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Histone H2B Ubiquitylation Controls Processive Methylation but Not Monomethylation by Dot1 and Set1

Short Article

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Summary

Methylation is a relatively stable histone modification, yet regulation of the transition between mono-, di-, and trimethylation of lysine (K) residues may control dynamic processes such as transcription and DNA repair. Identifying factors that regulate the ability of methyltransferases to perform successive rounds of methylation on the same lysine residue is important for understanding the functions of histone methylation. Previous reports have indicated that ubiquitylation of histone H2B K123 is required for methylation of lysines 4 and 79 of histone H3 by the methyltransferases Set1 and Dot1, respectively. In contrast, by using chromatin immunoprecipitation and mass spectrometry, we find that ubiquitylation of H2B-K123 is dispensable for monomethylation of H3-K4 and H3-K79 but is required for the transition from monomethylation to subsequent methylation states. Dot1 binding to chromatin occurs normally in the absence of H2B-K123 ubiquitylation, suggesting that ubiquitylation does not regulate enzyme recruitment but does regulate the processive activity of the histone methyltransferase.

Introduction

The histone proteins, around which eukaryotic DNA is wound, play important roles in numerous cellular processes including condensing the DNA and protecting it from endonucleases as well as regulating processes such as DNA replication, repair, recombination, and transcription. One mechanism to diversify histone function to accomplish these multiple tasks involves their posttranslational modification by acetylation, ubiquitylation, methylation, and phosphorylation.

In *Saccharomyces cerevisiae*, histone H3 is known to be methylated at three residues (lysines 4, 36, and 79) by distinct enzymes (Set1, Set2, and Dot1, respectively). In accordance with their evolutionary conservation from *S. cerevisiae* to *H. sapiens*, these histone methylation marks regulate fundamental cellular processes. Set1 and Set2 specifically localize to and methylate actively transcribed genes, pointing to roles in transcription (Hampsey and Reinberg, 2003). Indeed, methylated H3-K4 appears to recruit the chromatin remodeling factors Isw1 and Chd1 (Pray-Grant et al., 2005; Santos-Rosa et al., 2003), and the loss of H3-K36 methylation at active genes leads to deficient transcription (Hampsey and Reinberg, 2003). Although Dot1 specifically localizes to active genes, its only supported role in transcription is an indirect effect on gene repression in heterochromatin (Ng et al., 2002a; van Leeuwen et al., 2002). It has been recently shown that H3-K79 methylation is important for survival after exposure to ionizing radiation in mammals (Game et al., 2005), most likely because it is required for the recruitment of the DNA repair protein p53 binding protein 1 (53BP1) to double-strand breaks (Huyen et al., 2004).

The fact that the ϵ-amino group of lysine residues can accept up to three methyl groups broadens the potential roles for each of the methylated residues. Evidence suggests, at least for H3-K4 and H3-K9 (K9 is methylated in higher eukaryotes), that there are indeed functional differences between di- and trimethylation. For example, H3-K4 trimethylation exhibits a strong preference for the 5′ end of active genes, whereas dimethylation is found at both inactive and active genes (Ng et al., 2003; Santos-Rosa et al., 2002). Furthermore, in *Neurospora*, H3-K9 trimethylation, but not dimethylation, causes transcriptional repression in a reconstituted chromatin transcription system (Wang et al., 2003). Methyltransferases also vary in their ability to mono-, di-, and trimethylate. Mammalian SET7/9 preferentially monomethylates substrates at H3-K4 (Zhang et al., 2003); the H3-K9 methyltransferase DIM-5, on the other hand, primarily trimethylates its substrate (Tamaru et al., 2003); and G9a, which also methylates H3-K9, is predominantly a dimethyltransferase (Rice et al., 2003). Other enzymes have a broader range of modification: Set1 and Dot1 perform mono-, di- and trimethylation of histone H3 at K4 (Santos-Rosa et al., 2002) and K79 (van Leeuwen et al., 2002), respectively.

