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The Rtf1 Component of the Paf1 Transcriptional Elongation Complex Is Required for Ubiquitination of Histone H2B*

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In yeast cells, the Rtf1 and Paf1 components of the Paf1 transcriptional elongation complex are important for recruitment of Set1, the histone H3-lysine 4 (H3-Lys^4) methylase, to a highly localized domain at the 5′ portion of active mRNA coding regions. Here, we show that Rtf1 is essential for global methylation of H3-Lys^4 and H3-Lys^{79}, but not H3-Lys^{36}. This role of Rtf1 resembles that of Rad6, which mediates ubiquitination of histone H2B at lysine 123. Indeed, Rtf1 is required for H2B ubiquitination, suggesting that its effects on H3-Lys^4 and H3-Lys^{79} methylation are an indirect consequence of its effect on H2B ubiquitination. Rtf1 is important for telomeric silencing, with loss of H3-Lys^4 and H3-Lys^{79} methylation synergistically reducing Sir2 association with telomeric DNA. Dot1, the H3-Lys^{79} methylase, associates with transcriptionally active genes, but unlike the association of Set1 and Set2 (the H3-Lys^{36} methylase), this association is largely independent of Rtf1. We suggest that Rtf1 affects genome-wide ubiquitination of H2B by a mechanism that is distinct from its function as a transcriptional elongation factor.

In a variety of eukaryotic organisms, histone H3 is methylated at lysines 4, 36, and 79 (respectively H3-Lys^4, H3-Lys^{36}, H3-Lys^{79}). In the budding yeast *Saccharomyces cerevisiae*, H3-Lys^4 is methylated by Set1 (1–4), H3-Lys^{36} is methylated by Set2 (5), and H3-Lys^{79} is methylated by Dot1 (6–8). Unexpectedly, genome-wide methylation of H3-Lys^4 and H3-Lys^{79} depends on Rad6, an enzyme that mono-ubiquitinates lysine 123 of histone H2B (9–12). H3-Lys^4 and H3-Lys^{79} methylation also depends on Bre1, an E3 ubiquitin ligase that is required for substrate selection of Rad6 (13, 14). The relationship between ubiquitination of H2B-Lys^{123} and methylation of H3 at lysines 4 and 79 is unidirectional, as the loss of H3 methylation does not influence H2B ubiquitination.

Set1 and Dot1 are important for heterochromatic silencing (1, 3, 6, 7, 15, 16), whereas Set2 does not appear to play a role in this process. Dot1-mediated methylation and H3-Lys^{79} itself are important for association of Sir silencing proteins at telomeres, with the effects being more dramatic at telomere-distal regions than at telomere-proximal regions (6, 7). It is likely, but not yet demonstrated, that Set1-mediated methylation of H3-Lys^4 affects Sir protein association. Interestingly, methylation of H3-Lys^4 (15, 17) and H3-Lys^{79} (18) is extremely low at heterochromatic loci, and H3-Lys^{79} methylation occurs at high levels in bulk chromatin (6). These observations suggest that Sir proteins preferentially associate with nucleosomes that are unmethylated at H3-Lys^{79} (6, 18) and that Sir proteins block the ability of Dot1 to methylate H3-Lys^{79} (18).

In addition to its role in heterochromatin silencing, Set1 has an important role in transcriptional elongation. Set1 mediates both di- and trimethylation of H3-Lys^4, and trimethylation correlates with transcriptional activity (19). Set1 is specifically recruited by elongating RNA polymerase II (pol II)[1] to a highly localized domain at the 5′ region of actively transcribed genes, thereby generating a highly localized domain of trimethylated H3-Lys^4 (20). Recruitment of Set1 depends on some, but not all, components of the Paf1 complex (20, 21). The Paf1 complex associates with elongating Pol II over the entire mRNA coding region and has a role in transcriptional elongation (22–26). The Paf1 complex is also required for recruitment of Set2 and elevated levels of H3-Lys^{36} methylation within mRNA coding regions of transcriptionally active genes (27).

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Here, we show that the Rtf1 component of the Paf1 complex is required for global dimethylation of H3-Lys^4 and H3-Lys^{79}, a result obtained independently (21). Furthermore, we show that Rtf1 is required for ubiquitination of bulk H2B, strongly suggesting that Rtf1 affects H3-Lys^4 and H3-Lys^{79} methylation through its effect on H2B ubiquitination. Rtf1 is required for silencing, and H3-Lys^4 and H3-Lys^{79} act synergistically for stability of Sir2 at the telomeres. Dot1 is also recruited to transcriptionally active mRNA coding regions, but unlike Set1 and Set2, this recruitment is largely independent of Rtf1. We suggest that Rtf1 affects genome-wide ubiquitination of H2B by a mechanism that is distinct from its function as a transcriptional elongation factor.

EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmids—Most experiments used strain UCC1111 (28), and derivatives containing *rtf1::Kan^R*, *dot1::Kan^R* and *set1::HIS5* deletion alleles that were generated by PCR-based gene replacement (29). FLAG-H2B, which contains the FLAG epitope immediately upstream of the ATG initiation codon of H2B, was expressed from the H2B promoter in the context of plasmid FB1251 (11). For analysis of Dot1 occupancy, we generated a derivative of ZGY005 (30) containing a version of Dot1 with 9 copies of the Myc epitope at the C terminus by PCR-based gene replacement (31) along with an isogenic strain containing the *rtf1::Kan^R* allele.

Western Blotting—For analyzing histone ubiquitination, exponentially growing cells were harvested by centrifugation and boiled immediately in sample buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 2%

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[1] The abbreviation used is: pol II, polymerase II.

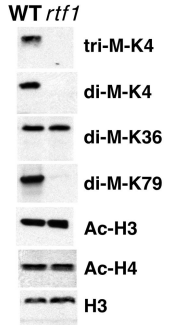

FIG. 1. **Rtf1 is required for methylation of H3-Lys^4 and H3-Lys^{79}, but not H3-Lys^{36}.** Western blots of total cellular protein from wild-type and *rtf1* deletion strains using the indicated antibodies.

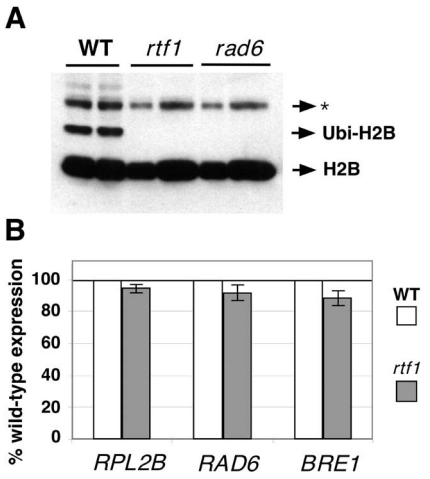

FIG. 2. **Rtf1 is required for ubiquitination of histone H2B.** *A*, Western blots of total cellular protein from wild-type, *rtf1*, and *rad6* strains containing FLAG-tagged H2B using monoclonal antibodies against the FLAG epitope. U-H2B is the slower migrating, ubiquitinated species of histone H2B, and the *asterisk* denotes an unknown protein that fortuitously cross-reacts with the FLAG antibody. *B*, levels of the indicated RNAs in wild-type (*white bars* and defined as 100%) and *rtf1* (*gray bars*) strains.

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SDS, 0.1 M dithiothreitol, 0.001% bromphenol blue, 2 mM EDTA, supplemented with Complete protease inhibitors) for 10 min with occasional vortexing, and the resulting lysates were clarified by centrifugation. For analyzing other histone modifications, cell-free extracts were prepared as described previously (7). The resulting proteins were electrophoretically separated on a 14% denaturing polyacrylamide gel and analyzed by Western blotting using antibodies against the following peptides: FLAG epitope (M2, Eastman Kodak Co.); dimethylated H3-lysine 4; dimethylated H3-lysine 36; di-acetylated H3-lysines 9 and 14; tetra-acetylated H4-lysines 5, 8, 12, and 16 (all obtained from Upstate Biotechnology); dimethylated H3-lysine 79 (7, 32).

Transcriptional Analysis—*RPL2B*, *BRE1*, and *RAD6* mRNA levels were determined with respect to *GLT1* mRNA levels by reverse transcription-PCR in real time (11, 33).

Chromatin Immunoprecipitation—Formaldehyde-cross-linked chromatin was immunoprecipitated with affinity-purified anti-Sir2 antibody (kindly provided by Danesh Moazed) or with a monoclonal antibody against the myc epitope (9e10, Upstate Biotechnology) and the resulting input and immunoprecipitated samples analyzed by quantitative PCR in real time (18). Sir2 and Dot1 occupancy values are presented in arbitrary units that are directly related to the apparent immunoprecipitation efficiency (*i. e.* the amount of material immunoprecipitated relative to that of the input sample), with a value of 1 being the experimental background.

