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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-

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 OD_{600} 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 OD_{600} of 1.0 at 30°C. Yeast strains were grown in YPD (yeast extract peptone plus 2% dextrose) up to an OD_{600} 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 OD_{600} 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 $CuSO_4$ for 15 min in synthetic complete medium at 30°C. For long induction of *CUP1*, yeast cells were treated with 1 mM $CuSO_4$ 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 plus agarose 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:

GAL1 (UAS):	5′-CGCTTAACTGCTCATTGCTATATTG-3′
	5′-TTGTTCGGAGCAGTGCGGCGC-3′
GAL1 (Core):	5′-ATAGGATGATAATGCGATTAGTTTTTTAGCCTT-3′
	5′-GAAAATGTTGAAAGTATTAGTTAAAGTGGTTATGCA-3′
GAL1 (ORF):	5′-CAGTGGATTGTCTTCTTCGGCCGC-3′
	5′-GGCAGCCTGATCCATACCGCCATT-3′
GAL1 (ORF1):	5′-CAGAGGGCTAAGCATGTGTATTCT-3′
	5′-GTCAATCTCTGGACAAGAACATTC-3′
GAL7 (Core):	5′-CTATGTTCAGTTAGTTTGGCTAGC-3′
	5′-TTGATGCTCTGCATAATAATGCCC-3′
GAL7 (ORF):	5′-TGAGACCTTGGTCATTTCAAAGAAG-3′
	5′-ATGGATACCCATTGAGTATGGGAAA-3′
GAL10 (Core):	5′-GCTAAGATAATGGGGGCTCTTTACAT-3′
	5′-TTTCACTTTGTAACTGAGCTGTCAT-3′
GAL10 (ORF):	5′-TTAATGCGAATCATAGTAGTATCGG-3′
	5′-TTACCAATAGATCACCTGGAAATTC-3′
CTT1 (Core):	5′-GGCTGCAGGCTAGCCTAGCCGAT-3′
	5′-GGAATAGAGGTAAAGCAACGACTTC-3′
CTT1 (ORF):	5′-TGCTGAACTGTCAGGCTCCCACC-3′
	5′-GGGGAATTCCTTGTGTGGCCATATT-3′
STL1 (Core):	5′-ACCTTTGATAGGGCTTTCATTGGGGC-3′
	5′-TCTAAGGCCAAGCAGCGTTGAAG-3′
STL1 (ORF):	5′-ACACTAGACGCGGATCCAAAT-3′
	5′-AGCGTTACAACCAGTAAATTGCTGG-3′
CUP1 (Core):	5′-TCTTCTAGAAGCAAAAAGAGCGATG-3′
	5′-CGCTGAACATTTTATGTGATGATTG3′
CUP1 (ORF):	5′-ACTTCCAAAATGAAGGTCATGAGTG-3′
	5′-AGCAGCATGACTTCTTGGTTTCTTC-3′
ADH1 (ORF):	5′-CGGTAACAGAGCTGACACCAGAGA-3′
	5′-ACGTATCTACCAACGATTTGACCC-3′

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 Na_2EDTA and 1% SDS) along with 100 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 *GAL1, GAL7, GAL10, ADH1, ACT1, CUP1, CTT1, 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′-CAGAGGGCTAAGCATGTGTATTCT-3′
	5′-GTCAATCTCTGGACAAGAACATTC-3′
GAL7:	5′-TGAGACCTTGGTCATTTCAAAGAAG-3′
	5′-ATGGATACCCATTGAGTATGGGAAA-3′
GAL10:	5′-TTAATGCGAATCATAGTAGTATCGG-3′
	5′-TTACCAATAGATCACCTGGAAATTC-3′
ADH1:	5′-CGGTAACAGAGCTGACACCAGAGA-3′
	5′-ACGTATCTACCAACGATTTGACCC-3′
RPS5:	5′-AGGCTCAATGTCCAATCATTGAAAG-3′
	5′-CAACAACTTGGATTGGGTTTTGGTC-3′
CUP1:	5′-ACTTCCAAAATGAAGGTCATGAGTG-3′
	5′-AGCAGCATGACTTCTTGGTTTCTTC-3′
CTT1:	5′-TGCTGAACTGTCAGGCTCCCACC-3′
	5′-GGGGAATTCCTTGTGTGGCCATATT-3′
STL1:	5′-ACACTAGACGCGGATCCAAAT-3′
	5′-AGCGTTACAACCAGTAAATTGCTGG-3′
ACT1:	5′-TCCACCACTGCTGAAAGAGAAATTG-3′
	5′-AATAGTGATGACTTGACCATCTGGA-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 OD_{600} of 0.9, and then induced for 90 min in YPG. 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 OD_{600} 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 OD_{600} of 0.2 at 30°C without dilution. Subsequently, yeast cells were switched to YPD at 30°C, and OD_{600} 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 OD_{600} of 0.9 at 30°C without dilution. Subsequently, yeast cells were switched to YPG at 30°C for 90 min, and OD_{600} 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 OD_{600} 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 Δ*rrd1* 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 Δ*rrd1* strain. To test this possibility, we analyzed the global levels of Rpb1p in the wild-type and Δ*rrd1* strains, using the western blot assay. We found that the global level of Rpb1p was not changed in the Δ*rrd1* 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 Δ*rrd1* strain (Figure 1C). Thus, reduced association of RNA polymerase II with the *GAL1* coding sequence following 90 min transcriptional induction in the Δ*rrd1* strain was not due to an impaired stability of Rpb1p. However, it is quite possible that the Δ*rrd1* strain grew slowly in galactose-containing growth medium during 90 min transcriptional induction, leading to a less number of the Δ*rrd1* cells and consequently decreased level of RNA polymerase II at *GAL1*. To test this possibility, we measured OD_{600} of the wild-type and Δ*rrd1* strains under the growth conditions used in the above ChIP experiments. We found that OD_{600} of the Δ*rrd1* 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 Δ*rrd1* 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 Δ*rrd1* 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 Δ*rrd1* strain. To test this, we analyzed *GAL1* mRNA levels in the wild-type and Δ*rrd1* strains following transcriptional induction in galactose-containing growth medium. We isolated total RNAs from the wild-type and Δ*rrd1* strains, and then performed the RT-PCR analysis which revealed significant reduction of *GAL1* mRNAs in the Δ*rrd1* strain (Figure 2A). As a loading control, we show that the mRNA level of a constitutively active *ADH1* gene is not altered in the Δ*rrd1* strain in comparison to the wild-type equiv-

alent (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 Δ*rrd1* strains. We found that the recruitment of TBP and TFIIH (Rad3p) to the *GAL1* core promoter was significantly decreased in the Δ*rrd1* strain in comparison to the wild-type equivalent (Figure 2B). Likewise, the recruitment of RNA polymerase II to the *GAL1* core promoter is decreased in the absence of Rrd1p (Figure 2C). However, the global levels of these proteins were not altered in the Δ*rrd1* 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 Δ*rrd1* 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 Δ*rrd1* strains in galactose-containing growth medium up to an OD_{600} of 1.0 prior to crosslinking/harvesting, and then performed RT-PCR and ChIP analyses. We found that transcription of *GAL1* in the Δ*rrd1* 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 Δ*rrd1* strain was almost same as that of the wild-type equivalent following long transcriptional induction (Figure 3B).
