Flag-tagged histone H2B. YKH045 was obtained from the Osley laboratory (Mary Ann Osley, University of New Mexico Health Science Center; (18)). Multiple Myc epitope tags were added at the original chromosomal locus of **RAD3** in W303a and SMY16 to generate ASY41 (Rad3p-Myc) and SMY18 (Δrrd1::HIS3, Rad3p-Myc), respectively. Similarly, multiple Myc epitope tags were added at the C-terminals of Rpb1p and Rrd1p in W303a to generate ZDY4 (Rpb1p-Myc) and SMY19 (Rrd1p-Myc), respectively (19). The endogenous **RRD1** gene in ZDY4 was knocked out to generate SFY1 (Δrrd1::HIS3, Rpb1p-Myc).

**Growth media**

For studies at **GAL1**, **GAL7** and **GAL10**, yeast cells were grown in YPR (yeast extract, peptone plus 2% raffinose) up to an OD600 of 0.9 at 30°C, and then switched to YPG (yeast extract, peptone plus 2% galactose) for different time periods (e.g. 20, 40, 60 and 90 min). Likewise, these genes were also induced for 2, 4 and 6 h in YPG. For continuous induction of **GAL1**, **GAL7** and **GAL10**, yeast cells were grown in YPG up to an OD600 of 1.0 at 30°C. Yeast strains were grown in YPD (yeast extract peptone plus 2% dextrose) up to an OD600 of 1.0 at 30°C for the studies at the constitutively active genes (e.g. **ADH1** and **RPS5**). For studies at the **CTT1** and **STL1** genes, yeast cells were initially grown in synthetic complete medium (yeast nitrogen base and complete amino acid mixture plus 2% dextrose) up to an OD600 of 0.9, and then were induced by 0.45 M NaCl for 7 min before crosslinking or harvesting for mRNA analysis. Similarly, these genes were also induced for 30 min in the presence of 0.45 M NaCl. The **CUP1** gene was induced by 1 mM CuSO4 for 15 min in synthetic complete medium at 30°C. For long induction of **CUP1**, yeast cells were treated with 1 mM CuSO4 for 1 h.

**Chromatin immunoprecipitation assay**

The chromatin immunoprecipitation (ChiP) assay for TATA-box binding protein (TBP), Rpb1p-Myc, Rad3p-Myc and Flag-tagged histone H2B was performed as described previously (20–30). For ChiP analysis of Myc-tagged Rrd1p, the ChiP protocol was modified as described previously (22,24,28,29). Briefly, a total of 800 µl lysate was prepared from 100 ml of yeast culture. Following sonication, 400 µl lysate was used for each immunoprecipitation (using 10 µl of anti-Myc antibody and 100 µl of protein A/G plusagarose beads from Santa Cruz Biotechnology, Inc.), and immunoprecipitated-DNA sample was dissolved in 10 µl TE 8.0 of which 1 µl was used for PCR analysis (a total of 23 cycles). In parallel, PCR analysis for input DNA was performed using 1 µl DNA that was prepared by dissolving purified DNA from 5 µl lysate in 100 µl TE 8.0. The primer pairs used for PCR analysis were as follows:

<table>
<thead>
<tr>
<th>Primer Pair</th>
<th>5′-Sequence-3′</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GAL1</strong> (UAS)</td>
<td>5′-GGTTGCTACCTGGCTGTGCCATATG-3′</td>
</tr>
<tr>
<td><strong>GAL1</strong> (Core)</td>
<td>5′-GGGTGACGTCCTGCTGCGTCATTTT-3′</td>
</tr>
<tr>
<td><strong>GAL1</strong> (ORF)</td>
<td>5′-CTTTGATAGATGCTATTTTGGTGCC-3′</td>
</tr>
<tr>
<td><strong>GAL1</strong> (ORF1)</td>
<td>5′-TTTCACTTGTGAAAGCAGTATATG-3′</td>
</tr>
<tr>
<td><strong>CTT1</strong> (Core)</td>
<td>5′-TCGAATGGAATGCGAATAATGGA-3′</td>
</tr>
<tr>
<td><strong>CTT1</strong> (ORF)</td>
<td>5′-TGCTGCACTCGCTGGTCCC-3′</td>
</tr>
<tr>
<td><strong>SLT1</strong> (Core)</td>
<td>5′-ATGGATGGTTTTATGGGTTTTG-3′</td>
</tr>
<tr>
<td><strong>SLT1</strong> (ORF)</td>
<td>5′-GGAATAGAGGTAAAGCAACGACTTC-3′</td>
</tr>
<tr>
<td><strong>CUP1</strong> (Core)</td>
<td>5′-GGCTGTACAAATTTTGGTATCTG-3′</td>
</tr>
<tr>
<td><strong>CUP1</strong> (ORF)</td>
<td>5′-CTTTGGGTGCTCTCTCTT-3′</td>
</tr>
<tr>
<td><strong>ADH1</strong> (ORF)</td>
<td>5′-CTTTGATAGATGCTATTTTGGTGCC-3′</td>
</tr>
</tbody>
</table>

** Autoradiograms were scanned and quantitated by the National Institutes of Health image 1.62 program. Immunoprecipitated-DNA was quantitated as the ratio of immunoprecipitate to input, and represented as a ChiP signal. The average ChiP signal of the biologically independent experiments is reported with standard deviation (SD; Microsoft Excel 2003). The Student’s t test of Microsoft Excel 2003 (with tail = 2 and types = 3) was used to determine the P values for statistical significance of the change in the ChiP signals. The changes were considered to be statistically significant at P < 0.05. ORF, open reading frame;
ORF1, ORF region toward the 3′-end; and Core, core promoter.