Previous studies have indicated that ubiquitylation of histone H2B at K123 is essential for both K4 and K79 methylation (Briggs et al., 2002; Dover et al., 2002; Ng et al., 2002b; Sun and Allis, 2002). In contrast, we demonstrate that the absence of H2B K123 ubiquitylation does not abolish the activity of Set1 and Dot1; these enzymes are able to monomethylate, but their ability to carry out subsequent di- and trimethylation is impeded. Consistent with this finding, Dot1 recruitment to chromatin occurs normally in the absence of H2B-K123 ubiquitylation. Therefore, our results indicate that H2B-K123 ubiquitylation regulates neither enzyme recruitment nor monomethylation but does regulate the processivity of the histone methyltransferase.

Results and Discussion

To investigate the role of histone H2B ubiquitylation in histone H3 lysine 79 methylation, we first raised antibodies against H3 peptides that contain either a di- or trimethylated form of K79. (An antibody to the monomethylated form of H3-K79 was obtained commer-

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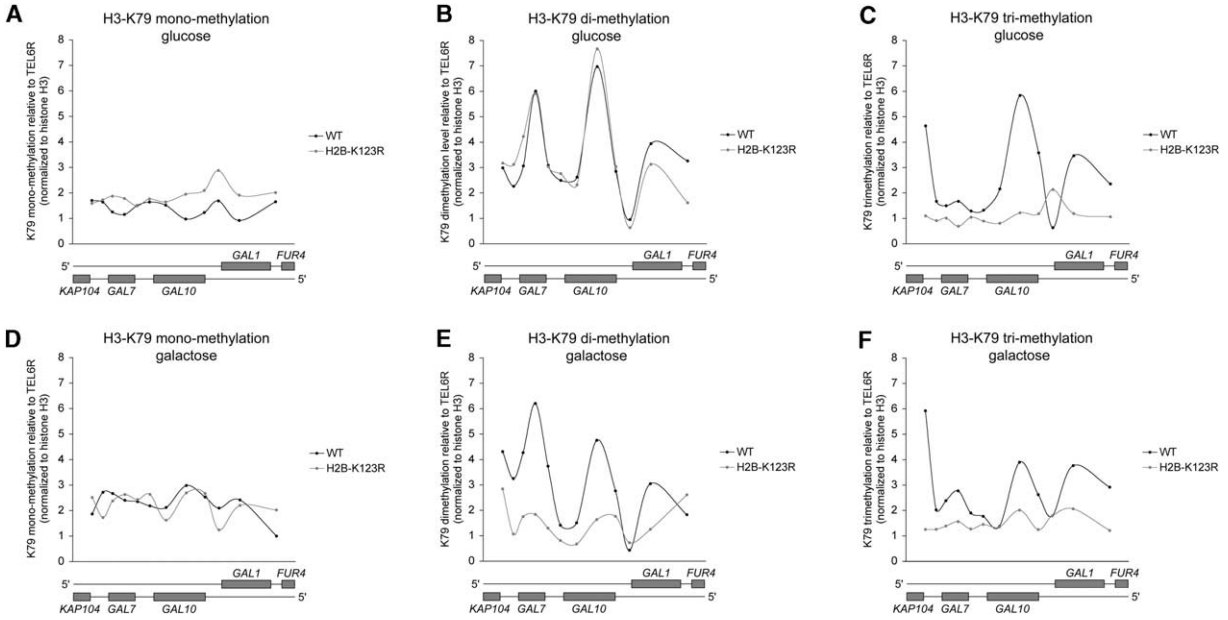

Figure 1. Chromatin Immunoprecipitation of K79 Mono-, Di-, and Trimethylated Histone H3 in the Region of the *GAL* Genes (with Normalization to Histone H3 Levels)

(A) H3-K79 monomethylation in a wild-type strain (wt) is present at low levels but is increased in the H2B-K123R mutant strain.

(B) H3-K79 dimethylation is enriched over the coding regions of the *GAL* genes in the repressed state and is not significantly decreased in the H2B-K123R strain.

