The Paf1 Complex Represses *ARG1* Transcription in *Saccharomyces cerevisiae* by Promoting Histone Modifications

Elia M. Crisucci and Karen M. Arndt*

Department of Biological Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania 15260

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The conserved multifunctional Paf1 complex is important for the proper transcription of numerous genes, and yet the exact mechanisms by which it controls gene expression remain unclear. While previous studies indicate that the Paf1 complex is a positive regulator of transcription, the repression of many genes also requires the Paf1 complex. In this study we used *ARG1* as a model gene to study transcriptional repression by the Paf1 complex in *Saccharomyces cerevisiae*. We found that several members of the Paf1 complex contribute to *ARG1* repression and that the complex localizes to the *ARG1* promoter and coding region in repressing conditions, which is consistent with a direct repressive function. Furthermore, Paf1 complex-dependent histone modifications are enriched at the *ARG1* locus in repressing conditions, and histone H3 lysine 4 methylation contributes to *ARG1* repression. Consistent with previous reports, histone H2B monoubiquitylation, the mark upstream of histone H3 lysine 4 methylation, is also important for *ARG1* repression. To begin to identify the mechanistic basis for Paf1 complex-mediated repression of *ARG1*, we focused on the Rtf1 subunit of the complex. Through an analysis of *RTF1* mutations that abrogate known Rtf1 activities, we found that Rtf1 mediates *ARG1* repression primarily by facilitating histone modifications. Other members of the Paf1 complex, such as Paf1, appear to repress *ARG1* through additional mechanisms. Together, our results suggest that Rtf1-dependent histone H2B ubiquitylation and H3 K4 methylation repress *ARG1* expression and that histone modifications normally associated with active transcription can occur at repressed loci and contribute to transcriptional repression.

The organization of eukaryotic DNA into chromatin presents a significant obstacle to transcription by RNA polymerase II (Pol II). To allow proper gene expression, a multitude of accessory factors associate with RNA Pol II to facilitate the transcription of a chromatin template. A conserved, multifunctional protein complex that enables proper RNA Pol II transcription is the Paf1 complex. In *Saccharomyces cerevisiae*, the Paf1 complex consists of Paf1, Ctr9, Cdc73, Rtf1, and Leo1 (36, 45, 64, 66). Many physical and genetic interactions and phenotypes implicate the Paf1 complex in regulating the elongation stage of transcription. Specifically, strains lacking Paf1 complex members exhibit phenotypes associated with transcription elongation defects, such as sensitivity to 6-azauracil and mycophenolic acid (12, 66). During transcription elongation, the Paf1 complex associates with RNA Pol II on open reading frames (ORFs) (36, 54), where it orchestrates modifications to the chromatin template (11, 35, 49, 50, 78) and influences the phosphorylation state of the RNA Pol II carboxy-terminal domain (CTD) (46, 51). In addition, the Paf1 complex genetically and physically interacts with elongation factors such as the Spf4-Spt5 (yDSIF) and Spt16-Pob3 (yFACT) complexes, suggesting coordinated functions of these elongation factors during transcription (12, 36, 66).

Appropriate transcription by RNA Pol II depends on the dynamic regulation of chromatin structure, which is modulated by histone modifications. Members of the Paf1 complex are required for the establishment of several histone modifications that are associated with active genes. Specifically, Paf1 and Ctr9 are required for histone H3 lysine (K) 36 trimethylation by the histone methyltransferase Set2 (11), and Paf1 and Rtf1 are needed for methylation of histone H3 K4 and K79 by the histone methyltransferases Set1 and Dot1, respectively (35, 49, 50). Di- and trimethylation of histone H3 K4 and K79 is dependent on the monoubiquitylation of histone H2B K123 by the ubiquitin conjugating enzyme Rad6 and the ubiquitin ligase Bre1 (7, 68). Because Paf1 and Rtf1 are also required for histone H2B ubiquitylation, the Paf1 complex most likely regulates histone H3 K4 and K79 methylation indirectly through histone H2B ubiquitylation (49, 78). Both histone H3 K4 methylation and H2B ubiquitylation correlate with active transcription. These modifications are enriched on the coding regions of active genes (5, 60, 80), and the necessary histone-modifying enzymes are recruited to active genes in a Paf1 complex-dependent manner (35, 50, 80). Importantly, Rad6 and Bre1 are evolutionarily conserved, and the interconnections between the Paf1 complex, histone H2B ubiquitylation, and gene expression observed in yeast extend to other eukaryotes, including humans (87).

Due to its multiple roles during transcription elongation, it is not surprising that the Paf1 complex is required for the proper expression of many genes (53). However, it is unclear how the Paf1 complex regulates the expression of most genes or whether the complex uses similar mechanisms to effect gene activation and repression. To investigate the repressive function of the Paf1 complex, we focused on *ARG1*, a gene whose expression is negatively regulated by Paf1 (53) and whose cis- and trans-regulatory factors are well characterized. *ARG1* transcription is repressed by the ArgR/Mcm1 complex in rich me-
dia (2, 4, 13, 14, 17, 19, 20, 55) and induced by Gcn4 in conditions of amino acid starvation (17, 24). Interestingly, although histone H2B ubiquitination is generally associated with active transcription, this modification has been implicated in ARGI repression. Deletion of RAD6, mutation of the Rad6 ubiquitin conjugation site, or mutation of histone H2B K123 results in derepression of an ARGI-lacZ reporter construct (73). Consistent with these observations, both gene-specific and genome-wide studies found increased ARGI expression in hbt1-K123R cells (38, 48, 84). Therefore, it is possible that the Paf1 complex may promote transcriptional activation and repression through the very same histone modifications. However, it is unknown whether the Paf1 complex or Paf1 complex-dependent modifications are enriched at repressed loci such as ARGI or contribute to their repression. Therefore, we investigated the role of the Paf1 complex in transcriptional repression, with a particular focus on characterizing the contributions of Paf1 complex-dependent histone modifications to repression. Our results indicate that the Paf1 complex associates with and determines the histone modification state at ARGI under repressing conditions, that Rtf1-dependent histone H2B ubiquitination can both activate and repress transcription, and that Paf1 has roles in ARGI repression beyond its known roles in facilitating histone modifications.