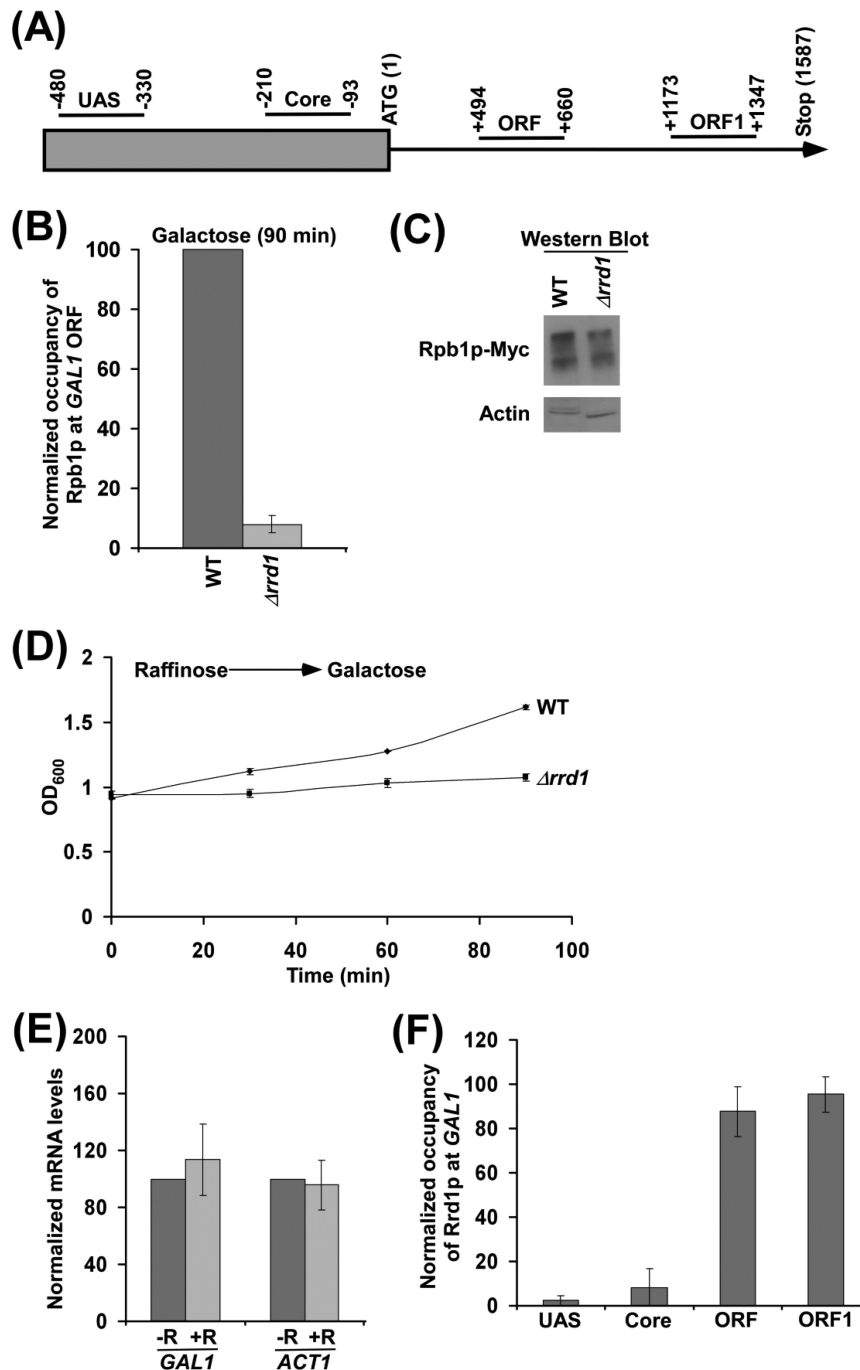
Figure 1. Rrd1p associates with the coding sequence of a rapamycin non-responsive *GAL1* gene, and promotes the association of RNA polymerase II following 90 min transcriptional induction. (**A**) Schematic diagram showing the locations of different primer pairs at *GAL1* for the ChIP analysis. The numbers are presented with respect to the position of the first nucleotide of the initiation codon (+1). (**B**) Analysis of Rpb1p association with the *GAL1* coding sequence in the wild-type and *Δrrd1* strains. Both wild-type and *Δrrd1* strains expressing Myc-tagged Rpb1p were grown in YPR at 30°C up to an OD$_{600}$ of 0.9, and then switched to YPG for 90 min prior to formaldehyde-based *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 *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 *Δrrd1* strains after switching to YPG from YPR (i.e. during 90 min induction time period). (**E**) RT-PCR analysis of the *GAL1* 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**) Rrd1p associates with the coding sequence of *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 *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.