(C) H3-K79 trimethylation is high over the coding regions of the *GAL* genes under repressed conditions and is reduced to background levels in the H2B-K123R mutant.

(D) When the GAL genes are active, H3-K79 monomethylation is similar between wild-type and mutant strains.

(E) H3-K79 dimethylation is reduced in the H2B-K123R mutant compared to wild-type.

(F) H3-K79 trimethylation is lower in the H2B-K123R strain than wild-type at the transcriptionally active *GAL* genes. The mean values from four independent ChIPs with α-K79me1, α-K79me2, and α-K79me3 divided by the mean values from four ChIPs with α-H3 are plotted.

cially.) We will refer to the antibody directed against the dimethylated K79 peptide as α-K79me2 and the antibody for the trimethylated peptide as α-K79me3. Inhibition ELISA assays confirmed the specificity of the α-K79me2 and α-K79me3 antibodies as described in Figure S1 (see Figure S1 in the Supplemental Data available with this article online). To study the role of H2B-K123 ubiquitylation on H3-K79 methylation in detail, we used these antibodies in chromatin immunoprecipitation (ChIP) experiments with both a wild-type strain and a strain containing a histone H2B gene in which the lysine residue at position 123 has been replaced with arginine in order to block ubiquitylation (H2B-K123R) (Robzyk et al., 2000). H3-K79 mono-, di-, and trimethylation were analyzed at an ~8 kb region encompassing the *GAL* gene cluster, consisting of the *GAL1*, *GAL7*, and *GAL10* genes, because the transcription (and chromatin modifications) of these genes are coordinately regulated depending on the carbon source. Additionally, H2B is known to become ubiquitylated at the *GAL1* promoter during gene activation (Henry et al., 2003). Wild-type and H2B-K123R cells were grown in the presence of raffinose (in which the *GAL* genes are neither repressed nor activated) and then transferred to medium containing either glucose (to repress transcription) or galactose (to activate transcription) for 1 hr. In the repressed condition, the wild-type strain demonstrates a low level of H3-K79 monomethylation throughout the *GAL* gene cluster. Monomethylation is modestly but reproducibly increased in the H2B-K123R mutant (Figure 1A). Enrichment values are relative to the unmethylated control situated near the telomere on the right arm of chromosome VI. Because nucleosomes are lost from the *GAL* genes upon activation (M.D.S. and M.G., unpublished data), the enrichment values for methylation were normalized to the relative level of histone H3 (data before normalization to histone levels are in Figure S2). In the wild-type strain, H3-K79 dimethylation peaks within the *GAL* gene-coding regions and is low within intergenic regions. The level of dimethylation is unaffected in the H2B-K123R mutant (Figure 1B). The wild-type pattern of K79 trimethylation is similar to that of dimethylation under these conditions, except that it is lower over the *GAL7* coding region (Figure 1C). In the H2B-K123R mutant, however, trimethylation is reduced to background levels throughout this region (Figure 1C). These results demonstrate that H2B ubiquitylation is critical for trimethylation but is not required for either mono- or dimethylation at the repressed *GAL* genes.

Because histone H2B is ubiquitylated preferentially at transcriptionally active genes (Henry et al., 2003; Kao et al., 2004), we asked if its effect on H3-K79 methylation was more severe at the *GAL* gene cluster during gene activation. H2B-K123 ubiquitylation at the *GAL1*

gene is known to peak 60 min after cells are switched from raffinose- to galactose-containing medium (Henry et al., 2003). Under these conditions, the lack of H2B-K123 ubiquitylation has no discernable effect on H3-K79 monomethylation at the *GAL* genes (Figure 1D). On the other hand, both di- and trimethylation of H3-K79 are markedly reduced in the H2B-K123R strain compared to wild-type (Figures 1E and 1F). We conclude that ubiquitylation at H2B-K123 is not required for monomethylation but is a key factor for both di- and trimethylation of H3-K79 at the active *GAL* genes.