MATERIALS AND METHODS

Yeast strains and media. Rich (yeast extract-peptone-dextrose [YPD]) and synthetic complete (SC) media were prepared as described previously (56). Yeast strains used in these studies are isogenic with FY2, a GAL2+ derivative of S288C, and listed in Table 1 (77). Because certain cellular autoxidations influence the level of ARGI repression (E. Crisucci and K. Arndt, unpublished observations), experiments were performed with prototrophic strains where possible. Mating types of prototrophic strains were assigned through visual examination of mating with MATa and MATa tester strains. Gene disruptions were created through PCR-mediated gene replacement via transformation and/or mating, sporulation, and tetrad dissection and confirmed by PCR or Southern analysis (5, 56). PCR fragments for gene replacement with KanMX were generated by amplification of the KanMX cassette on pRS400 (6). Strains containing an integrated copy of hbt1-K123R as the only source of H2B were constructed and verified as described previously (72). Strains containing rtf1 internal deletion mutations were created through a two-step gene replacement method in which constructs encoding the N-terminally triple hemagglutinin (HA)-tagged Rtf1 derivatives were integrated to replace endogenous histone H2A. Comparison of tagged and untagged Rtf1 derivatives revealed that the HA tag did not alter Rtf1 function or interfere with ARGI repression. A yeast strain containing an integrated, tagged copy of RPB1, RPB1-13αMYC::KanMX, was constructed as previously described and generously provided by Joe Martens (22).

Northern analysis. Unless stated otherwise, 10 μg of total RNA, isolated from cells grown in YPD at 30°C to a density of 1 × 107 to 2 × 107 cells/ml, were subjected to Northern analysis with random-prime-labeled, PCR-amplified DNA probes for ARGI (+34 to +121), SNZ1 (+79 to +80), GAP1 (+133 to +1213), and SCR1 (+242 to +283) as described previously (70). Signals were quantified by using phosphorimag and ImageQuant software. ARGI signals were normalized to the loading control SCR1. To facilitate comparisons between samples and avoid introducing errors from the very low ARGI transcript levels in wild-type strains, normalized ARGI transcript levels in experimental samples are presented relative to normalized ARGI transcript levels in an arg80A control strain, which was processed in parallel. The normalized ARGI transcript levels in arg80A samples (not shown) were set equal to 1. Relative signals for at least three independent samples were averaged and plotted with the standard deviation.

Western analysis. Whole-cell extracts were prepared by a rapid boiling method as described previously (67). Briefly, cells were grown in YPD to a density of approximately 4 × 106 cells/ml. A 1.5-ml portion of culture was harvested by centrifugation and resuspended in 20-μl sample buffer (80 mM Tris pH 6.8, 2% sodium dodecyl sulfate [SDS], 1% β-mercaptoethanol, 10% glycerol, 2 mM phenylmethylsulfonyl fluoride, and 0.1% bromophenol blue) and immediately boiled for 2 min at 100°C. After glass bead lysis, an additional 80-μl sample buffer was added, and 20 μl of this lysate was separated on a SDS–10% polyacrylamide gel. Membranes were probed with a 1:2,500 dilution of anti-HA antibody (Roche catalog no. 11666606001), followed by a 1:5,000 dilution of sheep anti-mouse horseradish peroxidase-coupled secondary antibody (GE Healthcare). As a loading control, membranes were probed with a 1:100,000 dilution of anti-glucose-6-phosphate dehydrogenase antibody (GE Healthcare; Sigma catalog no. A9521), followed by a 1:5,000 dilution of donkey anti-rabbit horseradish peroxidase-coupled secondary antibody (GE Healthcare).

Chromatin immunoprecipitation (ChIP) assays. Cells were grown in YPD to a density of 107 cell/ml and harvested or washed and resuspended in minimal media and then incubated for an additional 30 min. Chromatin was prepared as described previously (65). Sonicated chromatin was incubated with antibodies at 4°C overnight. Agarose-conjugated anti-HA (Santa Cruz Biotechnology sc-7392 AC) or anti-MYC (Santa Cruz Biotechnology sc-40 AC) was used to precipitate HA-Paf1 or Rpb1-Myc, respectively. Polyclonal anti-Rtf1 antibody (66), anti-H3 trimethyl K36 (Abcam catalog no. ab1791), anti-H3 trimethyl K4 (Active Motif catalog no. 39159), anti-H3 dimethyl K4 (Millipore catalog no. 07-030), or anti-H3 (Abcam catalog no. ab1791), followed by incubation with protein A-coupled Sepharose beads (GE Healthcare catalog no. 17-2800-01), were used to precipitate Rtf1 or the appropriate histone protein. Precipitated DNA was purified by using PCR purification columns (Qiagen). For HA-Paf1 and Rtf1 ChIP assays, two dilutions of input and immunoprecipitated (IP) DNA from three independent chromatin preparations were amplified by PCR in the presence of [α-32P]dATP. PCR products were separated on 6% native polyacrylamide gels, and signals were quantified by using a phosphorimag and ImageQuant software. After signals were multiplied by their dilution factor, the average input was divided by the average IP signal. IP/input signals for ARGI were normalized to a subtelomeric control region on chromosome VI (75). For ChIP assays exam-

