Small region of Rtf1 protein can substitute for complete Paf1 complex in facilitating global histone H2B ubiquitylation in yeast

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Histone modifications regulate transcription by RNA polymerase II and maintain a balance between active and repressed chromatin states. The conserved Paf1 complex (Paf1C) promotes specific histone modifications during transcription elongation, but the mechanisms by which it facilitates these marks are undefined. We previously identified a 90-amino acid region within the Rtf1 subunit of Paf1C that is necessary for Paf1C-dependent histone modifications in *Saccharomyces cerevisiae*. Here we show that this histone modification domain (HMD), when expressed as the only source of Rtf1, can promote H3 K4 and K79 methylation and H2B K123 ubiquitylation in yeast. The HMD can restore histone modifications in *rtf1* Δ cells whether or not it is directed to DNA by a fusion to a DNA binding domain. The HMD can facilitate histone modifications independently of other Paf1C subunits and does not bypass the requirement for Rad6–Bre1. The isolated HMD localizes to chromatin, and this interaction requires residues important for histone modification. When expressed outside the context of full-length Rtf1, the HMD associates with and causes Paf1C-dependent histone modifications to appear at transcriptionally inactive loci, suggesting that its function has become deregulated. Finally, the Rtf1 HMDs from other species can function in yeast. Our findings suggest a direct and conserved role for Paf1C in coupling histone modifications to transcription elongation.

transcription-coupled histone modifications | nucleosome

In eukaryotes, transcription occurs within the context of a restrictive, yet dynamic, chromatin environment. The posttranslational modification of histones represents a major mechanism by which cells control the structure of chromatin. Some modifications of histones include acetylation, methylation, and ubiquitylation. These modifications can alter the structural properties of nucleosomes and serve as specific effectors for the recruitment of proteins that further modify the chromatin template and regulate transcription (1).

Monoubiquitylation of histone H2B on lysine (K) 123 in *Saccharomyces cerevisiae* is a conserved modification that is enriched on active genes but plays roles in both transcriptional repression and activation (2–4). Consistent with a repressive role, H2B monoubiquitylation stabilizes nucleosomes at yeast promoters (5), inhibits the association of the RNA polymerase (pol) II kinase Ctk1 with genes in yeast (6), and interferes with the recruitment of the elongation factor TFIIS to genes in human cells (7). In other studies, H2B monoubiquitylation has been shown to stimulate transcription of chromatin templates (8), promote nucleosome reassembly during transcription elongation (9), and inhibit chromatin compaction (10). H2B monoubiquitylation is also a prerequisite for other histone modifications that mark active genes. Ubiquitylation of H2B K123 by the Rad6–Bre1 ubiquitin conjugase–ligase proteins in yeast (11–13) is required for dimethylation and trimethylation of H3 K4 and K79 by the Set1/COMPASS and Dot1 methyltransferases, respectively (14–16). Histone H3 K4 dimethylation and trimethylation subsequently stimulate the recruitment and activity of histone

acetyltransferase and deacetylase complexes, thereby governing histone acetylation patterns on genes (17–20).

From its position at the top of a histone modification cascade that determines the methylation and acetylation state of active chromatin, H2B monoubiquitylation and the factors that establish this mark are key regulators of gene expression. In addition to Rad6 and Bre1, the conserved Paf1 complex (Paf1C) is required for H2B K123 ubiquitylation. Paf1C—which in budding yeast consists of the subunits Paf1, Ctr9, Cdc73, Rtf1, and Leo1—impacts RNA synthesis at multiple stages (21). Paf1C associates with RNA pol II from the 5' end of a gene to the poly(A) site (22, 23); interacts functionally and physically with the transcription elongation factors Spt4–Spt5/DSIF, Spt16–Pob3/FACT, and TFIIS (8, 24–27); regulates the phosphorylation state of RNA pol II (28, 29); and is required for proper 3' end formation of certain transcripts (30–32). Important to this study, deletion of *RTF1* from yeast cells causes dramatic reductions in H2B K123 ubiquitylation and H3 K4 and K79 methylation (33–36), and this role in histone modification, like other Paf1C functions, is conserved in higher eukaryotes (37–40).