To confirm that the observed effects of H2B ubiquitylation on H3-K79 methylation were not specific to the *GAL* gene cluster, we analyzed H3-K79 mono-, di- and trimethylation at an ~5 kb region surrounding the ribosomal protein gene *RPS31*. *RPS31* is highly transcribed under standard growth conditions (~130 mRNAs/hr), whereas the surrounding genes, *APS1*, *YLR168C*, and *YLR171W,* have low to moderate transcriptional frequencies (~1–4 mRNAs/hr) (Holstege et al., 1998). The general pattern of H3-K79 modifications is similar to the *GAL* gene cluster in that the wild-type level of monomethylation is low, whereas di- and trimethylation generally peak at the coding regions and dip at intergenic regions (Figures 2A–2C). The effect of the H2B-K123R mutation in this chromosomal location is also similar to that at the *GAL* genes. H3-K79 monomethylation is consistently higher at several of the genes surrounding *RPS31* in the mutant strain compared to wild-type (Figure 2A). Levels of H3-K79 dimethylation are diminished in the mutant at the *RPS31* gene as well as the surrounding genes, except for *SEC10* (Figure 2B). K79 trimethylation in the H2B-K123R strain is severely reduced at all genes in this region (Figure 2C). We conclude that the loss of H2B-K123 ubiquitylation causes an increase in H3-K79 monomethylation and decreases in di- and trimethylation. Whereas a marked reduction in H3-K79 trimethylation is observed at all of the studied genes, the effects of H2B-K123 ubiquitylation on H3-K79 mono- and dimethylation appear to be more dependent on the chromosomal context or environmental factors. Transcriptional differences do not appear to be the cause of the gene-specific effects. For example, at the *GAL* genes, H2B ubiquitylation affects H3-K79 dimethylation when the genes are active, but not when they are repressed, whereas within the region of the *RPS31* gene, the only unaffected gene is *SEC10*, which is unlikely to be repressed because it is an essential gene in standard growth conditions. It is thus likely that other chromatin-associated factors besides H2B-K123 ubiquitylation, which are independent of transcription, influence the enzymatic ability of Dot1 to carry out mono-, di-, and trimethylation.

To examine the effect of H2B-K123 ubiquitylation on H3-K79 methylation on a global scale, we isolated histones from wild-type and H2B-K123R strains and analyzed their methylation profile by western blot and mass spectrometry. Western blotting analysis demonstrated that as observed by chromatin immunoprecipitation, monomethylation of H3-K79 is slightly increased in the mutant strain whereas di- and trimethylation are notably decreased (Figure S3). These results shed some light on why previous studies of the effect of H2B-K123 ubiquitylation on methylation came to the conclusion