RESULTS AND DISCUSSION

Rtf1 Is Important for Genome-wide Methylation of H3-Lys^4 and H3-Lys^{79}, but Not H3-Lys^{36}—Previously, we showed that Set1 recruitment to mRNA coding regions in *rtf1* and *paf1* deletion strains is ~15–30% of the wild-type level, even though bulk H3-Lys^4 trimethylation is abolished (20). This apparent discrepancy suggested the possibility that the Paf1 complex has a more general effect on methylation of histone H3. Western blot analysis on bulk histones shows that loss of Rtf1 abolishes dimethylated H3-Lys^4 and H3-Lys^{79}, whereas it has no significant effect on H3-Lys^{36} methylation or acetylation of H3 and H4 (Fig. 1). After this work was completed, similar observations were reported for the Rtf1, Paf1, and Ctr9 subunits of the Paf1 complex (21). Interestingly, levels of trimethylated H3-Lys^4, dimethylated H3-Lys^4, and dimethylated H3-Lys^{79} are unaffected in strains lacking the Leo1 and Cdc73 components of the Paf1 complex (20, 21).

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Rtf1 Is Required for H2B Ubiquitination—Ubiquitination of H2B-Lys^{123} is required for genome-wide methylation of H3-Lys^4 and H3-Lys^{79} (9–14). This suggested the possibility that Rtf1 might affect ubiquitination of H2B-Lys^{123}, which in turn would account for the failure to observe methylation of H3-Lys^4 and H3-Lys^{79}. Western blot analysis of wild-type and *rtf1* deletion strains containing FLAG-tagged H2B indicates that the ubiquitinated, slow migrating form of H2B is absent in the *rtf1* mutant strain (Fig. 2*A*). The levels of *RAD6* and *BRE1* mRNA are not significantly altered in the *rtf1* deletion strain (Fig. 2*B*), excluding the possibility that Rtf1 indirectly affects the expression of the enzymes that mediate H2B ubiquitination. Thus, Rtf1 is required for H2B ubiquitination.

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Rtf1 Is Important for Telomeric Silencing—Methylation of H3-Lys^4, methylation of H3-Lys^{79}, and ubiquitination of H2B-Lys^{123} are important for telomeric silencing, because strains individually lacking Dot1 (6, 7), Set1 (1, 3, 15, 16), Rad6 (9, 34), and Bre1 (13) are defective in telomeric silencing. As Rtf1 is essential for H2B ubiquitination, and hence for methylation of H3-Lys^4 and H3-Lys^{79}, it should be important for telomeric silencing. Indeed, in a standard telomeric silencing assay in which the *URA3* gene is located within the telomeric region (35), *rtf1* mutant strains show decreased growth in the presence of 5-fluoro-orotic acid, indicating that loss of Rtf1 impairs telomeric silencing. Similar results were reported after the work described here was completed (21).

H3-Lys^4 and H3-Lys^{79} Methylation Synergistically Affect Sir Protein Association at Telomeres—Dot1-mediated methylation and H3-Lys^{79} itself are important for association of Sir silencing proteins at telomeric regions (6, 7). However, the reduction in Sir protein association in *dot1* mutant strains is marginal at the telomere itself and becomes more pronounced at a region 3.5 kb from the telomere (7). This distinction is likely to reflect the fact that Sir proteins are directly recruited to the telomere by Rap1 (and associated proteins), whereas Sir protein association at telomere-distal regions is mediated primarily through interactions with histones (36). As with H3-Lys^{79}, loss of Set1-mediated H3-Lys^4 methylation results in a very marginal reduction in Sir2 occupancy at the telomeres (Fig. 3). In contrast, Sir2 association at the telomere is reduced 3-fold in the *set1*, *dot1* double mutant strain (Fig. 4), indicating that H3-Lys^4 and H3-Lys^{79} methylation synergistically affects the association of silencing proteins at heterochromatic loci. A similar 3-fold reduction in Sir2 occupancy at the telomere is observed in the *rtf1* mutant strain, suggesting that the Paf1 complex and H2B ubiquitination indirectly affect Sir protein association by virtue of their effects on H3 methylation.