The mechanisms by which Paf1C promotes histone modifications have yet to be elucidated. Deletion of *RTF1*, *CTR9*, or *PAF1* reduces the occupancies of Rad6 and Set1/COMPASS on coding regions (33, 35, 41), and purified Paf1C interacts with Bre1 in vitro (42, 43), suggesting that Paf1C serves as a platform for recruiting histone-modifying enzymes to RNA pol II during elongation. Whether Paf1C has a more direct role in promoting histone modifications is not known. We previously reported that deletions and amino acid substitutions within a small region of the *S. cerevisiae* Rtf1 protein dramatically reduce global levels of H3 K4 and K79 dimethylation and trimethylation and H2B K123 ubiquitylation, leading us to define residues 62–152 as the histone modification domain (HMD) of Rtf1 (32, 44). Here we show that the Rtf1 HMD interacts with chromatin and is sufficient to promote H2B K123 ubiquitylation and H3 K4 and K79 methylation. Our findings suggest that Paf1C plays an active role in promoting conserved, transcription-coupled histone modifications.

Results

Rtf1 HMD Is Sufficient to Promote Paf1C-Dependent Histone Modifications. To investigate the role of Paf1C in histone modification, we asked whether the *S. cerevisiae* Rtf1 HMD is sufficient to promote histone modifications in the absence of all other parts of the Rtf1 protein. Because the HMD is genetically separable

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Therefore, although overexpression of the HMD is likely required for high levels of histone modifications, it does not bypass the canonical pathway for H2B K123 ubiquitylation and downstream H3 modifications.

Null alleles of *RTF1* or mutations within the HMD-coding region suppress the transcriptional defects caused by a Ty- δ element insertion mutation within the *HIS4* promoter, *his4-912 δ* , and thus confer a Suppressor of Ty or Spt⁻ phenotype (32, 44). We examined the Spt⁻ phenotype of an *rtf1 Δ his4-912 δ* strain that was transformed with the NLS-Myc-HMD or Myc-Rtf1 2-micron expression plasmids. As expected, the *rtf1 Δ* strain

transformed with the NLS-Myc vector exhibited a strong Spt⁻ (His⁺) phenotype (Fig. 2E). The NLS-Myc-HMD plasmid complemented the Spt⁻ phenotype of the *rtf1 Δ* strain, albeit to a lesser extent than the Myc-Rtf1 plasmid. This result is consistent with the conclusion that the HMD constitutes a functional domain in vivo.

HMD Can Support H2B Ubiquitylation in the Absence of Other Paf1C Members.

We investigated the extent to which other Paf1C subunits are required for the activity of the HMD. Previous studies showed that Paf1 and Ctr9 are important for Paf1C stability and integrity (26, 28). To eliminate the residual Paf1C that forms in the absence of Rtf1 (29) and test the effect on HMD activity, we transformed the NLS-Myc-HMD expression plasmid into *rtf1 Δ paf1 Δ* and *rtf1 Δ ctr9 Δ* double mutants. Remarkably, even in the absence of Paf1 or Ctr9, the HMD behaved similarly to full-length Rtf1 and was sufficient to direct H2B K123 ubiquitylation, H3 K79 dimethylation/trimethylation, and H3 K4 dimethylation, although at a reduced level compared with strains containing both Paf1 and Ctr9 (Fig. 3A and B). In contrast, H3 K4 trimethylation was greatly reduced in the *paf1 Δ* and *ctr9 Δ* backgrounds (Fig. 3A). Although a slight reduction in Rtf1 and HMD protein levels in the *paf1 Δ* and *ctr9 Δ* strains may contribute to this effect (Fig. 3B), these results suggest a stimulatory role for other Paf1C subunits in the pathway toward H3 K4 trimethylation. Expression of the NLS-Myc-HMD or full-length Rtf1 in *cdc73 Δ rtf1 Δ* strains also partially restored H3 K79 dimethylation/trimethylation and H3 K4 dimethylation, but not H3 K4 trimethylation, whereas deletion of *LEO1* had no obvious effect (Fig. S5). We conclude that the HMD is the principal effector of H2B ubiquitylation-dependent histone modifications within Paf1C and that other subunits play an important regulatory role.