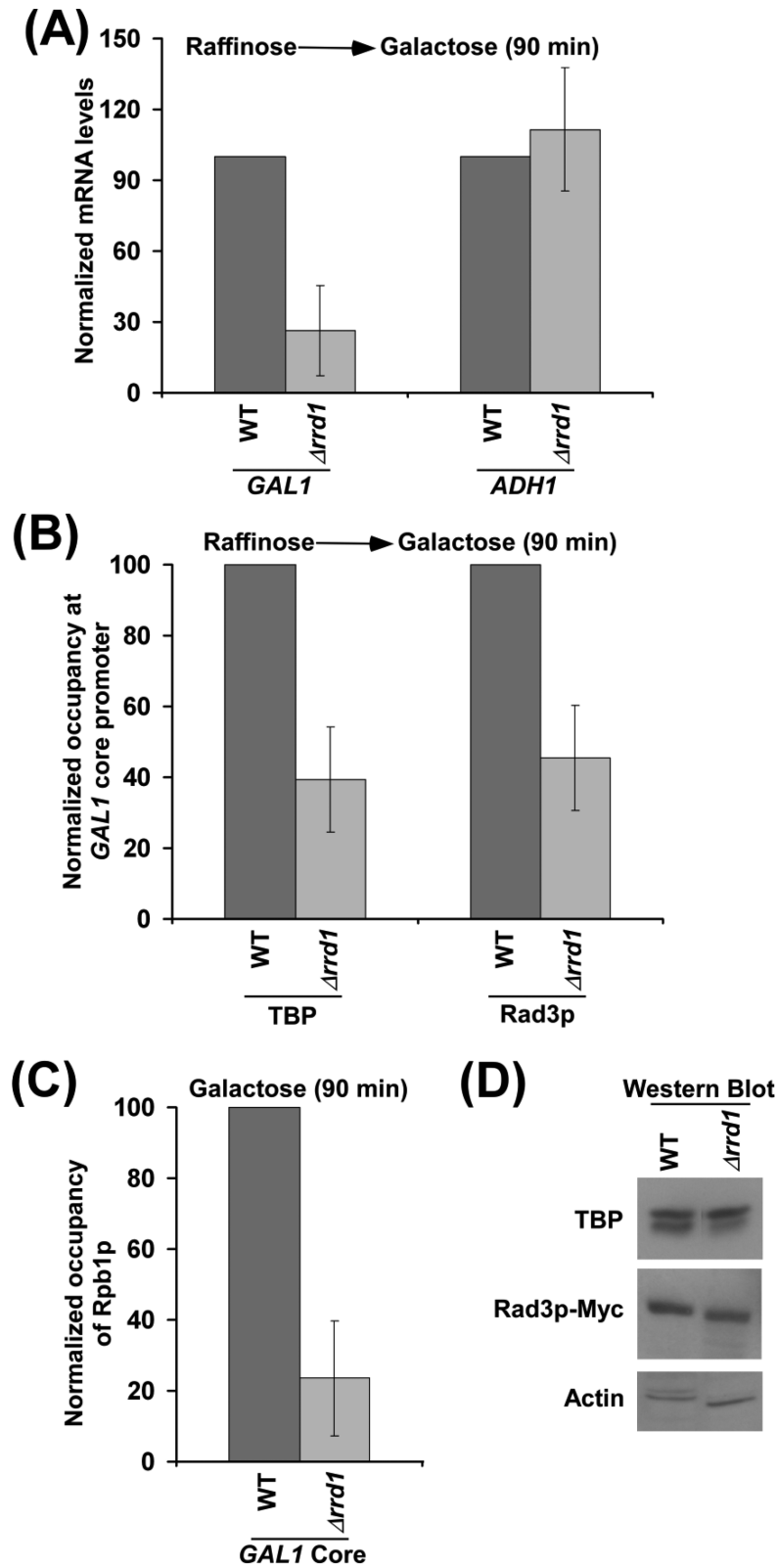
Figure 2. Rrd1p facilitates the PIC formation at the *GAL1* promoter, and enhances transcription following 90 min transcriptional induction. (**A**) RT-PCR analysis of *GAL1* and *ADH1* mRNA levels in the wild-type and *Δrrd1* strains following 90 min transcriptional induction in YPG. (**B** and **C**) 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.