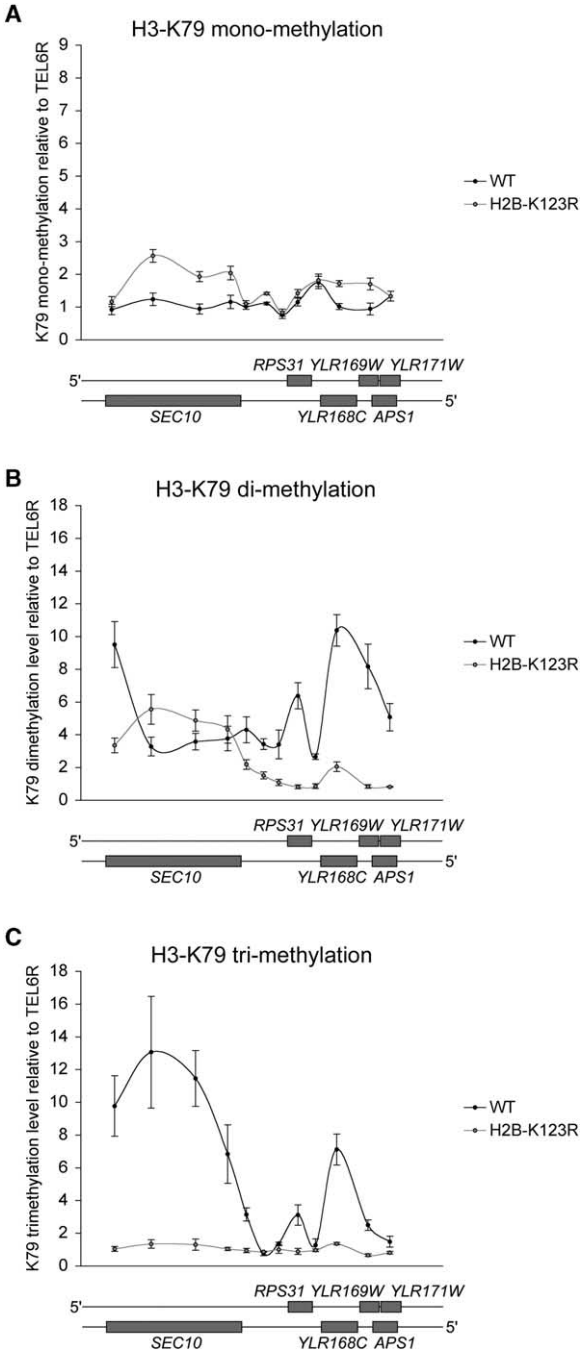

Figure 2. H3-K79 Mono-, Di-, and Trimethylation Levels in a Region Containing the Highly Active *RPS31* Gene

(A) H3-K79 monomethylation is increased in the H2B-K123R strain at genes surrounding *RPS31* compared to wild-type.

(B) H3-K79 dimethylation is enriched in the *RPS31* coding region and in the coding regions of the surrounding genes in a wild-type strain. Although in the H2B-K123R strain H3-K79 dimethylation levels are relatively similar within the *SEC10* gene, severe reductions are seen within *RPS31* and the other surrounding genes.

(C) H3-K79 trimethylation levels undergo a marked reduction in the H2B-K123R strains compared to wild-type. All data points are plotted as the mean value from three to five independent ChIPs, and error bars represent the standard error.