Rtf1 HMD Associates with Chromatin. Two strategies were used to investigate whether the HMD promotes H2B ubiquitylation by interacting with chromatin. First, we performed ChIP analysis of HMD localization at two active genes using *rtf1 Δ* strains transformed with the 2-micron NLS-Myc-HMD or Myc-Rtf1 expression plasmids. Fortuitously, our Rtf1 antiserum (25) recognizes the HMD and reacts equivalently with the isolated HMD and full-length Rtf1 in immunoblots (Fig. S2A and B). Therefore, we used the Rtf1 antiserum to immunoprecipitate the NLS-Myc-HMD and Myc-Rtf1 proteins, and the NLS-Myc vector provided a measure of Rtf1-independent background signals. As expected for a subunit of Paf1C, the full-length Myc-Rtf1 protein showed high levels of occupancy on both genes (Fig. 4A and B). Interestingly, levels of NLS-Myc-HMD occupancy were also significantly enriched over background levels at *PYK1* and *PMI1*, demonstrating that the isolated HMD can localize to chromatin.

In a second approach, a nucleosome-pulldown assay was used to analyze the chromatin interaction properties of the HMD. Strains deleted for *RTF1* and containing either untagged or FLAG-tagged H2B were transformed with plasmids that expressed the NLS-Myc tag (vector) or the NLS-Myc-HMD (Fig. 4C, lanes 1–4). To preserve the nucleosomes, cells were exposed to formaldehyde and then mononucleosome- and dinucleosome-enriched samples were prepared by micrococcal nuclease digestion of extracts (Fig. S6A). Following the immunoprecipitation of FLAG-H2B and reversal of cross-links by heat treatment, coprecipitated proteins were examined by immunoblotting using Rtf1 antiserum. By using this approach, the NLS-Myc-HMD specifically coprecipitated with FLAG-H2B (Fig. 4C, lane 4). The coprecipitation of H3 with FLAG-H2B indicated that the FLAG-H2B was incorporated into nucleosomes. Importantly, two mutant forms of the HMD, NLS-Myc-HMD E104K and NLS-Myc-HMD F123S—which abrogate the histone modification activity of full-length Rtf1 (32) and are expressed at levels similar to the

Fig. 2. An HMD protein lacking a sequence-specific DNA binding domain can globally promote histone modifications. (A and B) ChIP analysis of H3 K4 trimethylation (A) and H3 K79 dimethylation and trimethylation (B) at the *PYK1* 5' region in an *rtf1 Δ* strain transformed with NLS-Myc vector or plasmids that express NLS-Myc-HMD or Myc-Rtf1. (C) Immunoblot analysis of histone modifications in *rtf1 Δ* strains containing the indicated plasmids. (D) Anti-FLAG immunoblot analysis of strains expressing FLAG-H2B, as indicated, and Myc-Rtf1, NLS-Myc vector, NLS-Myc-HMD, or endogenous Rtf1 (lane 7). A FLAG-H2B-K123R strain provided a specificity control. Rtf1 was detected with Rtf1 antiserum. A consistently observed Rtf1 breakdown product may be seen in lanes 1 and 2. The epitope(s) for the Rtf1 antiserum maps within the HMD (Fig. S2). (E) An *rtf1 Δ his4-912 δ* strain, transformed with indicated plasmids, was tested for complementation of the Spt⁻ phenotype by growth on SC-Trp-His (4 d) or SC-Trp control (2 d) medium at 30 °C.