**Total RNA preparation**

The total RNA was prepared from yeast cell culture as described previously (26,29,31,32). Briefly, 10 ml yeast culture was harvested, and suspended in 100 µl RNA preparation buffer (500 mM NaCl, 200 mM Tris–HCl, 100 mM Na₂EDTA and 1% SDS) along with 100 µl volume equivalent of glass beads (acid washed; Sigma). Subsequently, yeast cell suspension was vortexed with a maximum speed (10 in a VWR mini-vortexer; cat. no. 58816-121) five times (30 s each). After vortexing, 150 µl RNA preparation buffer and 150 µl phenol/chloroform/isoamyl alcohol and 100 µl volume equivalent of glass beads (acid washed; Sigma). Subsequently, yeast cell suspension was vortexed with a maximum speed (10 in a VWR mini-vortexer; cat. no. 58816-121) five times (30 s each). After vortexing, 150 µl RNA preparation buffer and 150 µl phenol/chloroform/isoamyl alcohol were added to the yeast cell suspension followed by vortexing for 15 s with a maximum speed on a VWR mini-vortexer. The aqueous phase was collected for isolation of total RNA by precipitation with ethanol.

**Reverse transcription PCR analysis**

Reverse transcription PCR (RT-PCR) analysis was performed as described previously (29,33,34). Briefly, RNA was treated with RNase-free DNase (M610A, Promega), and then reverse-transcribed into cDNA using oligo(dT) as described in the protocol supplied by Promega (A3800, Promega). PCR was performed using synthesized first strand as template and the primer pairs targeted to GAL7, GAL10, ADHI, ACTI, CUP1, CTTI, STL1 and RPS5 ORFs. RT-PCR products were separated by 2.2% agarose gel electrophoresis and visualized by ethidium bromide staining. The average signal of the biologically independent RT-PCR experiments is reported with SD (Microsoft Excel 2003). The Student’s t test (with tail = 2 and types = 3) was used to determine P-values for statistical significance of the change in the RT-PCR signals. The changes were considered to be statistically significant at P < 0.05. The primer pairs used in the PCR analysis of cDNAs were as follows:

**GAL1**: 5′-CAGAGGGCTAAGCATGTATCTT-3′
5′-CTCAACACTCGGAACGAACATTT-3′

**GAL7**: 5′-TTGGAAGCTTGGCTTATCCAAAGAG-3′
5′-ATGGAGCGACGCCCTATTTTCTAA-3′

**GAL10**: 5′-TTAATGCGAATCATAGTAGTATCGG-3′
5′-ATGGATACCCATTGAGTATGGAAA-3′

**ADHI**: 5′-CTTTTACGTACCAACAGTAGCTACG-3′
5′-AGGCTCAATGTCCAATCATTGAAAG-3′

**CUP1**: 5′-TTCCACAAATGAGGGTGCTAGTG-3′
5′-AGCAGCATGACTTCTTGGTTTCTC-3′

**CTTI**: 5′-TGGCTATCTTCTGGCTTATCTT-3′
5′-AGCAGCATGACTTCTTGGTTTCTC-3′

**STL1**: 5′-ACATCTGAGGGGATCAAAAT-3′
5′-ATATTGAGTACCTGGACACTGAT-3′

**ACTI**: 5′-ACGTATCTACCAACGATTTGACCC-3′
5′-TGAGACCTTGGTCATTTCAAAGAAG-3′

Whole cell extract preparation and western blot analysis

For analysis of global levels of TBP, Rpb1p, Rad3p and actin in the RRD1 deletion mutant and its isogenic wild-type equivalent, yeast cells were grown in YPR up to an OD₆₀₀ of 0.2 at 30°C, and then switched to galactose-containing medium (non-inducing) up to an OD₆₀₀ of 0.9, and then switched to galactose-containing growth medium for 90 min prior to formaldehyde-based in vivo crosslinking. Using crosslinked cells, we performed the harvested cells were lysed and sonicated to prepare the whole cell extract with solubilized chromatin following the protocol as described previously for the ChIP assay (20-24). The whole cell extract was run on SDS-polyacrylamide gel, and then analyzed by western blot. The anti-TBP (obtained from Michael R. Green, University of Massachusetts Medical School), anti-Myc (9E10; Santa Cruz Biotechnology) and anti-actin (A2066; Sigma) antibodies against TBP, Myc-tagged Rpb1p, Myc-tagged Rad3p and actin were used in the western blot analysis.