| Strain | Genotype |
|--------|----------|
| K1Y1302 | MATa RPBI-13×MYC::KanMX |
| K1Y1698 | MATa |
| K1Y1699 | MATa |
| K1Y1700 | MATa paf1::KanMX |
| K1Y1703 | MATa rtf1::KanMX |
| K1Y1704 | MATa rtf1::KanMX |
| K1Y1705 | MATa cre9::KanMX |
| K1Y1706 | MATa cdc73::KanMX |
| K1Y1709 | MATa arg80A::KanMX |
| K1Y1711 | MATa rad6::KanMX |
| K1Y1713 | MATa brel1::KanMX |
| K1Y1714 | MATa brel1::KanMX ura3Δ0 |
| K1Y1715 | MATa set1Δ::KanMX |
| K1Y1716 | MATa set2Δ::KanMX |
| K1Y1717 | MATa dot1::KanMX |
| K1Y1721 | MATa 3×HA-PAF1 |
| K1Y1722 | MATa 3×HA-rtf1Δ1 |
| K1Y1723 | MATa 3×HA-rtf1Δ3 |
| K1Y1724 | MATa 3×HA-rtf1Δ4 |
| K1Y1725 | MATa 3×HA-rtf1Δ7 |
| K1Y1726 | MATa 3×HA-rtf1Δ12 |
| K1Y1727 | MATa 3×HA-rtf1Δ13 |
| K1Y1731 | MATa HTA1-hbt1-K123R (hta2-hbt2)Δ::KanMX paf1Δ::KanMX |
| K1Y1732 | MATa HTA1-hbt1-K123R (hta2-hbt2)Δ::KanMX paf1Δ::KanMX |
| K1Y1755 | MATa set2Δ::KanMX |
| K1Y1805 | MATa leo1Δ::KanMX |
| K1Y1821 | MATa set1Δ::KanMX set2Δ::KanMX |
| K1Y1826 | MATa set1Δ::KanMX dot1Δ::KanMX |
| K1Y1832 | MATa set2Δ::KanMX dot1Δ::KanMX |
| K1Y1847 | MATa set1Δ::KanMX set2Δ::KanMX dot1Δ::KanMX |
| K1Y1890 | MATa 3×HA-rtf1Δ5 |
| K1Y1981 | MATa 3×HA-rtf1Δ102-104A |
| K1Y1982 | MATa 3×HA-rtf1Δ104 |
| K1Y1983 | MATa 3×HA-rtf1Δ108-110A |
| K1Y1984 | MATa 3×HA-rtf1Δ80/F123S |
| K1Y2074 | MATa HTA1-hbt1-K123R (hta2-hbt2)Δ::KanMX rtf1Δ::KanMX |
| K1Y2082 | MATa 3×HA-RTF1 leu2Δ1 trp1Δ63 lys2-128Δ5 |
ining histone modification levels, immunoprecipitated DNA from three independent chromatin preparations was used in quantitative real-time PCR with SYBR green detection (Fermentas). IP/input values for the histone modifications were normalized to those for total histone H3. Error bars represent the standard error of the mean.

RESULTS

Members of the Paf1 complex repress \textit{ARG1} transcription. The Paf1 complex was first implicated in \textit{ARG1} repression by microarray analyses investigating changes in gene expression in \textit{paf1}\(\Delta\) cells (53). To determine whether other members of the Paf1 complex are required for \textit{ARG1} repression in rich media (YPD), \textit{ARG1} transcript levels were examined by Northern analysis in wild-type strains and strains lacking individual members of the Paf1 complex (Fig. 1A and B). Early in our analysis, we discovered that certain cellular auxotrophies influence the degree of \textit{ARG1} repression in otherwise wild-type cells, even when grown in rich media (data not shown). Therefore, to eliminate any effects of auxotrophies on our measurements, we analyzed \textit{ARG1} transcript levels in prototrophic strains wherever possible. Under these conditions, wild-type cells had very low \textit{ARG1} transcript levels (Fig. 1A and B). In contrast, strains lacking Paf1, Ctr9, Cdc73, or Rtf1 exhibited high levels of \textit{ARG1} expression relative to the isogenic wild-type control strain. Of these strains, \textit{paf1}\(\Delta\) and \textit{ctr9}\(\Delta\) mutants were most defective in \textit{ARG1} repression. These results indicate that although Leol does not appear to have a very significant role, Paf1, Ctr9, and to a lesser extent Cdc73 and Rtf1 each contribute to \textit{ARG1} repression in nutrient-rich conditions.

To determine whether the repressive functions of the Paf1 complex are specific to nutrient-rich conditions or whether the Paf1 complex also negatively regulates \textit{ARG1} expression in nutrient-limiting conditions, when \textit{ARG1} is activated, we examined the effects of deleting Paf1 complex members on \textit{ARG1} transcript levels in minimal media. First, we examined the timing of \textit{ARG1} induction in wild-type cells and found that \textit{ARG1} expression was fully induced 30 min after the cells were transferred to minimal media from rich media (Fig. 1C). Consequently, we measured \textit{ARG1} expression in Paf1 complex mutant strains that were grown in YPD and shifted to minimal media for 30 min. We found that deletion of genes encoding Paf1 complex members resulted in slightly higher \textit{ARG1} expression in inducing conditions (Fig. 1D and E). Consistent with our results, it was shown previously that \textit{paf1}\(\Delta\) strains exhibit more \textit{ARG1} expression than wild-type cells when \textit{ARG1} transcription is induced with sulfoacetamid methyl, which increases cellular levels of the \textit{ARG1} activator Gcn4 (69). These results suggest that the Paf1 complex acts as a transcriptional repressor of \textit{ARG1} in both repressing and inducing conditions.