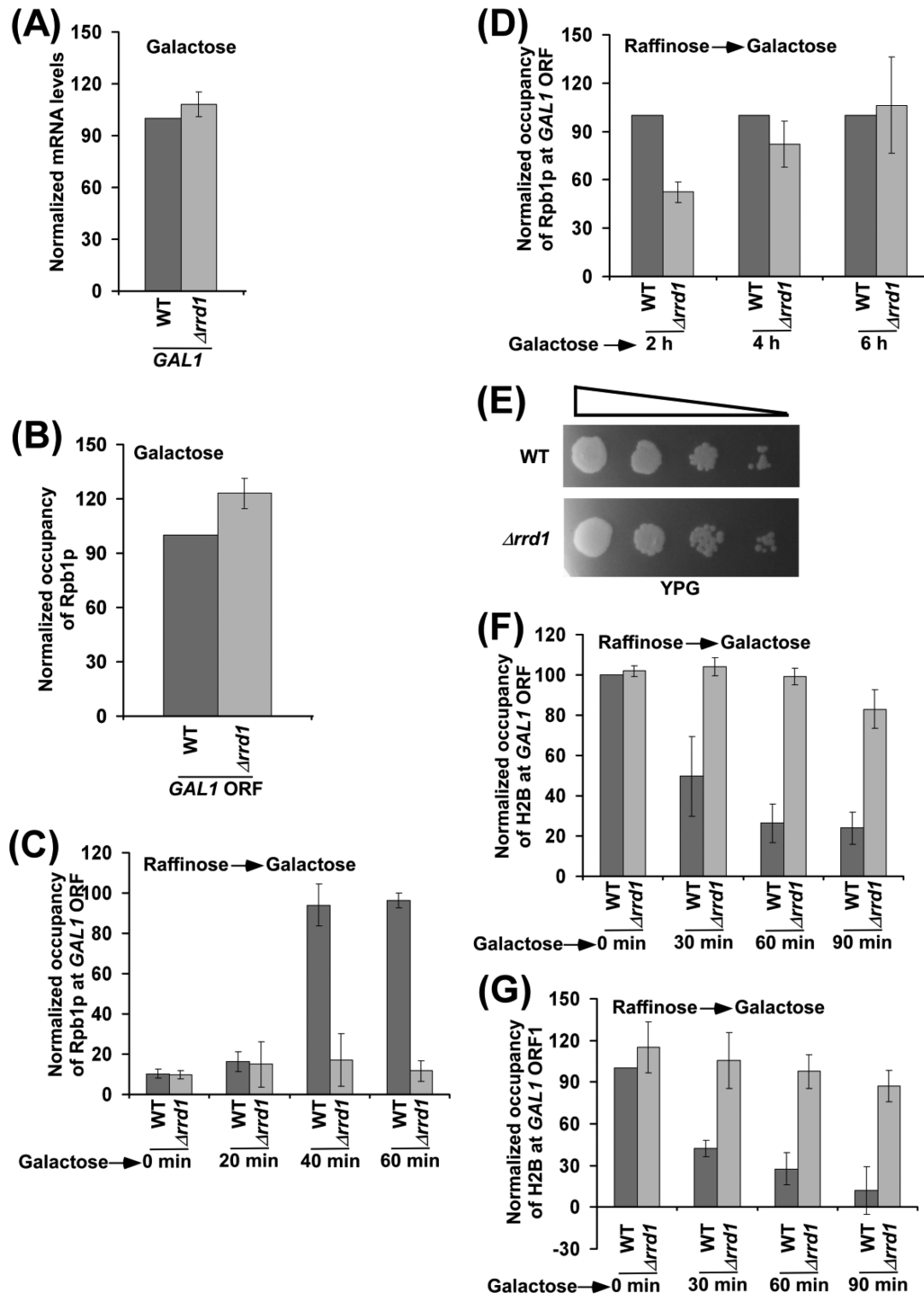
Figure 3.   Rrd1p has no effect on the steady-state level of *GAL1* transcription. (**A**) RT-PCR analysis of *GAL1* mRNA levels in the wild-type and *Δrrd1* strains following continuous growth in YPG. (**B**) ChIP analysis for the association of RNA polymerase II with the *GAL1* coding sequence following 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 above 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 an 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 TFIIH (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 additional 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 OD$_{600}$ 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).

Rrd1p associates with the coding sequences of rapamycin-responsive non-*GAL* genes such as *CUP1*, *STL1* and *CTT1*, and promotes their transcription in the absence of rapamycin treatment

We find above that Rrd1p promotes transcription of *GAL* genes independently of rapamycin treatment. To determine whether Rrd1p has similar effects on transcription of non-*GAL* genes, we analyzed transcription of *CUP1*, *STL1* and *CTT1* following transcriptional induction in the presence and absence of Rrd1p. *CTT1* and *STL1* are induced by NaCl, while Cu^{2+} induces *CUP1*. We found that transcription of *CUP1*, *STL1* and *CTT1* was impaired following transcriptional induction in the absence of Rrd1p (Figure 7A