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Ubiquitination of Histone H2B by Rad6 Is Required for Efficient Dot1-mediated Methylation of Histone H3 Lysine 79*

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Dot1 is a non-SET domain protein that methylates histone H3 at lysine 79, a surface-exposed residue that lies within the globular domain. In the context of a nucleosome, H3 lysine 79 is located in close proximity with lysine 123 of histone H2B, a major site for ubiquitination by Rad6. Here we show that Rad6-mediated ubiquitination of H2B lysine 123 is important for efficient methylation of lysine 79, but not lysine 36, of histone H3. In contrast, lysine 79 methylation of H3 is not required for ubiquitination of H2B. Our study provides a new example of *trans*-histone regulation between modifications on different histones. In addition, it suggests that Rad6 affects telomeric silencing, at least in part, by influencing methylation of histone H3.

Histones are subjected to post-translational modifications such as acetylation, phosphorylation, ubiquitination, and methylation. Methylation on specific lysine or arginine residues is carried out by distinct classes of enzymes. The CARM1/PRMT1 class of enzymes mediates arginine methylation, while the SET domain-containing enzymes mediate lysine methylation (1, 2). In *Saccharomyces cerevisiae*, arginine methylation of histones has not been described, but the N-terminal tail of histone H3 is methylated at lysines 4 and 36. Set1 is the lysine 4 methylase because lysine 4 methylation is abolished in a *set1* deletion strain and the Set1 complex methylates lysine 4 *in vitro* (3–6). Set1 methylation of H3 lysine 4 is important for rDNA silencing (3, 7). Lysine 36 methylation of H3 is mediated by Set2, and artificial recruitment of Set2 to a promoter results in transcriptional repression (8). Unlike the case in higher eukaryotes, methylation of histone H3 at lysines 9 and 27 and methylation of histone H4 at lysine 20 are not observed in *S. cerevisiae* (1, 9, 10).

Recently *S. cerevisiae* Dot1 and the related human protein have been identified as histone methylases that specifically methylate lysine 79 within the globular domain of histone H3 (11–14). These studies demonstrate that histone H3 can be methylated outside the tail region, and they provide the first example of a non-SET domain protein that mediates lysine methylase activity. Based on the crystal structure of a nucleosome, lysine 79 is a surface-exposed residue that is located within loop 1 between helix 1 and helix 2 of histone H3 (15). Dot1 methylates lysine 79 only in the context of nucleosomes, indicating that a certain structural feature of the nucleosome is required for enzymatic activity (11, 12). A similar requirement for the nucleosomal configuration has been reported for Set2 and SET8/PR-SET7 (8–10).

In yeast cells, either loss or overexpression of Dot1 results in impaired telomeric silencing (16). Telomeric silencing is also disrupted by mutations of lysine 79 of histone H3 or by mutations that abolish the catalytic activity of Dot1, suggesting that Dot1 influences telomeric silencing largely through methylation of lysine 79 (11, 12). This defect in telomeric silencing might reflect an interaction between Sir proteins and lysine 79 because *dot1* and lysine 79 mutations weaken the interaction of Sir2 and Sir3 with the telomeric region *in vivo* (11, 12).

In *S. cerevisiae*, histone H2B is monoubiquitinated at lysine 123 by Rad6 (17), and Rad6 is important for telomeric silencing (18). Unlike higher eukaryotes, ubiquitination of histone H2A has not been reported (19), and mutations of the putative H2A ubiquitination site have no phenotypic effect (17). Interestingly, Rad6-mediated ubiquitination of H2B lysine 123 is required for methylation of H3 at lysine 4, a phenomenon termed *trans*-regulation (20, 21). Here we provide a second and related example of *trans*-histone regulation of histone modifications by showing that Rad6-mediated ubiquitination of H2B is also important for Dot1-mediated methylation of H3 lysine 79. A similar conclusion was reported in a recent paper that appeared while this manuscript was in preparation (22).