Paf1 and Rtf1 are present at \textit{ARG1} in repressing conditions. While it is known that the Paf1 complex associates with RNA Pol II during transcription elongation (36, 54), it is unclear whether the Paf1 complex localizes to repressed genes. Therefore, we examined whether the Paf1 complex localizes to \textit{ARG1} in repressing conditions by performing ChiP analysis using PCR primers that amplify the promoter, 5' middle, and 3' coding regions of \textit{ARG1} (Fig. 2A). Relative to the untagged control strain, we reproducibly detected a low level of HA-Paf1 occupancy at the \textit{ARG1} coding region in repressing conditions (Fig. 2B). Rtf1 occupancy, detected with polyclonal antiserum against Rtf1, mirrored that of HA-Paf1 and was enriched over an \textit{rtf1}\(\Delta\) control strain (Fig. 2C). These results indicate that members of the Paf1 complex localize to the \textit{ARG1} coding region in repressing conditions. When cells were shifted to minimal media, HA-Paf1 and Rtf1 occupancy increased across the \textit{ARG1} coding region indicating that, similar to other active genes, Paf1 complex occupancy correlates with gene expression levels at \textit{ARG1} (Fig. 2B and C) (43).

Since the Paf1 complex associates with RNA Pol II during transcription elongation (36, 54), Paf1 complex occupancy correlates with RNA Pol II levels on active genes (43). To determine whether Paf1 complex occupancy also correlates with RNA Pol II occupancy on a Paf1 complex-repressed gene, ChiP analysis was performed to examine Rpb1-Myc levels at \textit{ARG1} in strains grown in repressing or inducing conditions (Fig. 2D). In repressing conditions, Rpb1-Myc was enriched at the \textit{ARG1} promoter and coding region compared to the untagged control strain, indicating that low levels of RNA Pol II are present at \textit{ARG1} in repressing conditions (Fig. 2D). This is consistent with our finding that long exposures of Northern blots revealed low levels of \textit{ARG1} transcription in wild-type cells grown in rich media (data not shown). As expected, Rpb1-Myc occupancy increased across the \textit{ARG1} coding region when cells were shifted to minimal media (Fig. 2D). Therefore, similar to its association with activated genes, the Paf1 complex likely associates with \textit{ARG1} through its interaction with RNA Pol II even under repressing conditions.

Histone H3 methylation contributes to Paf1 complex-mediated \textit{ARG1} repression. The presence of the Paf1 complex at the \textit{ARG1} locus in repressing conditions suggests that the Paf1 complex may regulate the histone modification state at \textit{ARG1} under these conditions. To determine whether histone H3 methylation is present at the promoter, 5', middle, or 3' coding region of \textit{ARG1} in repressing conditions, we performed ChiP assays using antibodies that detect histone H3 K4 trimethylation, H3 K4 dimethylation, and H3 K36 trimethylation. While total histone H3 levels were similar in all strains examined (Fig. 3D), changes in the histone modification pattern at the \textit{ARG1} locus were detected in the absence of Paf1 complex members. Specifically, both histone H3 K4 di- and trimethylation marks were detected in wild-type strains at all four regions examined but were lost in strains deleted for Paf1, Rtf1, or Set1 (Fig. 3A and B). Similarly, whereas histone H3 K36 trimethylation was detected at all four locations in the wild-type strain, H3 K36 trimethylation was undetectable in the \textit{paf1}\(\Delta\) and \textit{set2}\(\Delta\) strains (Fig. 3C). Furthermore, histone H3 K36 trimethylation was specifically reduced at the promoter in the absence of Rtf1 (Fig. 3C). These results indicate that the histone H3 K4 and K36 methylation marks are present at the \textit{ARG1} locus in repressing conditions in a Paf1 complex-dependent manner.

To determine to what extent Paf1 complex-dependent histone H3 methylation is required for \textit{ARG1} repression, we performed Northern analysis of \textit{ARG1} transcript levels in strains lacking Set1, Set2, or Dot1. While previous work showed that deletion of \textit{SET2} or \textit{DOT1} caused increased expression of an \textit{ARG1-lacZ} reporter construct (48), these mutations did not lead to a change in repression of the native \textit{ARG1} gene that was statistically different from wild-type (Fig. 3E and F). The differing results may be due to increased
sensitivity of the \textit{ARG1} expression reporter or the presence of auxotrophies in the previously analyzed strains. In contrast, \textit{set1Δ} strains exhibited an increase in \textit{ARG1} expression (Fig. 3E and F). This result is consistent with the finding that loss of Bre2 or Swd3, components of the Set1-containing COMPASS complex, results in increased expression of an \textit{ARG1-lacZ} reporter construct (48). However, the increase in endogenous \textit{ARG1} transcript levels in \textit{set1Δ} cells was not as high as in \textit{paf1Δ} cells (Fig. 3E and F). Together, these results suggest that none of the methyltransferases examined individually are as important for \textit{ARG1} repression as Paf1. Since the Paf1 complex is important for multiple methylation marks, we tested whether the combined loss of multiple methyltransferases might derepress \textit{ARG1} to the same degree as deleting \textit{PAF1}. Surprisingly, no combination of double or triple mutations caused any more than an \(\sim 2.6\)-fold increase in \textit{ARG1} transcript levels, whereas deletion of \textit{PAF1} resulted in an \(\sim 12\)-fold increase in \textit{ARG1} transcript levels (Fig. 3E and F). Together, these results demonstrate that Paf1-dependent histone H3 K4 and K36 methylation are present at \textit{ARG1} in repressing conditions and histone H3 K4 methylation contributes to \textit{ARG1} repression; however, the Paf1 subunit has repressive functions in addition to facilitating histone H3 methylation.