Fig. 3. The HMD functions independently of other Paf1C members. Immunoblot analyses of H3 K4 and K79 methylation (A) or FLAG-H2B ubiquitylation (B) in *rtf1Δ*, *rtf1Δ paf1Δ*, and *rtf1Δ ctr9Δ* strains transformed with the indicated plasmids are shown.

wild-type HMD (Fig. S6B)—showed reduced association with FLAG-H2B (Fig. 4C, lanes 5–8). Of the two substitutions tested, E104K causes stronger defects, eliminating dimethylation and trimethylation of H3 K4 and K79, whereas F123S only reduces these marks (32). In three experiments, we consistently observed decreased nucleosome association of HMD-E104K relative to HMD-F123S. Therefore, the HMD can associate with chromatin, and this interaction requires residues important for histone modification.

We previously identified a central region of Rtf1 that is required for proper recruitment of the Paf1C to ORFs—presumably through an interaction with RNA pol II—and a C-terminal region of Rtf1 that is required for interactions with other Paf1C subunits (44). The NLS-Myc-HMD lacks these regions, raising the possibility that the HMD can associate with chromatin independently of transcription. In support of this idea, we detected enrichment of the NLS-Myc-HMD and HMD-mediated histone modifications at transcriptionally inactive loci (Fig. 4D and E and Fig. S6C). In contrast to the ChIP patterns observed at the active *PYK1* and *PMA1* genes, the levels of Myc-Rtf1 occupancy at *TELVI* and an intergenic region of chromosome V were appreciably lower than those observed for the NLS-Myc-HMD (Fig. 4D and E). Consistent with the ability of the GBD-Myc-HMD to direct histone modifications at a region lacking Gal4 binding sites (Fig. 1D and E), we also detected enrichment of the GBD-Myc-HMD at *PYK1* (Fig. 4F) and *TELVI* (Fig. S6D). These results suggest that the HMD has chromatin-association activity, but other

Fig. 4. The HMD interacts with chromatin. (A, B, D, and E) ChIP analysis of NLS-Myc, NLS-Myc-HMD, and Myc-Rtf1 localization to the 5' and 3' regions of *PYK1* (A) and *PMA1* (B), a *TEL* *VIR* proximal region (D), and chromosome V intergenic region (E) using Rtf1 antiserum. Mean values and SDs from three biological replicates are shown. (C) Immunoblot analysis of a pull-down assay measuring the association of the indicated HMD proteins with nucleosomes containing FLAG-H2B. The figure is representative of three independent experiments. (F) ChIP analysis of GBD-Myc-HMD localization at the *PYK1* 5' region using Rtf1 antiserum.

regions of Rtf1 normally direct its association and function to transcribed genes.

Histone Modification Function of the HMD Is Conserved. Sequence conservation of the HMD (44) prompted us to test the activities of Rtf1 HMD sequences from other species. Using predicted structural features as a guide, we deleted the HMD coding sequence from a plasmid-borne *S. cerevisiae* *RTF1* gene, removing amino acids 74–187, and replaced it with those of the *Schizosaccharomyces pombe*, *Drosophila melanogaster*, *Danio rerio*, and *Homo sapiens* *RTF1* genes. The *S. cerevisiae* Rtf1ΔHMD deletion protein was expressed to the same level as wild-type Rtf1 but was completely defective in supporting histone modifications (Fig. 5, lane 2). Upon introduction of the predicted HMD sequence from *S. pombe* into the *S. cerevisiae* Rtf1 protein, recovery of H2B ubiquitylation, H3 K4 dimethylation and trimethylation, and H3 K79 dimethylation/trimethylation was observed (Fig. 5, lane 5). Insertion of predicted HMD sequences from other Rtf1 homologs resulted in stable proteins that rescued the histone modification defect of the Rtf1ΔHMD protein to varying degrees. All of the chimeric proteins restored some level of H3 K4 dimethylation and H3 K79 dimethylation/trimethylation (Fig. 5, lanes 6–9), although the recovery of H2B ubiquitylation was apparently below detection levels. These results indicate that the histone modification functions of the HMD are evolutionarily conserved.