**Growth analysis in solid and liquid media**

The growth of the Δrrd1 and wild-type cells was analyzed on plates containing solid YPG and YPD. Yeast cells were inoculated in YPR, and grown up to an OD₆₀₀ of 1.0 at 30°C without dilution. Both the wild-type and mutant strains grew similarly in YPR medium. Yeast cells were then spotted on solid growth media following serial dilutions. Yeast cells were grown at 30°C, and photographed after 2 or 3 days. For analysis of growth in liquid YPD medium, both the wild-type and Δrrd1 cells were inoculated in YPR, and grown up to an OD₆₀₀ of 0.2 at 30°C without dilution. Subsequently, yeast cells were switched to YPD at 30°C, and OD₆₀₀ was measured at different times. For analysis of growth in liquid medium following 90 min induction of GAL genes, both the wild-type and Δrrd1 cells were inoculated in YPR, and grown up to an OD₆₀₀ of 0.9 at 30°C without dilution. Subsequently, yeast cells were switched to YPG at 30°C for 90 min, and OD₆₀₀ was measured at different times within 90 min.

**RESULTS**

Rrd1p associates with the coding sequence of a rapamycin non-responsive GAL1 gene, and promotes the association of RNA polymerase II with GAL1 (and hence transcription) independently of rapamycin treatment

Since Rrd1p interacts with RNA polymerase II (9), and is involved in regulation of proline isomerization (9) and phosphorylation of CTD of RNA polymerase II (10), it may regulate the association of RNA polymerase II with the coding sequence of active gene, and hence transcriptional elongation. However, previous studies (10) have demonstrated the dispensability of Rrd1p in regulation of transcription of rapamycin-responsive as well as non-responsive genes in the absence of rapamycin treatment under vegetative growth conditions. It is quite possible that Rrd1p promotes initial rounds/cycles of transcription via its RNA polymerase II-specific prolyl isomerase activity independently of rapamycin response, and the function of Rrd1p in transcription becomes minimal (or absent) when the steady-state is reached. To test this possibility, we analyzed the association of RNA polymerase II with the coding sequence of a galactose-inducible GAL1 gene following transcriptional induction in the absence of rapamycin treatment. In this direction, we grew both the wild-type and Δrrd1 strains in raffinose-containing growth medium (non-inducing) up to an OD₆₀₀ of 0.9, and then switched to galactose-containing growth medium for 90 min prior to formaldehyde-based in vivo crosslinking. Using crosslinked cells, we performed the
ChIP experiments to analyze the level of RNA polymerase II (Rpb1p) at the GAL1 coding sequence. We found that the association of RNA polymerase II with the GAL1 coding sequence was dramatically impaired in the Δrrd1 strain in the absence of rapamycin treatment (Figure 1A and B). Such reduction in the association of RNA polymerase II with the GAL1 coding sequence could be due to decreased stability of Rpb1p in the Δrrd1 strain. To test this possibility, we analyzed the global levels of Rpb1p in the wild-type and Δrrd1 strains, using the western blot assay. We found that the global level of Rpb1p was not changed in the Δrrd1 strain as compared to the wild-type equivalent (Figure 1C). The level of actin was monitored as a loading control, and its level was not altered in the Δrrd1 strain (Figure 1C). Thus, reduced association of RNA polymerase II with the GAL1 coding sequence following 90 min transcriptional induction in the Δrrd1 strain was not due to an impaired stability of Rpb1p. However, it is quite possible that the Δrrd1 strain grew slowly in galactose-containing growth medium during 90 min transcriptional induction, leading to a less number of the Δrrd1 cells and consequently decreased level of RNA polymerase II at GAL1. To test this possibility, we measured OD600 of the wild-type and Δrrd1 strains under the growth conditions used in the above ChIP experiments. We found that OD600 of the Δrrd1 strain was less than that of the wild-type strain at 90 min following the switch of the growth medium containing raffinose to galactose (Figure 1D). Thus, the relatively smaller number of the Δrrd1 cells following 90 min transcriptional induction in the ChIP experiments might have led to decreased amount of immunoprecipitated-DNA in comparison to the wild-type equivalent. However, we rule out this possibility as we normalized the immunoprecipitated-DNA signal with respect to its input DNA (referred to as ChIP signal), and then compared the ChIP signal of the Δrrd1 strain with that of the wild-type equivalent. Thus, our results support that Rrd1p promotes the association of RNA polymerase II with the GAL1 coding sequence. Further, we found that transcription of GAL1 was not regulated by rapamycin (Figure 1E), consistent with previous studies (10). Transcription of ACT1 was analyzed as a control (Figure 1E), since ACT1 is not responsive to rapamycin (8,10). Taken together, our results (Figure 1B–E) demonstrate that Rrd1p promotes the association of RNA polymerase II with the coding sequence of a rapamycin non-responsive GAL1 gene following 90 min transcriptional induction in the absence of rapamycin treatment. Consistently, we observed predominant association of Rrd1p with the coding sequence of GAL1 (Figure 1A and F, Supplementary Figure S1).