EXPERIMENTAL PROCEDURES

Yeast Strains—Strains UCC1111 (*MAT* α, *ade2*::*his3*-Δ200, *leu2*-Δ0, *lys2*-Δ0, *met15*-Δ0, *trp1*-Δ63, *ura3*-Δ0, *adh4*::*URA3-TEL* (VII-L), *hhf2-hht2*::*MET15*, *hhf1-hht1*::*LEU2*, pRS412(*ADE2 CEN ARS*)-*HHF2-HHT2*) has been described previously (23). Strain FY406 (*MAT* **a** *hta1-htb1*Δ::*LEU2*, *hta2-htb2*Δ::*TRP1*, *leu2*-Δ1, *ura3*-52, *trp1*-Δ63, *his3*-Δ200, pSAB6(*URA3*, *CEN*, *ARS*, *HTA1-HTB1*)) was obtained from Fred Winston. Derivatives of UC1111 and FY406 containing *rad6*::Kan^R and *dot1*::Kan^R deletion alleles were generated by PCR-based gene replacement of the wild-type loci (24). Strains expressing FLAG-tagged or K123R derivatives of H2B were generated by standard plasmid shuffling in strain FY406. The plasmid expressing FLAG-H2B was generated by using PCR to insert DNA encoding the FLAG epitope (DYKDDDDK) immediately upstream of the ATG translational initiation codon of the H2B gene in the context of plasmid FB1251 (*HIS3 CEN ARS HTA1-HTB1*), which was kindly provided by Fred Winston. DNA encoding the H2B-K123R mutation was generated by PCR-mediated mutagenesis (QuikChange site-directed mutagenesis kit, Stratagene) of plasmid FB1251. All DNA constructs generated by PCR were sequenced to verify their sequences.

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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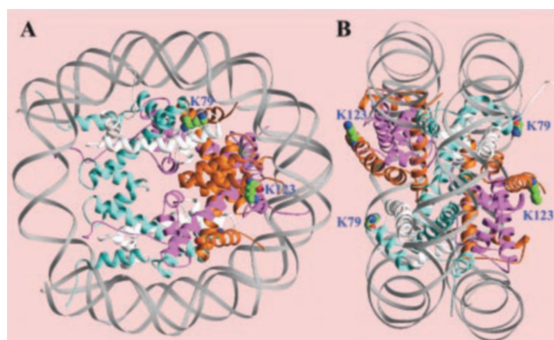

FIG. 1. **Histone H3 Lys-79 and H2B Lys-123 positions in the nucleosome.** *A*, the structure of the yeast nucleosome core particle (26), viewed along the superhelical axis, is shown in a *ribbon* representation (H3, *cyan*; H4, *white*; H2A, *magenta*; H2B, *brown*; DNA, *gray*). Lysine 79 of H3 and lysine 123 of H2B are shown in a *ball* model (carbon, *green*; oxygen, *red*; nitrogen, *blue*). *B*, the nucleosome core structure viewed in a direction perpendicular to that in *A*. The figures were generated using the Ribbons program (29).

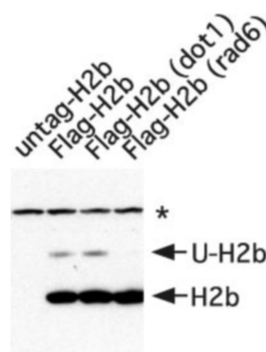

FIG. 2. **Dot1 is not required for ubiquitination of histone H2B.** Total cellular protein from strains containing untagged-H2B (FY406) or FLAG-tagged H2B (FY406 and *dot1* and *rad6* derivatives) were analyzed by Western blotting using monoclonal antibodies against the FLAG epitope. *U-H2B* is the slower migrating, ubiquitinated species of histone H2B, and the *asterisk* denotes an unknown protein that fortuitously cross-reacts with the FLAG antibody.

SDS, 0.1 M dithiothreitol, 0.001% bromphenol blue, 2 mM EDTA supplemented with Complete protease inhibitors (Roche Molecular Biochemicals)) 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 (12). 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, Kodak); diacetylated H3 lysines 9 and 14; tetraacetylated H4 lysines 5, 8, 12, 16; dimethylated H3 lysine 4; dimethylated H3 lysine 36; dimethylated H3 arginine 17; dimethylated H3 arginine 26 (all obtained from Upstate Biotechnology); and dimethylated H3 lysine 79 (12, 13). Dot1 protein levels were analyzed with monoclonal antibodies against the Myc epitope (Santa Cruz Biotechnology, 9e10) in strains expressing $(Myc)_5$-Dot1 from the natural *DOT1* promoter (12).

Transcriptional Analysis—*DOT1* mRNA levels were determined with respect to *GLT1* mRNA levels by reverse transcription-PCR in real time as described previously (25). For both genes, two primer pairs at different positions (*DOT1* positions 1383–1632 and 1188–1497 and *GLT1* positions 5786–6045 and 3244–3468) were used.