Discussion

To investigate how Paf1C stimulates transcription-coupled histone modifications, we characterized an 89-amino acid HMD of the *S. cerevisiae* Rtf1 protein. We show that this small fragment of Rtf1 can largely substitute for a complete Paf1C in promoting global H2B ubiquitylation and H3 K4 and K79 methylation. The HMD interacts with chromatin in a manner dependent on amino acids that are required for histone modification, indicating that HMD activity and chromatin association are correlated. The isolated HMD associates with and causes Rtf1-dependent histone modifications to appear at poorly transcribed regions of the genome, suggesting that the functions of the HMD are normally constrained by other parts of Paf1C. Finally, we found that HMD-like sequences from Rtf1 homologs are partially functional in *S. cerevisiae*.

Studies in yeast and metazoans have revealed a critical role for Paf1C in promoting histone modifications during transcription elongation. Defects in Paf1C cause dramatic reductions in H2B ubiquitylation, H3 K4 methylation, and H3 K79 methylation, as well as decreased occupancy of Rad6–Bre1 and Set1/COMPASS on coding regions (33, 35–38, 40, 41). In addition, in vitro assays on chromatin templates demonstrated a requirement for Paf1C in transcription-coupled H2B ubiquitylation (8, 42). Together with direct physical interactions between Paf1C and Bre1 (42, 43), these observations provide support for a model in which Paf1C mediates the interaction between histone modifiers and the RNA pol II elongation machinery. However, the retention of Rad6 at yeast promoters in *rtf1Δ* cells without concomitant H2B ubiquitylation previously led to the conclusion that Paf1C is required for the activity of Rad6–Bre1 at promoters in addition to its role in recruiting Rad6–Bre1 to transcribed regions (36, 41). Our discovery of a small region within Rtf1 that is competent to promote H2B ubiquitylation is in agreement with the activation model.

Through ChIP and nucleosome pulldown assays, we found that the HMD can interact, directly or indirectly, with chromatin. Amino acid substitutions that impair the histone modification function of Rtf1 (32) also impair the interaction of the HMD with nucleosomes (Fig. 4). This observation suggests a coupling between the histone modification and chromatin binding functions of the HMD and suggests that Paf1C, through the HMD, may transiently associate with chromatin during transcription. Whether the HMD, either alone or in the context of full-length Rtf1, interacts directly with nucleosomes or whether this association is mediated by factors such as Rad6, Bre1, or Set1/COMPASS remains to be elucidated. However, our current data indicate that deletion of these factors does not eliminate the HMD–chromatin interaction (Fig. S7). Although they do not exclude direct interactions between the HMD and the histone modifiers, our results are also consistent with the possibility that an interaction between the HMD and nucleosomes, even if transient, may prepare nucleosomes for catalysis.

Our results on the HMD provide insights into the functions of other parts of Paf1C. The isolated HMD associated with and promoted histone modifications at transcriptionally inactive loci (Fig. 4 and Fig. S6C). In contrast, full-length Rtf1 preferentially occupied transcribed genes. Our HMD constructs lack regions of Rtf1 required for its interactions with other Paf1C subunits and transcribed regions of genes (44). We conclude that, because of the absence of these interactions, the localization of the HMD is deregulated and its histone modification function appears to be uncoupled from RNA pol II. Most likely, other regions of Paf1C, such as the Rtf1 ORF association/Plus3 domain (44, 48), are dominant to the HMD in controlling the localization of Paf1C. Based on our analysis of *paf1Δ*, *ctr9Δ*, and *cdc73Δ* mutants, Rtf1 is the key member of Paf1C with respect to the establishment of H2B K123 ubiquitylation and downstream marks. However, the absence of H3 K4 trimethylation in these strains shows that the