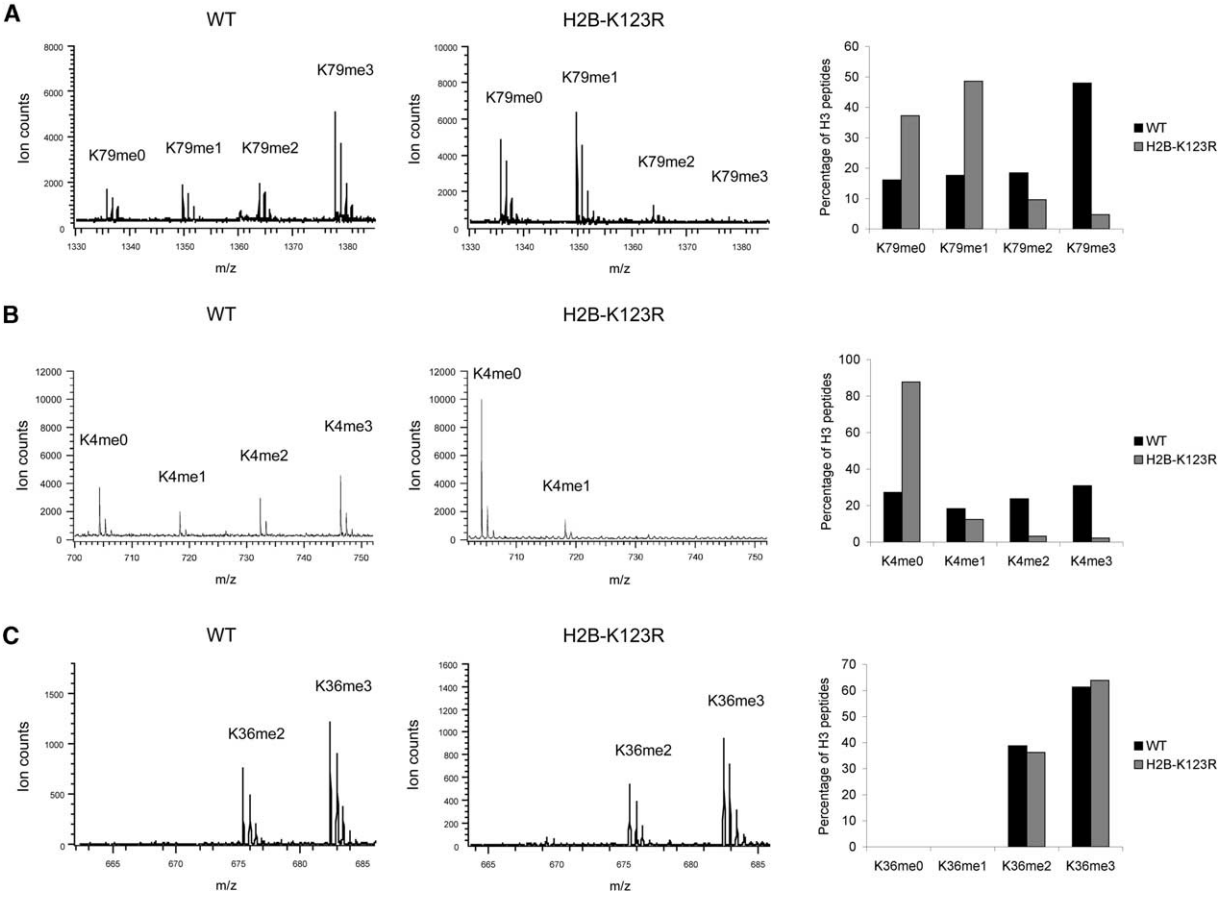

Figure 3. Mass Spectra of Histone H3 Peptides Demonstrate the Relative Levels of Lysine Methylation in Wild-Type and H2B-K123R Strains

(A) MALDI-TOF mass spectra of peptides EIAQDFK(me0–3)TDLR, where lysine 79 is un-, mono-, di-, or trimethylated corresponding, respectively, to peaks at *m/z* 1335.7 (K79me0), 1349.7 (K79me1), 1363.7 (K79me2), and 1377.7 (K79me3). Quantification of the data is shown in the graph on the right.

(B) MALDI-TOF mass spectra of peptides TK(me0–3)QTAR, where lysine four is un-, mono-, di-, or trimethylated corresponding respectively to peaks at *m/z* 704.4 (K4me0), 718.4 (K4me1), 732.4 (K4me2), and 746.4 (K4me3).

(C) ESI-MS mass spectra of peptides SAPSTGGVK(me2–3)KPHR, where lysine 36 is di- or trimethylated corresponding, respectively, to peaks at *m/z* 675.4 (K36me2) and 682.4 (K36me3). Negligible amounts of un- and monomethylated K36 were detected. The ions are doubly charged.

that ubiquitylation is required for H3-K4 and H3-K79 methylation. These previous analyses used antibodies to only the dimethylated forms of H3-K4 and H3-K79 in western blots of whole cell or nuclear extracts and, thus, would not have detected unchanged or increased monomethylation (Briggs et al., 2002; Dover et al., 2002; Ng et al., 2002b; Sun and Allis, 2002).

Mass spectrometry further demonstrated that in the absence of H2B ubiquitylation, H3-K79 trimethylation is severely reduced; dimethylation is reduced to half the wild-type level, consistent with retention of this modification at some genes; and monomethylation is increased compared to wild-type (Figure 3A). Because H2B ubiquitylation has also been shown to affect H3-K4 methylation, we also analyzed K4 methylation levels by mass spectrometry. Similar to the situation with H3-K79 methylation, we found that monomethylation of H3-K4 is retained in the H2B-K123R strain but that di- and trimethylation are reduced below the level of detection (Figure 3B). These results indicate that H2B-K123 ubiquitylation is important genome-wide for di- and especially trimethylation, but not for monomethylation of H3-K4 and H3-K79.