RESULTS AND DISCUSSION

Lysine 123 of Histone H2B and Lysine 79 of Histone H3 Are in Close Proximity in the Context of a Nucleosome—The crystal structure of a nucleosome (15, 26) reveals that lysine 123 of H2B and lysine 79 of histone H3 are in close proximity (Fig. 1*A*). Both modification sites are found on solvent-accessible surfaces of the nucleosome (Fig. 1*B*). These structural considerations and the fact that ubiquitination of H2B lysine 123 is required for methylation of H3 at lysine 4 (20) prompted us to investigate the relationship between Rad6-mediated ubiquitination of H2B lysine 123 and H3 methylation of lysine 79.

Dot1 Is Not Required for Ubiquitination of Lysine 123 of Histone H2B—To detect the ubiquitinated form of histone H2B, we constructed a yeast strain with FLAG-tagged H2B as the only copy of H2B gene. The ubiquitinated histone H2B can be differentiated from the non-modified form by Western blot analysis using an antibody against the FLAG epitope (Fig. 2). Deletion of *DOT1* in this strain background does not alter the level of ubiquitinated histone H2B, while deletion of *RAD6* leads to total loss of the slow migrating ubiquitinated form. Thus, lysine 79 methylation of H3 is not required for ubiquitination of bulk H2B *in vivo*.

Ubiquitination of Histone H2B Is Required for Efficient Methylation of Lysine 79 of Histone H3—To address whether ubiquitination of lysine 123 of histone H2B affects methylation of H3 lysine 79, we analyzed strains harboring a *rad6* deletion or a K123R mutation in H2B. Using an antibody that specifically recognizes dimethylated lysine 79 of H3 (12, 13), we observed a drastic loss in lysine 79 methylation of bulk histone H3 in both *rad6* and K123R mutant strains (Fig. 3*A*, *lanes 2* and *4*). However, the level of lysine 79 methylation in these mutant strains is clearly above that of the *dot1* mutant strain (*lane 6*) and is ~5% of the level observed in wild-type strains (*lanes 1*, *3*, and *5*). Importantly, the levels of Dot1 mRNA (Fig. 3*B*) and Myc-tagged Dot1 protein (Fig. 3*C*) are not significantly changed in both *rad6* and K123R mutants, indicating the reduction of lysine 79 methylation in the absence of histone H2B ubiquitination is not due to an indirect effect on Dot1 levels. Thus, Dot1 methylation of H3 lysine 79 strongly depends on Rad6-mediated ubiquitination of H2B lysine 123.

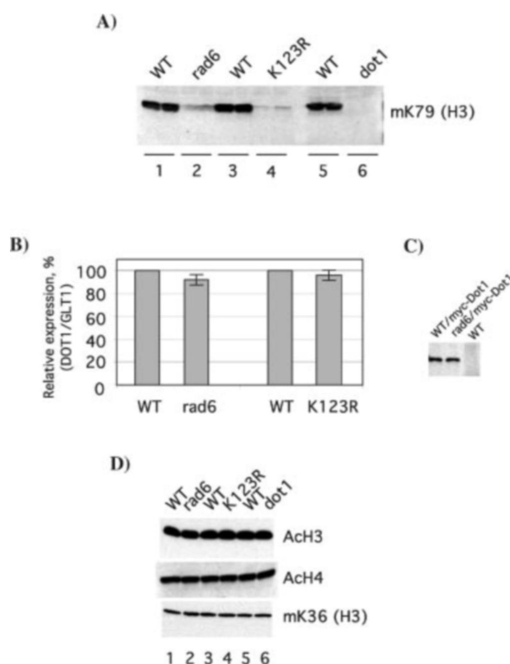

FIG. 3. **Histone H2B ubiquitination is required for methylation of lysine 79 of histone H3.** *A*, total cellular protein from the indicated strains (UCC1111 derivatives, *lanes 1* and *2* and *5* and *6*; FY406 derivatives, *lanes 3* and *4*) were analyzed by Western blotting using antibodies against dimethylated lysine 79 (*mK79*). *B*, relative *DOT1* mRNA levels (expressed as a percentage of *GLT1* levels in the same sample) in *rad6*, H2B-K123R, and isogenic wild-type strains. *C*, levels of $(Myc)_5$-Dot1 protein in wild-type and *rad6* derivatives (UCC1111 background) that do or do not express the protein. *D*, Western blotting of samples in *A* using antibodies against acetylated H3 (*AcH3*), acetylated H4 (*AcH4*), and dimethylated lysine 36 (*mK36*). *WT*, wild type.