Since Rrd1p associates with the coding sequence of GAL1, and promotes the association of RNA polymerase II, transcription of GAL1 would be impaired in the Δrrd1 strain. To test this, we analyzed GAL1 mRNA levels in the wild-type and Δrrd1 strains following transcriptional induction in galactose-containing growth medium. We isolated total RNAs from the wild-type and Δrrd1 strains, and then performed the RT-PCR analysis which revealed significant reduction of GAL1 mRNAs in the Δrrd1 strain (Figure 2A). As a loading control, we show that the mRNA level of a constitutively active ADH1 gene is not altered in the Δrrd1 strain in comparison to the wild-type equivalent (Figure 2A), consistent with previous studies (10) that demonstrated the dispensability of Rrd1p in regulation of transcription under vegetative growth conditions. Thus, our results demonstrate a new role of Rrd1p in promoting transcription of a rapamycin non-responsive GAL1 gene following 90 min transcriptional induction independently of rapamycin treatment (or TOR pathway).

Rrd1p facilitates the formation of pre-initiation complex at the GAL1 core promoter

We find above that Rrd1p promotes the association of RNA polymerase II with the GAL1 coding sequence following 90 min transcriptional induction. However, such stimulation of RNA polymerase II association with the GAL1 coding sequence could be mediated via the pre-initiation complex (PIC) formation at the promoter. To test this, we analyzed the recruitment of TBP and Transcription factor II H (TFIIH) components of the PIC to the GAL1 core promoter in the wild-type and Δrrd1 strains. We found that the recruitment of TBP and TFIIH (Rad3p) to the GAL1 core promoter was significantly decreased in the Δrrd1 strain in comparison to the wild-type equivalent (Figure 2B). Likewise, the recruitment of RNA polymerase II to the GAL1 core promoter was decreased in the absence of Rrd1p (Figure 2C). However, the global levels of these proteins were not altered in the Δrrd1 strain in comparison to the wild-type equivalent (Figures 1C and 2D). These results support the role of Rrd1p in promoting transcriptional initiation of GAL1 in the absence of rapamycin response. Intriguingly, the decrease in the recruitment of the PIC components (∼2.5-fold; Figure 2B) is much less as compared to the reduction in the association of RNA polymerase II with the GAL1 coding sequence (∼10-fold; Figure 1B). Such a dramatic decrease in the association of RNA polymerase II with the GAL1 coding sequence as compared to the reduction in the recruitment of the PIC components at the promoter in the Δrrd1 strain suggests the role of Rrd1p in promoting transcriptional elongation in addition to its function in transcription initiation of GAL1.

Rrd1p does not regulate the steady-state level of RNA polymerase II association with GAL1 (and hence steady-state/constitutive transcription)