**Rtf1 represses \textit{ARG1} by promoting histone modifications.**

Our results demonstrate that histone H3 K4 methylation is present at \textit{ARG1} in cells grown in rich media and can contribute to \textit{ARG1} repression. Because histone H3 K4 methylation is dependent on histone H2B ubiquitylation (68, 78), we tested whether the Paf1 complex mediates \textit{ARG1} repression by promoting histone H2B K123 ubiquitylation. Indeed, as previously reported, we found that \textit{rad6Δ} and \textit{bre1Δ} strains and strains in which the histone H2B ubiquitylation site is mutated (\textit{htb1-K123R}) exhibited \textit{ARG1} derepression (Fig. 4A and B) (26, 38, 48, 73, 84). Note that \textit{rad6Δ} cells exhibited higher levels of \textit{ARG1} derepression than either \textit{bre1Δ} or \textit{htb1-K123R} strains. These results are consistent with reports that Rad6 may function with another ubiquitin ligase that is required for \textit{ARG1} repression and further suggest that Rad6 has additional targets.

**FIG. 1.** Members of the Paf1 complex are required for repression of \textit{ARG1} in both repressing and inducing conditions. Representative Northern analysis (A) and quantitation (B) of \textit{ARG1} transcript levels in wild-type (KY1699), \textit{paf1Δ} (KY1700), \textit{ctr9Δ} (KY1705), \textit{cdc73Δ} (KY1704), and \textit{leo1Δ} (KY1805) strains were performed. \textit{SCR1} served as a loading control. Transcript levels were quantified and normalized to the levels detected in an \textit{arg80Δ} (KY1709) control strain (not shown) as described in Materials and Methods. The values shown are the means of three independent experiments. Error bars represent one standard deviation of the mean. (C) Northern analysis of \textit{ARG1} transcript levels in cells shifted from YPD to minimal media for various times. Transcript levels were quantified and normalized to the levels detected in an \textit{arg80Δ} (KY1709) control strain (not shown) as described in Materials and Methods. The values shown are the means of three independent experiments. Error bars represent one standard deviation of the mean.
important for ARG1 repression (73). Together, these results suggest that histone H2B ubiquitylation and downstream histone H3 K4 methylation are important for ARG1 repression. We previously identified a region within Rtf1 that is essential for these histone modifications (76); therefore, we decided to further examine the role of Rtf1 in ARG1 repression.

In addition to defining the region of Rtf1 required for histone modifications, we assigned other Rtf1 functions, including ORF association, Paf1 complex assembly, and interaction with the chromatin remodeling factor Chd1, to specific regions of the Rtf1 protein using deletion analysis (76). To determine which region and thus which function of Rtf1 is important for ARG1 repression, ARG1 transcript levels were examined in rtf1 deletion strains that define different functional classes (Fig. 4C). Mutations were chosen because they delete a region of Rtf1 with a known function (rtf1Δ1, rtf1Δ3, rtf1Δ4, rtf1Δ7, rtf1Δ12, and rtf1Δ13) or because they cause a phenotype that indicates a defect in transcription (rtf1Δ5). While Western analysis confirmed that the internal Rtf1 deletion mutant proteins were expressed to similar levels as full-length Rtf1 (Fig. 4D), Northern analysis indicated that the internal rtf1 deletions had differential effects on ARG1 transcript levels. Deletion of Rtf1 region 1 (amino acids 3 to 30), which is required for an interaction between Rtf1 and Chd1 (76), did not cause ARG1 derepression, suggesting that Rtf1-dependent recruitment of Chd1 is not required for ARG1 repression (Fig. 4E and F). Consistent with this result, a chd1Δ mutation did not alter ARG1 repression (data not shown). Similarly, cells lacking Rtf1 region 7 (amino acids 251 to 300), which is required for the association of Rtf1 with ORFs (76), showed only a slight increase in ARG1 transcription under repressing conditions (Fig. 4E and F). This result suggests that stable association with the ARG1 coding region may not be required for full repression of ARG1 by Rtf1. Furthermore, deletion of Rtf1 regions 12 (amino acids 491 to 535) or 13 (amino acids 536 to 558), which are required for the interaction between Rtf1 and other Paf1 complex members, Paf1 and Ctr9 (76), did not result in ARG1 derepression, suggesting that a stable interaction between Rtf1 and other Paf1 complex members is not required for ARG1 repression (Fig. 4E and F).

Rtf1 regions 3 (amino acids 62 to 109) and 4 (amino acids 112 to 152) are required for Rtf1-dependent histone modifications, leading us to define these regions collectively as the Rtf1 histone modification domain (HMD) (72, 76). Interestingly, deletion of Rtf1 region 3 or 4 resulted in significant ARG1 derepression, suggesting that Rtf1 mediates ARG1 repression primarily through promoting histone modifications (Fig. 4E and F). We recently identified a set of specific amino acid substitutions within the Rtf1 HMD that impair its histone modification functions (72). Therefore, we examined whether these substitutions, which greatly diminish histone H2B K123 ubiquitylation, also result in ARG1 derepression. While Western analysis demonstrated that wild-type Rtf1 and Rtf1 point mutant proteins were expressed to similar levels (Fig. 4G), cells expressing the Rtf1 point mutants, Rtf1-102-104A, Rtf1-E104K, Rtf1-108-110A, and Rtf1-F80V,F123S, exhibited ARG1 derepression similar to strains lacking Rtf1 entirely (Fig. 4H and I). Our results strongly suggest that Rtf1
mediates ARG1 repression by promoting histone H2B ubiquitylation and subsequent H3 K4 methylation. Complete deletion of RTF1 causes a suppressor-of-Ty (Spt) phenotype, indicating that deletion of RTF1 suppresses defects in transcription caused by the insertion of Ty transposons or their long terminal repeats within the promoters or 5' ends of genes (67). Strains lacking any of Rtf1 regions 3 to 9 (spanning amino acids 62 to 395) individually have an Spt phenotype, suggesting that regions 3 to 9 are each important for transcriptional regulation (76). Of these regions, only region 5 has yet to be assigned a specific function. We found that rtf1Δ strains exhibited levels of ARG1 derepression that were higher than an rtf1Δ strain (Fig. 4E and F), indicating that region 5 may have a negative effect on the function of the rest of the protein. Because our current data have not defined an obvious role for region 5 in regulating histone modifications or other known activities of Rtf1, future analysis of this region may reveal new insights on the regulation or functions of the Paf1 complex.