and B). Consistently, Rrd1p associates with *CTT1*, *STL1* and *CUP1* (Figure 7C, Supplementary Figure S1), and the association of RNA polymerase II with *CUP1*, *STL1* and *CTT1* was decreased in the *Δrrd1* strain in comparison to the wild-type equivalent (Figure 7D and E). Thus, like the results at *GAL* genes, Rrd1p associates with non-*GAL* genes and facilitates their transcription in the absence of rapamycin. Intriguingly, transcription of these genes was decreased following rapamycin treatment (Figure 8A and B). Thus, unlike *GAL* genes, these genes are regulated by rapamycin. Although these genes are controlled
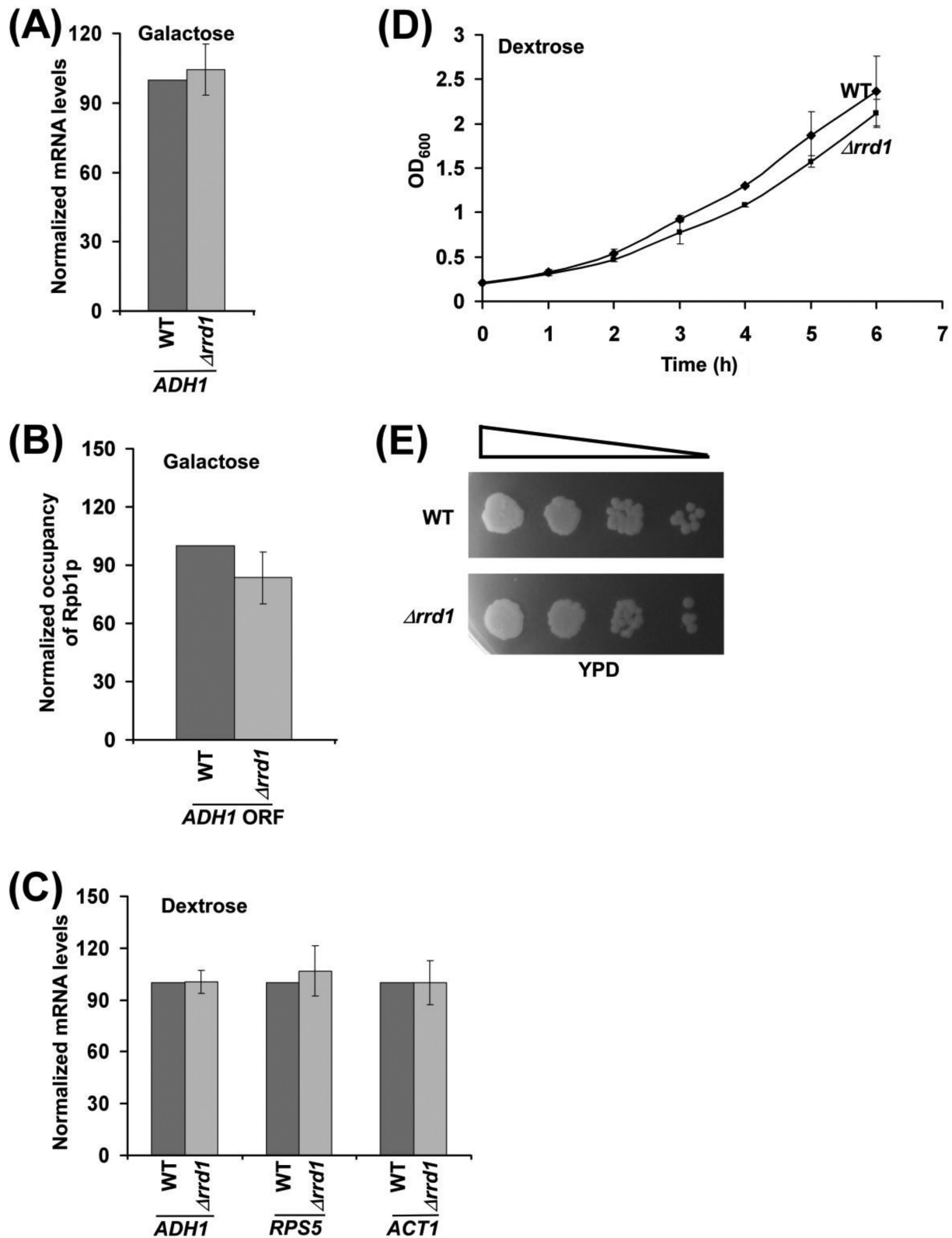
Figure 4. Rrd1p does not regulate transcription of the constitutively active genes. (**A**) RT-PCR analysis of *ADH1* mRNA levels in the wild-type and *Δrrd1* strains following continuous growth in YPG. (**B**) ChIP analysis for the association of RNA polymerase II with the *ADH1* coding sequence following 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.
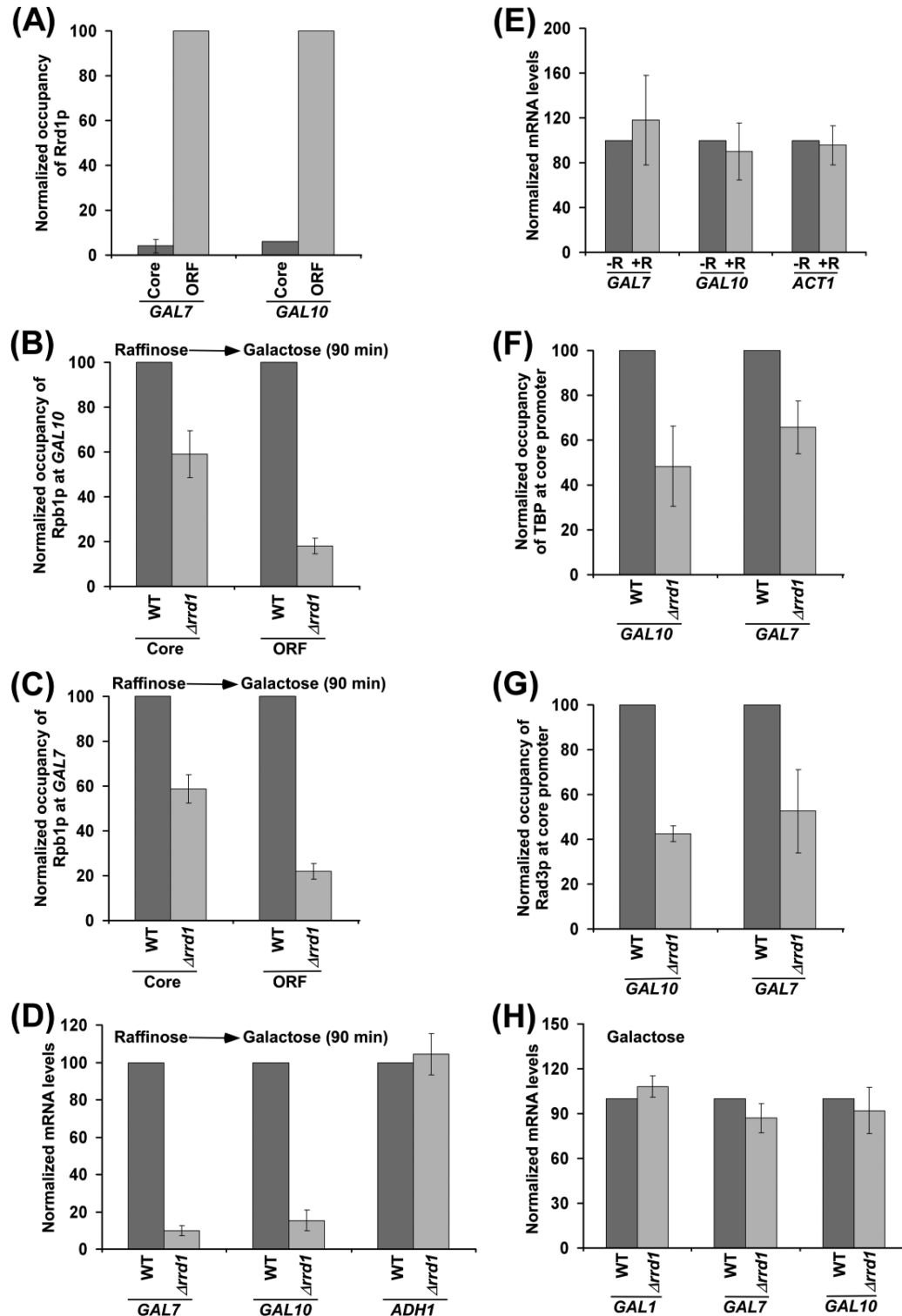
Figure 5. Rrd1p facilitates transcription of *GAL7* and *GAL10* following 90 min transcriptional induction. (**A**) Rrd1p associates with the coding sequences of *GAL7* and *GAL10*. Yeast strain expressing Myc-tagged Rrd1p was grown, crosslinked and immunoprecipitated as in Figure 1F. The ChIP signal 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.