Our above results at GAL1 reveal that Rrd1p promotes the PIC formation, association of elongating RNA polymerase II, and hence transcription following 90 min transcriptional induction. We next asked whether Rrd1p has any effect on GAL1 transcription when the steady-state is reached after a long induction in galactose-containing growth medium. To address this, we have continuously grown both the wild-type and Δrrd1 strains in galactose-containing growth medium up to an OD600 of 1.0 prior to crosslinking/harvesting, and then performed RT-PCR and ChIP analyses. We found that transcription of GAL1 in the Δrrd1 strain reached the wild-type level when the steady-state is reached after a long transcriptional induction (Figure 3A). Consistently, the level of RNA polymerase II at the GAL1 coding sequence in the Δrrd1 strain was almost same as that of the wild-type equivalent following long transcriptional induction (Figure 3B).
Figure 1. Rrd1p associates with the coding sequence of a rapamycin non-responsive \textit{GAL1} gene, and promotes the association of RNA polymerase II following 90 min transcriptional induction. (A) Schematic diagram showing the locations of different primer pairs at \textit{GAL1} for the ChIP analysis. The numbers are presented with respect to the position of the first nucleotide of the initiation codon (+1). (B) Analysis of Rpb1p association with the \textit{GAL1} coding sequence in the wild-type and \textit{Δrrd1} strains. Both wild-type and \textit{Δrrd1} strains expressing Myc-tagged Rpb1p were grown in YPR at 30°C up to an OD\textsubscript{600} of 0.9, and then switched to YPG for 90 min prior to formaldehyde-based \textit{in vivo} cross-linking. Immunoprecipitation was carried out using an anti-Myc antibody (9E10; Santa Cruz Biotechnology, Inc.) against Myc-tagged Rpb1p. Immunoprecipitated-DNA was analyzed by PCR using the primer pair targeted to the coding sequence of \textit{GAL1}. The ratio of immunoprecipitate over the input in the autoradiogram (i.e. ChIP signal) was measured. The ChIP signal of the wild-type strain was set to 100, and the ChIP signal of the mutant strain was normalized with respect to 100. The normalized ChIP signal (represented as normalized occupancy) is plotted in the form of a histogram. (C) Western blot analysis under similar growth conditions as in panel B. (D) Growth analysis of the wild-type and \textit{Δrrd1} strains after switching to YPG from YPR (i.e. during 90 min induction time period). (E) RT-PCR analysis of the \textit{GAL1} and \textit{ACT1} mRNA levels in the presence and absence of rapamycin. Yeast cells were grown in YPR up to an OD\textsubscript{600} of 0.9, transferred to YPG for 60 min, and then treated with 100 nM rapamycin (Sigma) for next 30 min prior to harvesting for RNA analysis. (F) Rrd1p associates with the coding sequence of \textit{GAL1}. Yeast strain expressing Myc-tagged Rrd1p was grown as in panel B. Immunoprecipitation was carried out using an anti-Myc antibody against Myc-tagged Rrd1p. Immunoprecipitated-DNA was analyzed by PCR using the primer pairs targeted to the UAS, core promoter and two different locations (ORF and ORF1) of the coding sequence of \textit{GAL1}. Maximum ChIP signal was set to 100, and other ChIP signals were normalized with respect to 100. The normalized ChIP signal (represented as normalized occupancy) is plotted in the form of a histogram.
Figure 2. Rrd1p facilitates the PIC formation at the GAL1 promoter, and enhances transcription following 90 min transcriptional induction. (A) RT-PCR analysis of GAL1 and ADH1 mRNA levels in the wild-type and Δrrd1 strains following 90 min transcriptional induction in YPG. (B) and (C) ChIP analysis for the recruitment of TBP, Rad3p and Rpb1p to the GAL1 core promoter in the wild-type and Δrrd1 strains following 90 min transcriptional induction in YPG. Immunoprecipitation was performed using anti-TBP antibody (obtained from Michael R. Green, University of Massachusetts Medical School) against TBP, and anti-Myc antibody against Myc-tagged Rad3p and Myc-tagged Rpb1p. Immunoprecipitated-DNA was analyzed using the primer pair targeted to the GAL1 core promoter. (D) Western blot analysis of TBP, Rad3p and actin in the wild-type and Δrrd1 strains. Yeast strains were grown as in Figure 1B.
Figure 3. Rrd1p has no effect on the steady-state level of GAL1 transcription. (A) RT-PCR analysis of GAL1 mRNA levels in the wild-type and Δrrd1 strains following continuous growth in YPG. (B) ChIP analysis for the association of RNA polymerase II with the GAL1 coding sequence following continuous growth in YPG. (C) ChIP analysis of RNA polymerase II association with the GAL1 coding sequence at different time points (20, 40 and 60 min) following transcriptional induction. Maximum ChIP signal was set to 100, and other ChIP signals were normalized with respect to 100. The normalized ChIP signal (represented as normalized occupancy) is plotted in the form of a histogram. (D) ChIP analysis of RNA polymerase II association with the GAL1 coding sequence following 2, 4 and 6 h transcriptional induction in YPG. The ChIP signal for wild-type strain was set to 100, and the ChIP signal for the Δrrd1 strain was normalized with respect to 100. (E) Growth analysis of the wild-type and Δrrd1 strains in solid YPG medium. (F and G) ChIP analysis of histone H2B level at the GAL1 coding sequence in the wild-type and Δrrd1 strains expressing Flag-tagged histone H2B. Yeast cells were grown and crosslinked as in panel C, and immunoprecipitation was performed using an anti-Flag antibody (F1804, Sigma) against Flag-tagged histone H2B.
Further, we performed the kinetic analysis for the association of RNA polymerase II with GAL1, and found that Rrd1p has significant stimulatory effects on RNA polymerase II association with GAL1 during initial stages of transcriptional induction, but not after long induction (Figure 3C and D). Thus, Rrd1p promotes the initial rounds of GAL1 transcription, but has no effect on steady-state transcription. Therefore, the growth defect of the Δrrd1 strain was not observed in the solid medium containing galactose (Figure 3E). Furthermore, we find that the role of Rrd1p in stimulation of RNA polymerase II association with GAL1 (and hence transcription) is correlated with facilitated nucleosomal disassembly as the eviction of histone H2B from GAL1 is impaired in the Δrrd1 strain following transcriptional induction (Figure 3F and G).

Based on our results at GAL1, we expect that Rrd1p would not have an effect on transcription of the constitutively active genes. Indeed, transcription of a constitutively active gene, ADH1, was not altered in the absence of Rrd1p (Figure 4A). Likewise, the association of RNA polymerase II with ADH1 was not changed in the Δrrd1 strain as compared to the wild-type equivalent (Figure 4B). Similarly, transcription of other constitutively active genes, RPS5 and ACT1, was not altered in the Δrrd1 strain (Figure 4C). In agreement with our results, a recent study (10) has also demonstrated the dispensability of Rrd1p in regulating transcription of the constitutively active genes under vegetative growth conditions. Therefore, the growth of the Δrrd1 strain would not be altered in comparison to the wild-type equivalent in dextrose-containing growth medium. Indeed, the growth of the Δrrd1 strain was not altered in the liquid and solid growth media containing dextrose (Figure 4D and E). Collectively, our results support that Rrd1p promotes initial rounds of GAL1 transcription, but has no effect on steady-state level or constitutive transcription.