Histone H2B K123 is required for full derepression in paf1Δ cells. Our results suggest that Rtf1 mediates ARG1 repression primarily through histone H2B ubiquitylation and H3 K4 methylation. To test this hypothesis, we examined the effect of
FIG. 4. Rtf1 mediates ARG1 repression primarily through histone H2B ubiquitylation. Representative Northern analysis (A) and quantitation (B) measuring ARG1 transcript levels in wild-type (KY1698), htb-K123R (KY1732), rad6Δ (KY1711), and bre1Δ (KY1713) strains were performed. The y axis was shortened to facilitate comparison between lower values. The value for rad6Δ is indicated above the bar. (C) Schematic of the 13 regions of Rtf1 defined by internal deletion mutations and their associated function (62). Regions that were examined for effects on ARG1 repression are indicated in gray. (D) Western analysis of wild-type and mutant Rtf1 protein levels using an anti-HA antibody in strains expressing untagged Rtf1 (KY1698), HA-Rtf1 (KY2082), HA-rtf1Δ1 (KY1722), HA-rtf1Δ3 (KY1723), HA-rtf1Δ4 (KY1724), HA-rtf1Δ5 (KY1980), HA-rtf1Δ7 (KY1725), HA-rtf1Δ12 (KY1726), or HA-rtf1Δ13 (KY1727). G6PDH serves as a loading control. Note that a faster-migrating band observed for HA-rtf1Δ4 is likely a degradation product, which has been reproducibly observed with several forms of Rtf1 (72, 76). Representative Northern analysis (E) and quantitation (F) of relative ARG1 transcript levels in wild-type (KY1698), rtf1Δ (KY1703), HA-rtf1Δ1 (KY1722), HA-rtf1Δ3 (KY1723), HA-rtf1Δ4 (KY1724), HA-rtf1Δ5 (KY1980), HA-rtf1Δ7 (KY1725), HA-rtf1Δ12 (KY1726), and HA-rtf1Δ13 (KY1727) strains were performed. The means of three independent experiments are shown, quantified and normalized to the levels detected in an arg80Δ (KY1709) control strain (not shown) as described in Materials and Methods. Error bars represent one standard deviation of the mean. (G) Western analysis of wild-type and mutant Rtf1 protein levels using an anti-HA antibody in strains expressing untagged Rtf1 (KY1698), HA-Rtf1 (KY2082), HA-rtf1-102-104A (KY1981), HA-rtf1-E104K (KY1982), HA-rtf1-108-110A (KY1983), and HA-rtf1-F80V,F123S (KY1984). G6PDH serves as a loading control. The faster-migrating band for HA-rtf1-102-104A and HA-rtf1-108-110A has been previously observed and is likely a product of proteolysis (72, 76). Representative Northern analysis (H) and quantitation (I) of relative ARG1 transcript levels in wild-type (KY1698), rtf1Δ (KY1703), HA-rtf1-102-104A (KY1981), HA-rtf1-E104K (KY1982), HA-rtf1-108-110A (KY1983), and HA-rtf1-F80V,F123S (KY1984) strains were performed. Graphs depict the means of three independent experiments, quantified and normalized to the levels detected in an arg80Δ (KY1709) control strain (not shown) as described in Materials and Methods. Error bars represent one standard deviation of the mean.
mutating the histone H2B ubiquitylation site, alone or in combination with deletion of RTF1. We found that rtf1Δ and rtf1Δ hbt1-K123R cells had similar levels of ARG1 derepression (Fig. 5A and B), consistent with Rtf1 and histone H2B ubiquitylation functioning in the same pathway for ARG1 repression. However, rtf1Δ cells reproducibly showed significantly lower levels of ARG1 derepression than paf1Δ cells, suggesting that Paf1 has repressive functions aside from its role in promoting histone H2B ubiquitylation (Fig. 1A and B). To test whether Paf1 and H2B ubiquitylation have independent roles in ARG1 repression, we performed Northern analysis on paf1Δ hbt1-K123R double-mutant cells. If Paf1 and H2B ubiquitylation have completely independent effects on ARG1 repression, paf1Δ hbt1-K123R double-mutant strains should exhibit an elevated level of ARG1 derepression compared to paf1Δ and hbt1-K123R single-mutant strains. In contrast to this prediction, hbt1-K123R significantly reduced the level of ARG1 derepression in paf1Δ cells (Fig. 5C and D). This result suggests that histone H2B ubiquitylation is required for full ARG1 derepression in paf1Δ cells and argues that this modification can have both positive and negative effects on the same gene.

The Paf1 complex uses similar mechanisms to repress other genes. To determine whether the manner in which the Paf1 complex mediates repression of ARG1 extends to other genes, we examined the effects of deleting PAF1 and RTF1 on the expression of SNZ1 and GAP1, which encode a protein involved in vitamin B biosynthesis and a general amino acid permease, respectively. We chose to examine these genes because, like ARG1, GAP1 and SNZ1 have been shown by genome-wide expression studies to be derepressed in paf1Δ and hbt1-K123R strains (48, 53, 84). Using Northern analysis, we found that SNZ1 and GAP1 were repressed in wild-type cells...
and derepressed in the absence of Pafl or Rtf1 (Fig. 6A). Similar to ARG1, paflΔ cells exhibited higher derepression of these genes than rtf1Δ cells, suggesting that Pafl and Rtf1 may function in a similar manner at all three genes.