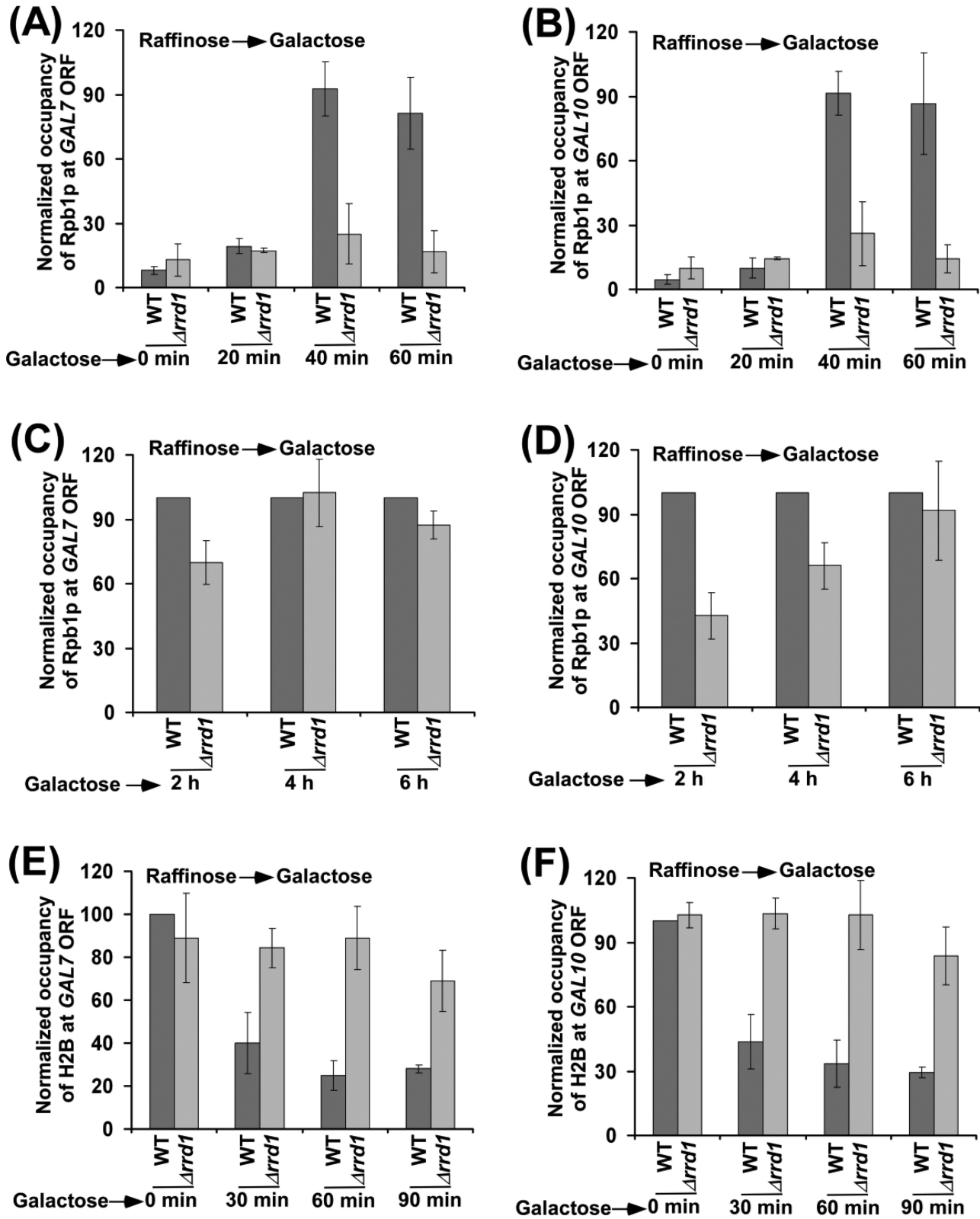
Figure 6. ChIP analysis of RNA polymerase II and histone H2B at *GAL7* and *GAL10* following transcriptional induction. (**A** and **B**) Analysis of RNA polymerase II association with the *GAL7* and *GAL10* coding sequence at different time points (20, 40 and 60 min) following transcriptional induction 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.