Fig. 5. The HMD is functionally conserved. Immunoblot analysis of histone modifications in strains expressing FLAG-H2B or untagged H2B and empty vector, an *S. cerevisiae* Myc-tagged Rtf1 protein deleted for the HMD (Rtf1ΔHMD), wild-type Myc-Rtf1 (lanes 3 and 4), or chimeric Myc-Rtf1 proteins in which amino acids 74–187 of *S. cerevisiae* Rtf1 were replaced with the predicted HMD of *S. pombe*, *D. melanogaster* (two HMD lengths tested), *D. rerio*, or *H. sapiens*.

residual Paf1C plays a role in enhancing the activities of Rad6–Bre1, Set1/COMPASS, or both.

Accounting for its broad effects on gene expression, H2B monoubiquitylation has been reported to alter the physical properties of chromatin (5, 10), influence the activities of transcription elongation factors (6–9), and ensure proper transcription termination (32). The balance between gene activation and repression mediated by H2B monoubiquitylation is especially important in human cells, where this modification promotes transcription of the p53 tumor suppressor gene and inhibits the expression of protooncogenes (49). Through its association with RNA pol II and its roles in histone modification, RNA pol II phosphorylation, Chd1 recruitment, and transcription termination, the multifunctional Paf1C also influences the expression of many genes (21). Underscoring the significance of understanding the molecular functions of this complex are its connections to development, cancer, and stem cell pluripotency (50–53). Our discovery that the function of the *S. cerevisiae* HMD can be partially replaced with those of other eukaryotes highlights the conserved nature of this domain.

Methods

Yeast Strains and Growth. Yeast strains (Table S1) are isogenic to FY2 (54) and were generated through transformation or tetrad analysis (55). Rich (YPD) and synthetic complete (SC) media were prepared as described (55). To measure the Spt⁺ phenotype, plasmid transformants of strain KY619 were grown in SC–Trp medium, harvested by centrifugation, and washed with sterile H₂O. Serially diluted cultures (10-fold; starting with 1 × 10⁸ cells per milliliter) were spotted onto solid medium.

Plasmids. Details of plasmid construction are provided in SI Methods.

ChIP Assays. ChIP assays were performed as described (ref. 44; SI Methods). Sonicated chromatin was incubated overnight with primary antibodies, followed

by incubation with Protein A- or G-coupled Sepharose (Amersham Biosciences). Following reversal of cross-links and purification of DNA, immunoprecipitated (IP) and total (input) DNA were analyzed by quantitative real-time PCR using SYBR green (Fermentas) detection or by PCR performed in the presence of [α - 32 P] dATP. For the latter, PCR products were resolved on native polyacrylamide gels and quantified by using a phosphorimager. Mean values of three biological replicates with SD are shown unless otherwise noted.

Immunoblotting Analysis. Yeast cultures were grown to a density of $\sim 3 \times 10^7$ cells per milliliter in selective medium, and extracts were prepared by glass bead lysis in radioimmunoprecipitation assay buffer or trichloroacetic acid as described (44, 56). Proteins were resolved by SDS/PAGE, transferred to nitrocellulose, and immunoblotted by standard methods (*SI Methods*). Extract preparation and immunoblot analysis of FLAG-H2B ubiquitylation were performed as described (32).

Nucleosome Pulldown Assay. Yeast cultures were grown to $\sim 2.5 \times 10^7$ cells per milliliter in selective medium. Cells were treated with formaldehyde, harvested, and transferred to MNase digestion buffer (*SI Methods*). Following glass bead lysis, cells were treated with MNase (Roche) for 30 min at 37 °C. After stopping the digestion with EDTA and centrifugation, the supernatant was used in an immunoprecipitation reaction with anti-FLAG M2 affinity resin (Invitrogen), and precipitated proteins were analyzed by immunoblotting.

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