To further test the requirements for SNZ1 and GAP1 repression, we performed Northern analyses of these genes in strains expressing the rtf1 internal deletion mutations. We found that the expression profile of SNZ1 mirrored that of ARG1 with rtf1Δ3, rtf1Δ4, and rtf1Δ5 cells exhibiting high levels of SNZ1 derepression (Fig. 6B). Consistent with a requirement for the Rtf1 HMD in repressing SNZ1 transcription, rtf1 point mutations within the HMD-coding region also caused SNZ1 derepression (Fig. 6C). Furthermore, the levels of SNZ1 derepression that occurred in these mutants closely mimicked the effects we observed at ARG1 (Fig. 4H and I), with rtf1-102-104A cells exhibiting the least dramatic derepression and rtf1-108-110A cells exhibiting the most dramatic derepression (Fig. 6C).

Similar to both ARG1 and SNZ1, repression of GAP1 requires a functional Rtf1 HMD, since rtf1Δ cells or strains expressing the rtf1 HMD point mutations showed significant derepression of GAP1 (Fig. 6D and E). However, unlike ARG1 and SNZ1, high levels of GAP1 derepression did not occur in rtf1Δ3 or rtf1Δ5, suggesting that Rtf1 regions 3 and 4 are not equivalent in all cases (Fig. 6D and E). Furthermore, while amino acid substitutions within the HMD resulted in GAP1 derepression, the relative levels of derepression caused by these substitutions differed from those observed at ARG1 and SNZ1. Specifically, rtf1-102-104A cells exhibited a high level of GAP1 derepression and rtf1-F80V/F123S cells exhibited a low level of GAP1 derepression (Fig. 6E). While the differences between GAP1 and the other genes examined will likely enrich further studies of the functions of the Pafl complex, the overall similarities point toward a common mechanism of gene repression by the Pafl complex in which Rtf1-dependent histone modifications play a prominent role.

DISCUSSION

In this study, we investigated the mechanisms by which the yeast Pafl complex negatively regulates transcription, using the well-characterized ARG1 gene as a framework for our studies. Although genome-wide expression patterns indicate that the repressive effects of the Pafl complex are widespread (53), an analysis of how the Pafl complex mediates gene repression has not been previously described. Here, we report that the Pafl, Rtf1, Ctr9, and Cdc73 subunits of the Pafl complex contribute to ARG1 repression. Consistent with a direct repressive role, the Pafl complex is present at the ARG1 coding region when cells are grown in conditions that strongly repress ARG1 transcription. Under these conditions, histone modifications primarily controlled by Rtf1 are present at ARG1 and contribute to repression. Interestingly, Pafl appears to have repressive
functions beyond its role in mediating known Pafl complex-dependent histone modifications. Finally, an analysis of two additional genes, SNZ1 and GAPI, indicates that the characteristics of Pafl complex-mediated transcriptional repression observed at ARG1 extend to other genes.

The correlation between Pafl complex occupancy and gene activity (43) raises the question of how the Pafl complex is recruited to a gene in repressing conditions. Our data indicate a modest but significant occupancy of both the Pafl complex and RNA Pol II at the ARG1 coding region in nutrient-rich media. In these conditions, a very low level of transcriptional activity can be detected by our Northern blot assays. Therefore, consistent with its known association with RNA Pol II during transcription elongation (36, 54), we hypothesize that the low levels of transcription occurring in repressing conditions are sufficient to result in enrichment of the Pafl complex across the ARG1 locus. Interestingly, an antisense transcript traversing the ARG1 coding region was detected by Steinmetz and coworkers (16, 82), raising the possibility that antisense transcription could contribute to RNA Pol II occupancy at ARG1. Consistent with transcriptional activity in the antisense direction, histone H3 K4 methylation and K36 methylation at ARG1 were highest at 3' and 5' locations, respectively, a histone methylation pattern that is opposite of the typical distribution (34, 35, 37, 40, 50, 61, 79). A reversed histone modification pattern has been observed at GAL10 (27), one of several genes recently shown to be regulated by antisense transcription (25, 27, 74, 81). Whether the Pafl complex and its associated histone modifications repress ARG1 expression by impacting antisense transcription at the ARG1 locus remains to be determined.

In accordance with the localization of the Pafl complex to ARG1 in repressing conditions, ChIP analysis demonstrated that histone H3 K4 and K36 methylation are significantly enriched at ARG1 in a Pafl complex-dependent manner. Both histone H3 K4 and K36 methylation have been shown to impact the levels of histone acetylation on genes through several established pathways of histone cross talk. In one well-studied pathway, histone H3 K36 dimethylation is required for the activity of the Rpd3S histone deacetylase complex (HDAC), which reduces histone acetylation on transcribed genes and inhibits transcription from cryptic promoters within coding regions (8, 31, 39, 41). We found that eliminating histone H3 K36 methylation by deleting SET2 had little impact on ARG1 expression in repressing conditions, suggesting that Set2-dependent histone deacetylation is unlikely to be involved in maintaining ARG1 repression. We also found no indications that the histone H3 K79 methyltransferase, Dot1, plays an important role in ARG1 repression.

In contrast to the effects of deleting SET2 and DOT1, deletion of SET1, the gene encoding the histone H3 K4 methyltransferase, caused a significant reduction in ARG1 repression. Interestingly, histone H3 K4 methylation has been implicated in pathways that direct either the acetylation or deacetylation of histones. By recruiting the NuA3 histone acetyltransferase (HAT) complex, histone H3 K4 methylation increases histone H3 K14 acetylation levels and gene activation (42, 71). By activating the Set3 HDAC, histone H3 K4 dimethylation lowers histone acetylation levels at the 5' ends of genes (21, 33). Because histone deacetylation has well-established links to gene repression, including the silencing of genes near telomeres (reviewed in reference 62), it is possible that histone deacetylation driven by histone H3 K4 methylation and the Set3 HDAC could be involved in repressing ARG1 and other loci. However, we did not observe a loss of ARG1 repression in set3Δ cells (data not shown). Therefore, although we cannot rule out the possibility that histone H3 K4 methylation leads to the recruitment of other HDACs, we currently have no experimental support for a model in which this modification represses ARG1 through activation of the Set3 complex.