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Several observations suggest that Sir proteins preferentially associate with nucleosomes that are unmethylated at H3-Lys^{79} (6, 18). The synergistic effects of *dot1* and *set1* mutations suggest that Sir proteins preferentially associate with nucleosomes in which H3 is unmethylated at both Lys^4 and Lys^{79}. Our results provide additional evidence for the similar roles of H3-Lys^4 and H3-Lys^{79} in heterochromatic silencing, and they suggest that reciprocal positive feedback loops such as pro-

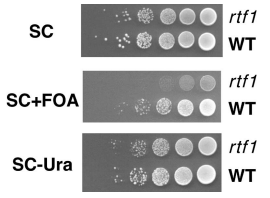

FIG. 3. **Rtf1 is important for telomeric silencing.** Equal numbers of wild-type (*WT*) and *rtf1* mutant strains were spotted at 10-fold dilution on synthetic complete (*SC*) medium in the absence of uracil (*Ura*) in the presence or absence of 0.12% 5-fluoro-orotic acid (*FOA*).

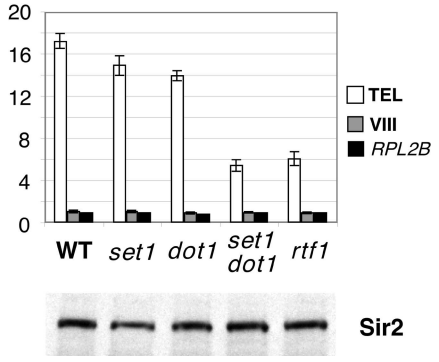

FIG. 4. **Synergistic effects of Set1 and Dot1 histone methylases on Sir2 protein association at the telomere.** Cross-linked chromatin from the indicated yeast strains was immunoprecipitated with affinity-purified Sir2 antibodies. Immunoprecipitated and input DNAs were quantified by PCR using primers for a telomeric region centered 300 bp from the chromosome end (*TEL*), an open reading frame-free region on chromosome VIII, and the *RPL2B* promoter. Sir2 occupancy at the indicated regions is presented in arbitrary units that are directly related to the apparent immunoprecipitation efficiency. Sir2 protein levels (analyzed by Western blotting) are comparable in all strains.

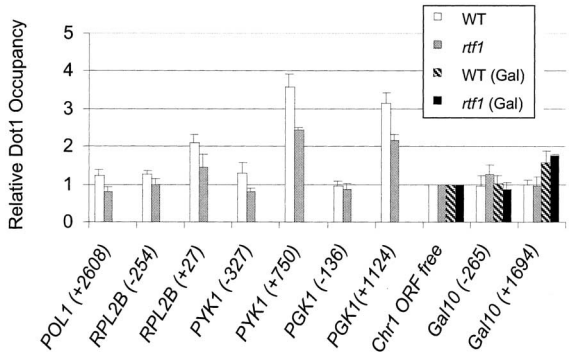

FIG. 5. **Dot1 associates with transcriptionally active genes in a manner largely independent of Rtf1.** Cross-linked chromatin from wild-type (*WT*) and *rtf1* mutant strains containing Myc-tagged Dot1 were immunoprecipitated with antibodies against the Myc epitope. Strains were grown in glucose medium except for the experiments involving *GAL10*, in which case strains were grown in raffinose or galactose medium. Immunoprecipitated and input DNAs were quantified by PCR using primers for the indicated promoter (negative numbers) and protein-coding (positive numbers) regions. Dot1 occupancy at the indicated regions is presented in arbitrary units that are directly related to the apparent immunoprecipitation efficiency, with a value of 1 being defined as the level observed on the open reading frame-free region of chromosome 1.

posed for H3-Lys^{79} (18) might also apply to Set1-mediated methylation of H3-Lys^{4}. By simultaneously affecting the H3-Lys^{4} and H3-Lys^{79} feedback loops, Rtf1 and Rad6-mediated ubiquitination of H2B would mutually reinforce the stability of the silenced state and significantly contribute to position-effect variegation.

Dot1 Associates with Transcriptionally Active Genes in an Rtf1-independent Manner—In vivo, Set1 and Set2 histone methylases associate with transcriptionally active genes in an Rtf1-dependent manner (20, 21, 27, 37). As Rtf1 is required for methylation of H3-Lys^{79}, we examined whether Dot1 also associates with active genes *in vivo* (Fig. 5). Dot1 associates with the *RPL2B*, *PYK1*, *PGK1*, and *GAL10* coding regions, but not with the corresponding promoters. In addition, Dot1 association with the *GAL10* coding region occurs when the gene is transcriptionally active (galactose medium), but not when it is quiescent (raffinose medium). In all these cases, Dot1 association is quantitatively modest (2–3-fold above the background), but clearly significant.