Rrd1p associates with the coding sequences of other rapamycin non-responsive GAL genes such as GAL7 and GAL10, and promotes their transcription independently of rapamycin treatment

We have shown above that Rrd1p associates with GAL1 and promotes its transcription. We next analyzed whether Rrd1p also associates with other GAL genes such as GAL7 and GAL10 to promote their transcription. In this direction, we first analyzed the association of Rrd1p with the core promoters and coding sequences of GAL7 and GAL10 following 90 min transcriptional induction. Like the results at GAL1, we find that Rrd1p predominantly associates with the coding sequences of GAL7 and GAL10 (Figure 5A, Supplementary Figure S1). Subsequently, we analyzed the association of RNA polymerase II with the core promoters and coding sequences of GAL7 and GAL10 following 90 min transcriptional induction in the Δrrd1 and wild-type strains. We find that Rrd1p promotes the association of RNA polymerase II with GAL7 and GAL10 (Figure 5B and C). Consistently, transcription of GAL7 and GAL10 was significantly decreased in the absence of Rrd1p (Figure 5D). Further, we show that transcription of GAL7 and GAL10 is not regulated by rapamycin (Figure 5E). Thus, our results demonstrate that Rrd1p promotes transcription of rapamycin non-responsive GAL7 and GAL10 genes following 90 min transcriptional induction independently of rapamycin treatment (or TOR pathway). Furthermore, similar to the results at GAL1, we find that Rrd1p promotes the recruitment of the PIC components such as TBP and TFIH (Rad3p) to the core promoters of GAL7 and GAL10 (Figure 5F and G), hence supporting the role of Rrd1p in stimulation of the PIC formation (and hence transcriptional initiation). However, the defect in formation of the PIC at GAL7 and GAL10 in the absence of Rrd1p is much less than the defect in RNA polymerase II association with the coding sequence. These results indicate that Rrd1p facilitates transcriptional elongation, in addition to its role in transcriptional initiation.