Ubiquitination of Histone H2B Is Not Generally Required for Methylation of Histone H3—Our results on Dot1-mediated methylation of H3 lysine 79 are similar to the Rad6-dependent effects on H3 lysine 4 methylation (20, 21) and hence provide a second example of *trans*-histone regulation of histone modifications. Given the importance of H2B ubiquitination for H3 methylation by two distinct histone methylases (Set1 and Dot1), we addressed whether histone H2B ubiquitination is generally required for histone methylation. In *S. cerevisiae*, H3 is methylated at lysines 4, 36, and 79, while histone H4 has not been reported to be methylated, and we are unable to detect arginine methylation of H3 at residues 17 and 26 using antibodies against methylated peptides (data not shown). Methylation of lysine 36 is mediated by Set2 methylase (8). Western blot analysis using an anti-dimethylated lysine 36 antibody indicates that the level of lysine 36 methylation is not altered in either *rad6* or K123R mutant cells (Fig. 3*D*). In addition, *rad6* and H2B-K123R mutations do not affect bulk acetylation of histones H3 and H4. Thus, ubiquitination of histone H2B specifically regulates methylation of lysines 4 and 79 but does not affect methylation of lysine 36. The effects of H2B ubiquitination on methylation of lysine 4 and lysine 79 are clearly independent because Set1 does not affect lysine 79 methylation and Dot1 does not affect lysine 4 methylation (12).

Mechanistic Implications—How does ubiquitination of H2B affect methylation of H3 lysine 79? *In vitro*, Dot1 is unable to methylate H3 on nucleosomes assembled from recombinant core histones, while nucleosomes reconstituted from HeLa core histones are excellent substrates,[1] suggesting that histone modifications *per se* may influence Dot1 activity. Given that H2B lysine 123 and H3 lysine 79 are in close proximity in the context of a nucleosome (Fig. 1), the simplest explanation is that Dot1 preferentially recognizes ubiquitin-modified nucleosomes. From a structural perspective, it is plausible that this might involve a direct interaction between Dot1 and the ubiquitin moiety on lysine 123 of H2B, although this remains to be shown. Although very important, H2B ubiquitination is not absolutely essential for Dot1 methylation of H3 lysine 79 *in vivo* because a low level of lysine 79 methylation is detected in a *dot1* mutant strain. This residual Rad6-independent methylation of lysine 79 might occur globally, or it could occur at specific locations to which Dot1 is recruited.

A potential problem with the explanation that Dot1 preferentially recognizes nucleosomes with ubiquitinated histone H2B is that, in yeast, the level of H2B ubiquitination is ~10% (17), whereas the level of lysine 79 methylation is as high as 90% (11). However, ubiquitination is a highly labile modification, and it is likely that the competing processes of histone ubiquitination and deubiquitination are in dynamic equilibrium as is the case for histone acetylation (27). In contrast, methylated lysines are stable, and it is unclear whether histones can be actively demethylated (28). Thus, we speculate that methylation of H3 lysine 79 occurs primarily during the relatively limited times when H2B in the same nucleosome is ubiquitinated but accumulates on most H3 molecules due to the stability of the modification. Alternatively, H2B ubiquitination might perturb the regional conformation within the nucleosome, which in turn serves as a favorable substrate for Dot1.

Loss of Rad6 and the H2B-K123R mutation of histone H2B lead to defective telomeric silencing (18, 20). Loss of Dot1 and mutation of H3 lysine 79 significantly reduces telomeric silencing and the association of the Sir proteins at the telomeric ends (11, 12). Our observation that *rad6* and H2B-K123R mutant strains are severely defective for H3 lysine 79 methylation suggests the possibility that Rad6 affects telomeric silencing, at least in part, by influencing methylation of histone H3. A similar suggestion has been made for H3 lysine 4 (20, 21). Thus, ubiquitination of H2B lysine 123 might be critical for silencing primarily (and perhaps exclusively) by virtue of *trans*-histone regulation of methylation at lysines 4 and 79.

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[1] H. H. Ng, R.-M. Xu, Y. Zhang, and K. Struhl, unpublished observation.

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