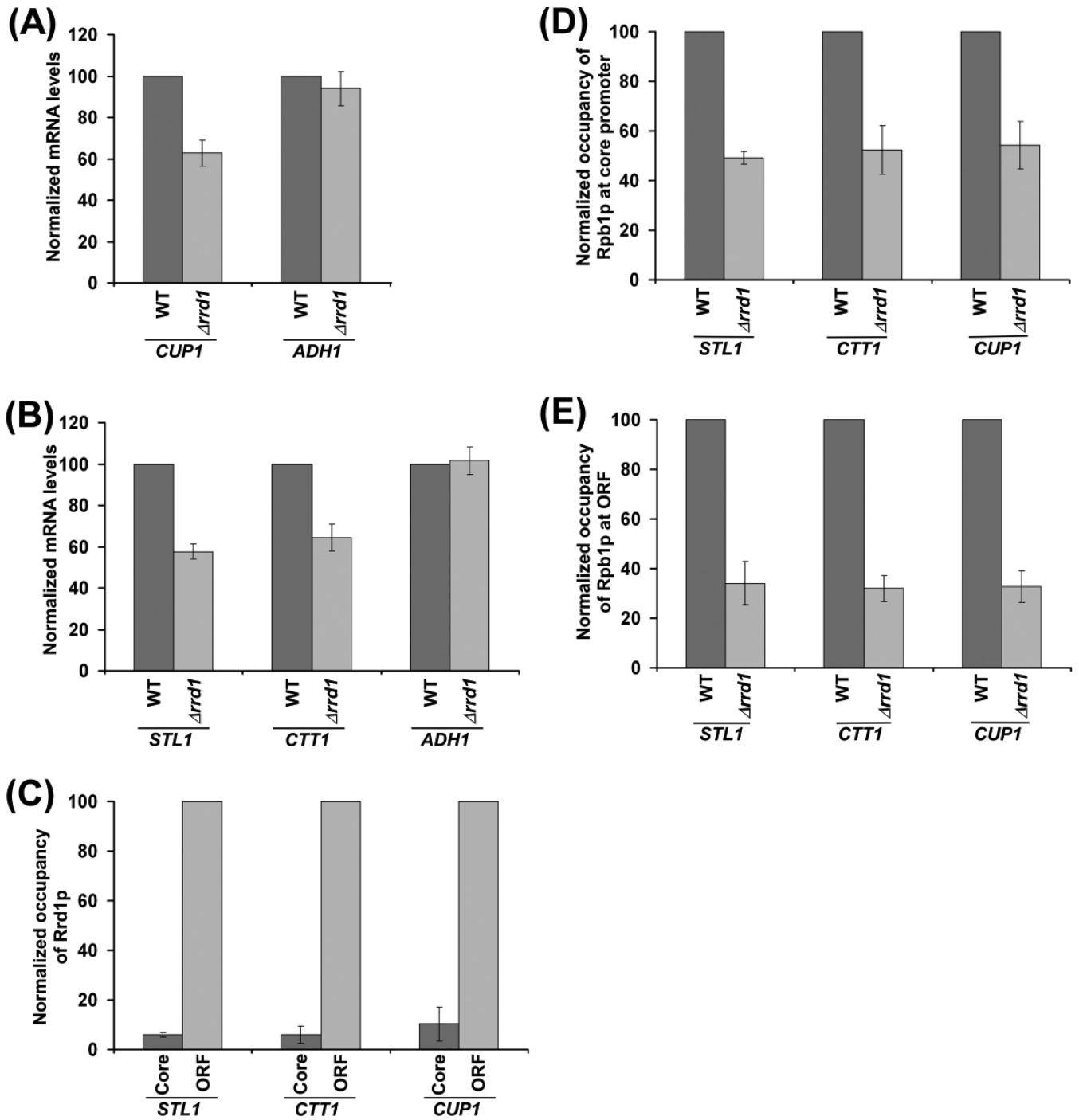
Figure 7. Rrd1p facilitates transcription of *CUP1*, *STL1* and *CTT1* following transcriptional induction. (**A** and **B**) RT-PCR analysis of *CUP1*, *STL1*, *CTT1* and *ADH1* mRNA levels in the wild-type and *Δrrd1* strains following transcriptional induction (7 min induction for *STL1* and *CTT1*; 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.
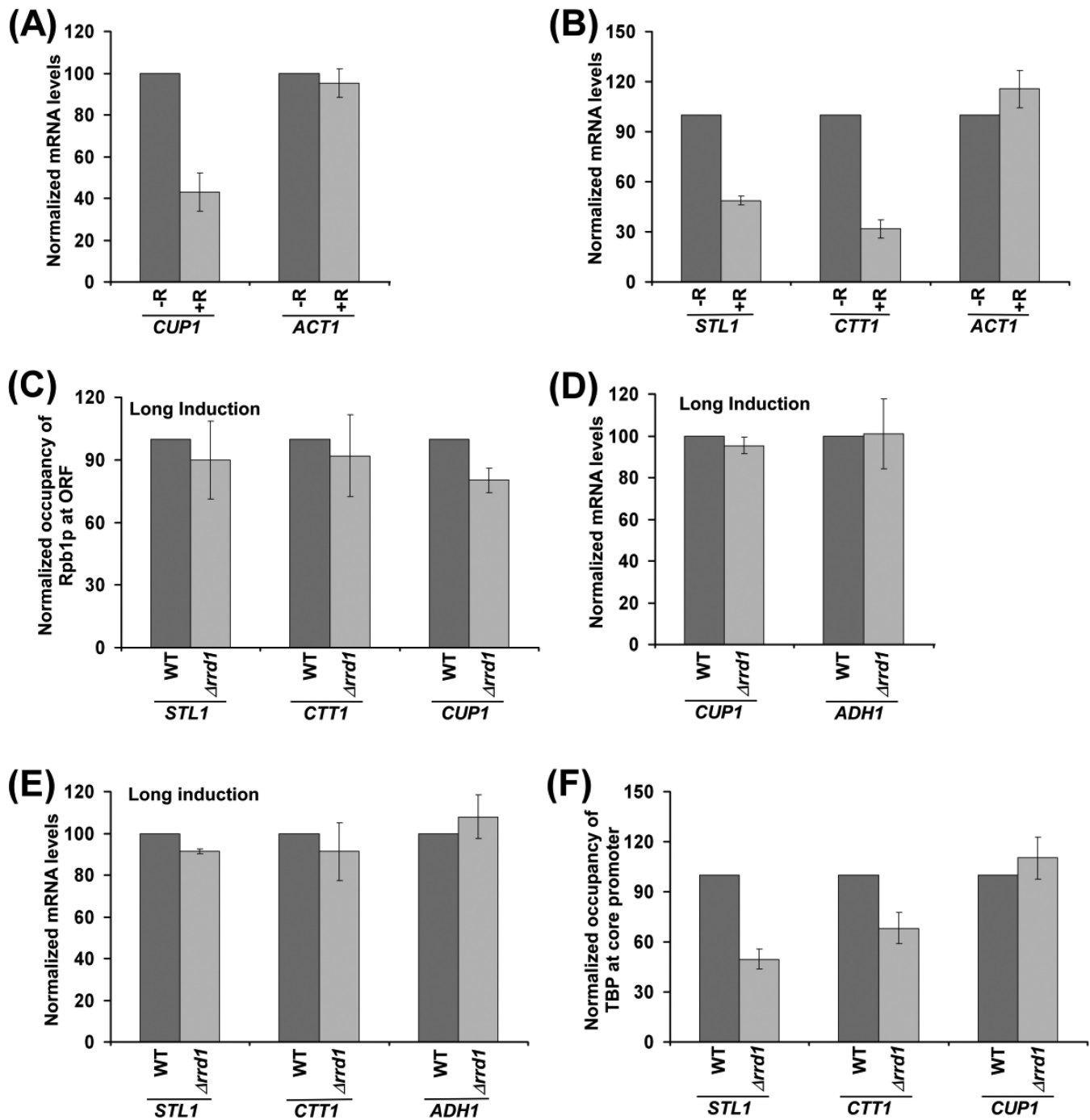
Figure 8. *CUP1*, *STL1* and *CTT1* are rapamycin-responsive genes, and are not regulated by Rrd1p following long transcriptional induction. (**A** and **B**) RT-PCR analysis of *CUP1*, *STL1, CTT1* and *ACT1* mRNA levels in the presence and absence of rapamycin treatment. (**C**) ChIP analysis of RNA 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 Rrd1p 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 Rrd1p 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, Rrd1p 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 Rrd1p (Figure 8F). Thus, Rrd1p appears to promote transcriptional elongation of *CUP1*, but not initiation. Consistently, Rrd1p 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, Rrd1p 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 Rrd1p in both transcriptional initiation and elongation of *CTT1* and *STL1*.

DISCUSSION

Rrd1p 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 Rrd1p 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 Rrd1p 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, Rrd1p promotes transcription of rapamycin non-responsive *GAL* genes in the absence of rapamycin. We also find here that Rrd1p 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 Rrd1p 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, Rrd1p 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 Rrd1p (Figure 4A–C). Collectively, our results demonstrate that Rrd1p promotes initial rounds of transcription, and has no effect on the steady-state level or constitutive transcription, thus providing a new role of Rrd1p in regulation of transcription independently of rapamycin response. Such function is medi-

ated via direct association of Rrd1p with active genes (Figures 1F, 5A and 7C, Supplementary Figure S1).

We find that Rrd1p 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 Rrd1p in comparison to the recruitment of the PIC components at the core promoter (Figures 1B, 2B, 5B, C, F, G, 7E and 8F). Further, Rrd1p interacts with RNA polymerase II for CTD phosphorylation involved in transcriptional elongation (9,10). Moreover, Rrd1p 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 Δ*rrd1* strain does not appear to be due to impaired expression of genes (including *URA* genes) as Rrd1p does not alter transcription under vegetative growth conditions in the absence of rapamycin treatment (10; Figure 4A–C). Moreover, the growth of the Δ*rrd1* 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 Rrd1p in transcriptional elongation of rapamycin-responsive and non-responsive genes independently of rapamycin treatment.