We next asked whether the effect of Rrd1p on transcription of GAL7 and GAL10 is minimal or absent when the steady-state is reached after a long induction in galactose-containing growth medium. To address this, both the wild-type and Δrrd1 strains were continuously grown in galactose-containing growth medium up to an OD600 of 1.0 prior to harvesting for RT-PCR analysis. We found that transcription of GAL7 and GAL10 in the Δrrd1 strain reached the wild-type level when the steady-state is reached after a long transcriptional induction (Figure 5H). Thus, Rrd1p promotes the initial rounds of GAL7 and GAL10 transcription, and has no effect on transcription when the steady-state is reached. This is further corroborated by the kinetic analysis of RNA polymerase II association with GAL7 and GAL10 following short or long transcriptional induction (Figure 6A–D). Moreover, the role of Rrd1p in stimulation of RNA polymerase II association with GAL7 and GAL10 is correlated with facilitated nucleosomal disassembly as the eviction of histone H2B from GAL7 and GAL10 is impaired in the Δrrd1 strain in comparison to the wild-type equivalent (Figure 6E and F).
Figure 4. Rrd1p does not regulate transcription of the constitutively active genes. (A) RT-PCR analysis of *ADH1* mRNA levels in the wild-type and Δrrd1 strains following continuous growth in YPG. (B) ChIP analysis for the association of RNA polymerase II with the *ADH1* coding sequence following continuous growth in YPG. (C) RT-PCR analysis of *ADH1*, *RPS5* and *ACT1* mRNA levels in the wild-type and Δrrd1 strains following continuous growth in dextrose-containing growth medium. (D and E) Growth analysis of the wild-type and Δrrd1 strains in both liquid and solid YPD media.
Figure 5. Rrd1p facilitates transcription of GAL7 and GAL10 following 90 min transcriptional induction. (A) Rrd1p associates with the coding sequences of GAL7 and GAL10. Yeast strain expressing Myc-tagged Rrd1p was grown, crosslinked and immunoprecipitated as in Figure 1F. The ChIP signal at the ORF was set to 100, and the ChIP signal at the promoter was normalized with respect to 100. (B and C) ChIP analysis for the association of RNA polymerase II with the GAL7 and GAL10 core promoters and coding sequences in the wild-type and Δrrd1 strains following 90 min transcriptional induction in YPG. (D) RT-PCR analysis of GAL7, GAL10 and ADH1 mRNA levels in the wild-type and Δrrd1 strains following 90 min transcriptional induction in YPG. (E) RT-PCR analysis of GAL7, GAL10, and ACT1 mRNA levels in the presence and absence of rapamycin. Yeast cells were grown in YPR up to an OD$_{600}$ of 0.9, transferred to YPG for 60 min, and then treated with 100 nM rapamycin (Sigma) for next 30 min prior to harvesting for RNA analysis. (F and G) ChIP analysis for the recruitment of TBP and Myc-tagged Rad3p to the core promoters of GAL7 and GAL10 following 90 min transcriptional induction in YPG. ChIP experiments were carried out as in Figure 2B. (H) RT-PCR analysis of GAL7 and GAL10 mRNA levels in the wild-type and Δrrd1 strains following continuous growth in YPG.
Figure 6. ChIP analysis of RNA polymerase II and histone H2B at GAL7 and GAL10 following transcriptional induction. (A and B) Analysis of RNA polymerase II association with the GAL7 and GAL10 coding sequence at different time points (20, 40 and 60 min) following transcriptional induction in YPG. (C and D) Analysis of RNA polymerase II levels at the GAL7 and GAL10 coding sequence following 2, 4 and 6 h transcriptional induction in YPG. (E and F) Analysis of histone H2B levels at the GAL7 and GAL10 coding sequences at different time points (30, 60 and 90 min) following transcriptional induction in YPG.
Figure 7. Rrd1p facilitates transcription of CUP1, STL1 and CTT1 following transcriptional induction. (A and B) RT-PCR analysis of CUP1, STL1, CTT1 and ADH1 mRNA levels in the wild-type and Δrrd1 strains following transcriptional induction (7 min induction for STL1 and CTT1; and 15 min induction for CUP1). (C) Rrd1p associates with the coding sequences of CUP1, STL1 and CTT1 following transcriptional induction. The ChIP signal at the coding sequence was set to 100, and the ChIP signal at the promoter was normalized with respect to 100. (D and E) ChIP analysis for the association of RNA polymerase II with the CUP1, STL1 and CTT1 core promoters and coding sequences in the wild-type and Δrrd1 strains following transcriptional induction.
Figure 8. CUP1, STL1 and CTT1 are rapamycin-responsive genes, and are not regulated by Rrd1p following long transcriptional induction. (A and B) RT-PCR analysis of CUP1, STL1, CTT1 and ACT1 mRNA levels in the presence and absence of rapamycin treatment. (C) ChIP analysis of RNA polymerase II association with STL1, CTT1 and CUP1 following long transcriptional induction (30 min induction for STL1 and CTT1; and 1 h induction for CUP1). (D and E) RT-PCR analysis of CUP1, STL1, CTT1 and ADH1 mRNA levels in the wild-type and Δrrd1 strains following long transcriptional induction. (F) ChIP analysis for TBP recruitment to the core promoters of CUP1, CTT1, STL1 in the wild-type and Δrrd1 strains following short transcriptional induction (i.e. 7 min induction for CTT1 and STL1; and 15 min induction for CUP1).
by rapamycin, they are positively regulated by Rrdl1p in the absence of rapamycin treatment (Figure 7A, B, D and E), similar to the results at the rapamycin non-responsive GAL genes. However, the effect of Rrdl1p on the association of RNA polymerase II with CUP1, STL1 and CTT1 following long transcriptional induction was not observed (Figure 8C). Consistently, transcription of these genes was not altered after long transcriptional induction (Figure 8D and E). Thus, similar to the results at GAL genes, Rrdl1p promotes initial rounds of transcription of non-GAL genes independently of rapamycin response (or TOR pathway), but has no effect on the steady-state level. However, unlike the results at the GAL genes, the recruitment of TBP to the CUP1 promoter is not altered in the absence of Rrdl1p (Figure 8F). Thus, Rrdl1p appears to promote transcriptional elongation of CUP1, but not initiation. Consistently, Rrdl1p has been recently implicated in regulation of transcriptional elongation, but not initiation, of rapamycin-responsive genes in the presence of rapamycin treatment (10). On the other hand, Rrdl1p facilitates TBP recruitment to the core promoters of CTT1 and STL1 (Figure 8F), but has more effect on RNA polymerase II association with the coding sequence, similar to the results at the GAL genes, thus indicating the role of Rrdl1p in both transcriptional initiation and elongation of CTT1 and STL1.

DISCUSSION

Rrdl1p has been previously implicated to promote transcriptional elongation of a set of rapamycin-responsive genes in the presence of rapamycin treatment (10). However, previous studies (10) demonstrated that Rrdl1p does not alter transcription of both rapamycin-responsive and non-responsive genes under vegetative growth conditions in the absence of rapamycin treatment. Intriguingly, we demonstrate here that Rrdl1p promotes transcription of galactose-inducible GAL genes following transcriptional induction in the absence of rapamycin treatment (Figures 1B, 2A, C, 5B–D). The GAL genes are not responsive to rapamycin (Figure 1E and 5E; 10). Thus, Rrdl1p promotes transcription of rapamycin non-responsive GAL genes in the absence of rapamycin. We also find here that Rrdl1p promotes transcription of rapamycin-responsive genes such as CTT1, STL1 and CUP1 following transcriptional induction in the absence of rapamycin treatment (Figures 7A, B, D, E, 8A and B). These results demonstrate for the first time a new role of Rrdl1p in promoting transcription of both rapamycin-responsive and non-responsive genes independently of rapamycin treatment (or TOR pathway). However, when the steady-state is reached after long transcriptional induction, Rrdl1p has no effect on transcription of these rapamycin-responsive and non-responsive genes (Figures 3A, B, D, 5H, 6C, D, 8C–E). Consistently, we find that transcription of the constitutively active genes such as ADH1, ACT1 and RPS5 is not altered in the absence of Rrdl1p (Figure 4A–C). Collectively, our results demonstrate that Rrdl1p promotes initial rounds of transcription, and has no effect on the steady-state level or constitutive transcription, thus providing a new role of Rrdl1p in regulation of transcription independently of rapamycin response. Such function is mediated via direct association of Rrdl1p with active genes (Figures 1F, 5A and 7C, Supplementary Figure S1).