By analogy with Set1 and Set2 histone methylases, the association of Dot1 with transcriptionally active genes strongly suggests that Dot1 interacts with the elongating Pol II machinery. However, Rtf1 is largely dispensable for Dot1 recruitment to active genes, although the level of Dot1 association in an *rtf1* deletion strain might be slightly reduced in comparison with the wild-type strain (Fig. 5). Thus, even though Rtf1 is extremely important for global H3-Lys^{79} methylation, it plays a minor role in recruitment of Dot1 to active coding regions.

How Does Rtf1 Affect Global Ubiquitination of H2B and Methylation of H3-Lys^{4} and H3-Lys^{79}?—It is likely that genome-wide H3-Lys^{4} and H3-Lys^{79} methylation is not directly affected by Rtf1, but rather is a consequence of global H2B-Lys^{123} ubiquitination. Unlike H3-Lys^{4} trimethylation, H3-Lys^{4} dimethylation is relatively constant over the entire genome (except for heterochromatic regions) in a manner that is unrelated to transcriptional activity and to Rtf1-dependent recruitment of Set1 to mRNA coding regions (20). Furthermore, loss of Rad6, and hence H2B ubiquitination, abolishes H3-Lys^{4} methylation at active mRNA coding regions even though it has no effect on the recruitment of Set1 histone methylase (20). Global H3-Lys^{79} methylation also occurs at a relatively constant level over euchromatic regions of the genome (18), and the physical proximity of H3-Lys^{79} and H2B-Lys^{123} within the nucleosome suggests that Dot1 activity *in vivo* might depend on ubiquitinated H2B (11). Human Dot1L can methylate native, but not recombinant nucleosomes (11), and the catalytic domain may interact with ubiquitinated H2B (38).

Our results suggest that Rtf1 has distinct global and elongation-related functions. First, although we have not directly assayed the genomic pattern of H2B ubiquitination, the localization of Rtf1 to actively transcribed genes (24, 25) is very discordant with the relatively constant levels of dimethylated H3-Lys^{4} and H3-Lys^{79}, both of which depend on H2B ubiquitination. In contrast, the localization pattern of Rtf1 is consistent with and important for targeted recruitment of Set1 and Set2 histone methylases to coding regions of actively transcribed genes and the corresponding high levels of H3-Lys^{4} trimethylation and H3-Lys^{36} methylation (20, 21, 27, 37). Second, Rtf1 is differentially required for recruitment of histone methylases to coding regions and genome-wide methylation. Genome-wide methylation of H3-Lys^{36} is not affected by Rtf1 (Fig. 1) (21), even though Rtf1 is critical for Set2 recruitment and elevated H3-Lys^{36} methylation at transcriptionally active genes (27). Conversely, Rtf1 is essential for genome-wide methylation of H3-Lys^{79} (Fig. 1) (21), but it plays a minimal role in recruitment of Dot1 to active genes (Fig. 5). Third, unlike Rtf1 association with active coding regions, the Rad6 and Bre1 enzymes mediating H2B ubiquitination are recruited to promoter regions in an activator-dependent manner (13).

We cannot exclude the possibility that Rtf1 might mediate global H2B ubiquitination in the context of the elongating Paf1-Pol II complex, but such a model would require additional features. For example, yeast pol II might elongate promiscuously over the entire genome at a low level, thereby permitting genome-wide, Rtf1-dependent ubiquitination of H2B. There is

no evidence for such a nonspecific function of elongating Pol II, although this might be difficult to detect by conventional chromatin immunoprecipitation or transcriptional analyses. Alternatively, it is formally possible that H2B ubiquitination may spread from transcribed regions to non-transcribed regions if ubiquitination at a given nucleosome facilitates ubiquitination of a neighboring nucleosome.

For these reasons, we suggest that the Paf1 complex, or a subcomplex containing Rtf1, functions independently of its association with elongating pol II to mediate genome-wide ubiquitination of H2B. Such an Rtf1-containing complex might interact with both the Rad6-Bre1 ubiquitinating activity and with histones, thereby acting as a bridge between the enzyme and substrate and facilitating H2B ubiquitination. Although our results are restricted to the Rtf1, it is likely that the Paf1 and Ctr9 components are also important for global H2B ubiquitination, because loss of Paf1 or Ctr9 abolishes H3-Lys^4 and H3-Lys^{79} methylation (20, 21). In contrast, the Leo1 and Cdc73 components of the Paf1 complex do not affect methylation of either Lys^4 or Lys^{79}, indicating that they also do not affect H2B ubiquitination. Although the mechanism remains to be resolved, our results demonstrate an unexpected link between specific components of the Paf1 complex and global ubiquitination of H2B.

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