Because Rtf1 is essential for histone H3 K4 di- and tri-methylation, we chose to investigate further the role of this Pafl complex subunit in gene repression. We previously showed that disruption of the Rtf1 HMD, either through deletion or substitution of conserved residues, dramatically reduces global levels of histone H2B K123 ubiquitylation and histone H3 K4 tri- and dimethylation (72, 76). Because these same rtf1 mutations alleviate ARG1 repression to approximately the same degree as an rtf1-null allele, we conclude that Rtf1 mediates ARG1 repression primarily through its histone modification functions. In support of this idea, a comparison of rtf1Δ cells and rtf1Δ htb1-K123R cells revealed approximately the same levels of ARG1 repression, strongly suggesting that Rtf1 and histone H2B ubiquitylation function in the same pathway for ARG1 repression. Therefore, we conclude that Rtf1 mediates repression by promoting histone H2B ubiquitylation and downstream H3 K4 methylation. Similar effects of the rtf1 mutations were obtained for two other genes, SNZ1 and GAPI, suggesting that Rtf1 can repress a subset of genes through similar mechanisms.

Microarray analysis of transcript levels in htb1-K123R cells revealed that the majority of affected genes exhibited increased expression, indicating that the repressive functions of histone H2B ubiquitylation are required at many genes (48). Providing a possible mechanism for gene repression by H2B K123 ubiquitylation, a recent study revealed that this modification enhances nucleosome stability at the promoters of repressed genes (9). Although we did not detect a reduction in histone H3 occupancy at the ARG1 promoter or coding region in rtf1Δ cells, it remains possible that our ChIP assays lacked the sensitivity to detect subtle changes in nucleosome stability. In addition to its role in nucleosome stability, histone H2B ubiquitylation is required for proper telomeric silencing (28, 68). Consequently, complete deletion of RTF1 (35, 49) or disruption of the Rtf1 HMD results in telomeric silencing defects (72, 76). The genome-wide loss of histone H3 K4 and K79 methylation in these cells has been proposed to cause a redistribution of telomeric silencing factors from their normal sites of action (reviewed in reference 59). Whether similar mechanisms can influence the occupancy of regulatory factors at genes such as ARG1 remains to be determined.

In addition to its repressive role, histone H2B K123 also positively regulates ARG1 expression under certain circumstances. For example, derepression of ARG1 in a paf1Δ strain is partially suppressed by the htb1-K123R substitution (Fig. 5). The histone H2B K123 residue itself may be important for full ARG1 derepression in paf1Δ cells through effects on nucleosome structure. Alternatively, the finding of an effect of htb1-K123R in a paf1Δ cells suggests that in paf1Δ cells, a low level of histone H2B ubiquitylation occurs that is required for full
levels of ARG1 derepression. In support of this idea, a bre1Δ mutation also partially suppresses ARG1 transcription in paf1Δ strains (data not shown). Another possibility is that histone H2B ubiquitylation and subsequent deubiquitylation, which is important for full expression of inducible genes, such as GAL1 and SUC2 (15, 23, 30), may be required for full ARG1 expression in the absence of Paf1. Consistent with this possibility, the loss of Ubp8, which deubiquitylates histone H2B, somewhat reduces ARG1 expression in inducing conditions (38). Histone H2B ubiquitylation in humans has also been shown to have both positive and negative influences on transcription. For example, histone H2B ubiquitylation facilitates transcription elongation in vitro (52) and preferentially associates with sites of active transcription in vivo (44). However, removal of histone H2B ubiquitylation by the deubiquitylating enzyme Usp22 inhibits heterochromatin silencing and facilitates gene activation (85, 86). Importantly, histone H2B ubiquitylation in human cells promotes transcription of tumor suppressor genes and represses several proto-oncogenes, indicating that both the positive and the negative transcriptional effects of histone H2B ubiquitylation are critical for cancer prevention (63).

In contrast to our observations on histone modifications, our data do not indicate strong repressive roles for other Rtf1 functions, including Chd1 interaction, ORF association, and Paf1 complex association. In agreement with previous studies (46, 76), these observations suggest that members of the Paf1 complex retain some functionality when their stable interactions with each other or elongating RNA Pol II are disrupted. Differing reports on whether human Rtf1 is absent from (58, 83, 87) or present in (32) the human Paf1 complex has led to the conclusion that, like Drosophila Rtf1 (1), human Rtf1 is a less stably associated member of the complex. However, despite its less stable association with the Paf1 complex, human Rtf1 retains its effects on gene expression (18, 47). Therefore, it may not be surprising that, in yeast, repression of a subset of genes by Rtf1 does not require stable association with other Paf1 complex members.

Although Rtf1 mediates repression primarily through histone H2B ubiquitylation and its downstream modifications, our results suggest that Paf1 has repressive functions aside from histone H2B ubiquitylation and other known Paf1-dependent histone modifications. Since most known roles for the Paf1 complex are intimately connected to histone modifications (reviewed in reference 29), it will be important to explore histone modification-independent functions of the complex. Interestingly, in a recent study, the human Paf1 complex was shown to stimulate in vitro transcription of a chromatin template independently of histone modifications (32). The extensive functional conservation between the yeast and human Paf1 complexes (reviewed in reference 29) strongly suggests that mechanistic studies of Paf1 complex-mediated gene repression in yeast will yield insights on the human complex, defects in which are associated with cancers (reviewed in reference 10) and the loss of stem cell identity (18).

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