We find that Rrdl1p associates with the coding sequences of GAL and non-GAL genes (Figures 1F, 5A and 7C, Supplementary Figure S1), and the association of RNA polymerase II with the coding sequences of these genes is greatly decreased in the absence of Rrdl1p in comparison to the recruitment of the PIC components at the core promoter (Figures 1B, 2B, 5B, C, F, G, 7E and 8F). Further, Rrdl1p interacts with RNA polymerase II for CTD phosphorylation involved in transcriptional elongation (9, 10). Moreover, Rrdl1p has been shown to be sensitive to 6-AU (6-Azauracil) (10). 6-AU decreases nucleotide pools, resulting in slow (or impaired) cellular growth upon deletion (or mutation) of the factors involved in transcriptional elongation (35). The 6-AU sensitivity of the Δrrd1 strain does not appear to be due to impaired expression of genes (including URA genes) as Rrdl1p does not alter transcription under vegetative growth conditions in the absence of rapamycin treatment (10); Figure 4A–C). Moreover, the growth of the Δrrd1 strain is not altered as compared to the wild-type equivalent in the growth medium lacking uracil (10) or YPD/YPG (Figures 3F, 4D and E). Collectively, these results implicate the role of Rrdl1p in transcriptional elongation of rapamycin-responsive and non-responsive genes independently of rapamycin treatment.

In addition to its role in transcriptional elongation, Rrdl1p promotes the PIC formation at the core promoters of GAL genes (and hence transcriptional initiation) (Figures 2B, 5F and G). Likewise, Rrdl1p promotes TBP recruitment at the core promoters of the non-GAL genes such as CTT1 and STL1, but not CUP1 (Figure 8F). Consistent with our results at CUP1, previous studies (10) have implicated the role of Rrdl1p in transcriptional elongation, but not PIC formation, of rapamycin-responsive genes in the presence of rapamycin treatment. However, the role of Rrdl1p in regulation of the PIC formation (and hence transcriptional initiation) was not known. Our results (Figures 2B, 5F, G and 8F) reveal that Rrdl1p facilitates PIC formation at the core promoters of CTT1, STL1 and GAL genes, in addition to its role in promoting transcriptional elongation.

Previous global genome-wide studies (10) show that Rrdl1p associates with the active coding sequences, but does not regulate transcription of the constitutively active genes under vegetative growth conditions. Consistently, we find here that Rrdl1p does not alter transcription of the constitutively active genes such as ADH1, ACT1 and RPS5 (Figure 4A–C). However, previous studies (10) did not analyze the role of Rrdl1p in expression of inducible genes following transcriptional induction. Thus, the role of Rrdl1p in regulation of initial rounds of transcription was not known. In this study, we analyzed the role of Rrdl1p in transcription of inducible GAL and non-GAL genes following transcriptional induction. We find that Rrdl1p promotes transcription of these genes following transcriptional induction (Figures 2A, 5D, 7A and B). However, the effect of Rrdl1p was not observed on the steady-state level of transcription after long transcriptional induction (Figures 3A, B, D, 5H, 6C, D, 8C–E), consistent with previous studies (10). Therefore, we provide a new role of Rrdl1p in promoting initial/early
The Δrdr1 strain has been shown to be sensitive to 4NQO (4-nitroquinoline 1-oxide) treatment (36). We have previously shown that RNA polymerase II is disassembled via degradation of its largest subunit Rpb1 in response to 4NQO-induced DNA damage (19). Such disassembly of RNA polymerase II allows the repair factors to access the lesion for repair. Further, DNA repair factor (e.g. Rad26p) is targeted to the lesion by elongating RNA polymerase II (24,37–40). Thus, the passage of elongating RNA polymerase II through the coding sequence plays an important role in repairing lesion at the active coding sequence. We find that the deletion of RRD1 dramatically decreases the association of RNA polymerase II with active coding sequence, hence impairing transcription-coupled DNA repair. Therefore, the Δrdr1 strain becomes sensitive to 4NQO treatment (36), as RNA polymerase II does not get efficiently engaged in transcriptional elongation. Thus, our results suggest how Rrd1p is connected to transcription-coupled repair or 4NQO sensitivity via regulation of transcriptional elongation.

In summary, we demonstrate here that Rrd1p promotes transcription independently of rapamycin response (or TOR pathway). However, the steady-state level of transcription is not altered in the absence of Rrd1p. Therefore, our results implicate Rrd1p as a new factor involved in promoting early rounds of transcription. Since Rrd1p is conserved from yeast to humans, Rrd1p’s human homologue is likely to facilitate early rounds of transcription of certain genes at right time in specific cell types, thereby contributing to temporal and spatial regulation of gene expression and hence cellular differentiation and development.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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