In addition to its role in transcriptional elongation, Rrd1p promotes the PIC formation at the core promoters of *GAL* genes (and hence transcriptional initiation) (Figures 2B, 5F and G). Likewise, Rrd1p 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 Rrd1p in transcriptional elongation, but not PIC formation, of rapamycin-responsive genes in the presence of rapamycin treatment. However, the role of Rrd1p in regulation of the PIC formation (and hence transcriptional initiation) was not known. Our results (Figures 2B, 5F, G and 8F) reveal that Rrd1p 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 Rrd1p 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 Rrd1 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 Rrd1p in expression of inducible genes following transcriptional induction. Thus, the role of Rrd1p in regulation of initial rounds of transcription was not known. In this study, we analyzed the role of Rrd1p in transcription of inducible *GAL* and non-*GAL* genes following transcriptional induction. We find that Rrd1p promotes transcription of these genes following transcriptional induction (Figures 2A, 5D, 7A and B). However, the effect of Rrd1p 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 Rrd1p in promoting initial/early

rounds of transcription. Since Rrd1p is conserved among eukaryotes, Rrd1p's homologue in higher eukaryotes might be playing similar functions in temporal and spatial regulation of gene expression involved in cellular differentiation and development. Thus, our results hint at possible involvement of Rrd1p's homologue in cellular differentiation and development, and hence disease pathogenesis.

In previous studies (10), *GAL1* was used as a control to show that its transcription was not altered in the *Δrrd1* strain. In contrast, we demonstrate here that transcription of *GAL1* requires Rrd1p. The reason for this discrepancy is likely to be the growth conditions. Our data demonstrate that Rrd1p has a dramatic effect on *GAL* transcription following transcriptional induction, and its effect is absent when cells were continuously grown in galactose-containing growth medium. In the previous study (10), the growth conditions for *GAL1* transcription analysis are not explicitly mentioned. Yeast cells might be continuously induced in galactose-containing growth medium in the previous study (10), and thus, the effect of Rrd1p on *GAL1* transcription was not observed.

The *Δrrd1* 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 *Δrrd1* 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, thus 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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