To address the possibility that the loss of H2B-K123 ubiquitylation affects methylation generally, for example, through decreased production of the S-adenosyl-L-methionine (AdoMet) cofactor, we asked whether all forms of methylation were disrupted in the H2B K123R strain. In opposition to this idea, the H2B-K123R strain demonstrated a wild-type profile of H3-K36 methylation by mass spectrometry (Figure 3C). The previous demonstration that H3-K36 methylation is increased at the *GAL1* gene in the absence of H2B K123 ubiquitylation (Henry et al., 2003) may point to gene-specific effects on H3-K36 methylation, but our data, together with previous data, demonstrate that this site of methylation is generally unaffected by H2B ubiquitylation on a genome-wide basis (Ng et al., 2002b; Briggs et al., 2002). In summary, H2B ubiquitylation is important on a global scale for di- and trimethylation of histone H3 at K4 and

K79, but not K36. It thus appears that H2B K123 ubiquitylation specifically alters the activities of the Dot1 and Set1 enzymes, rather than generally affecting histone methylation by a more indirect route.

Because it is thought that mono-, di-, and trimethylation arise from subsequent reactions by the histone methyltransferase (Patnaik et al., 2004), we asked whether the decreases in H3-K79 di- and trimethylation in the H2B K123R strain were due to diminished association of Dot1 with chromatin. To detect the Dot1 protein, we inserted a sequence encoding 13 tandem copies of the Myc epitope into the 3′ end of the *DOT1* coding region and carried out ChIP with an anti-Myc antibody. Epitope-tagged Dot1 is known to be catalytically active because a wild-type profile of H3-K79 methylation is observed in strains containing only the tagged version of Dot1 (M.D.S. and M.G., unpublished data). At the *GAL* genes, Dot1 is detectable at the coding regions when the genes are activated by galactose; both the pattern and level of Dot1 are similar between wild-type and H2B-K123R strains (Figure 4A). Dot1 binding is not detected in either the wild-type or mutant strain when the genes are repressed by glucose (Figure 4B). It is notable that although the *GAL* genes are methylated in both the repressed and active state, Dot1 binding is only detected in the active condition. A possible explanation is that Dot1 binds more stably or at higher levels at transcriptionally active genes in order to methylate new histones that are deposited through the process of histone turnover. At the highly transcribed *RPS31* gene, Dot1 is also found at similar levels in the wild-type and H2B-K123R strains (Figure 4C). These results suggest that the reduction of H3-K79 di- and trimethylation in the H2B-K123R mutant is not due to reduced binding of the methyltransferase. Moreover, we find that the expression level of *DOT1* is unaffected by the H2B-K123R mutation (data not shown). We conclude that in the absence of H2B ubiquitylation, Dot1 is recruited to and binds to chromatin normally, where it monomethylates histone H3 but is unable to efficiently perform successive rounds of methylation.

It is interesting that Dot1 and Set1, two methyltransferases that share no significant homology, are, in general, similarly affected by K123 ubiquitylation. Dot1 does share one notable structural feature with the SET domain-containing methyltransferases, the class of enzymes to which Set1 belongs. Crystallographic studies of Dot1 and members of the SET family have revealed the presence of a narrow channel that passes through these enzymes; this channel connects the target lysine side chain, which enters through one side of the enzyme, to the AdoMet cofactor, which enters through the opposite side (Min et al., 2003; Trievel et al., 2002). The internal structure of the channel is critical in determining the ability of enzymes to mono-, di-, and trimethylate. In the case of enzymes that exclusively monomethylate substrates, the side chains of residues within the channel are thought to sterically exclude lysines with methyl groups because single amino acid changes within the catalytic site can alter the specificity of the enzyme. For example, a Y305F mutation in SET7/9 converts it from a monomethyltransferase to a predominantly dimethyltransferase (Zhang et al., 2003). Additionally, DIM-5, which primarily trimethylates its

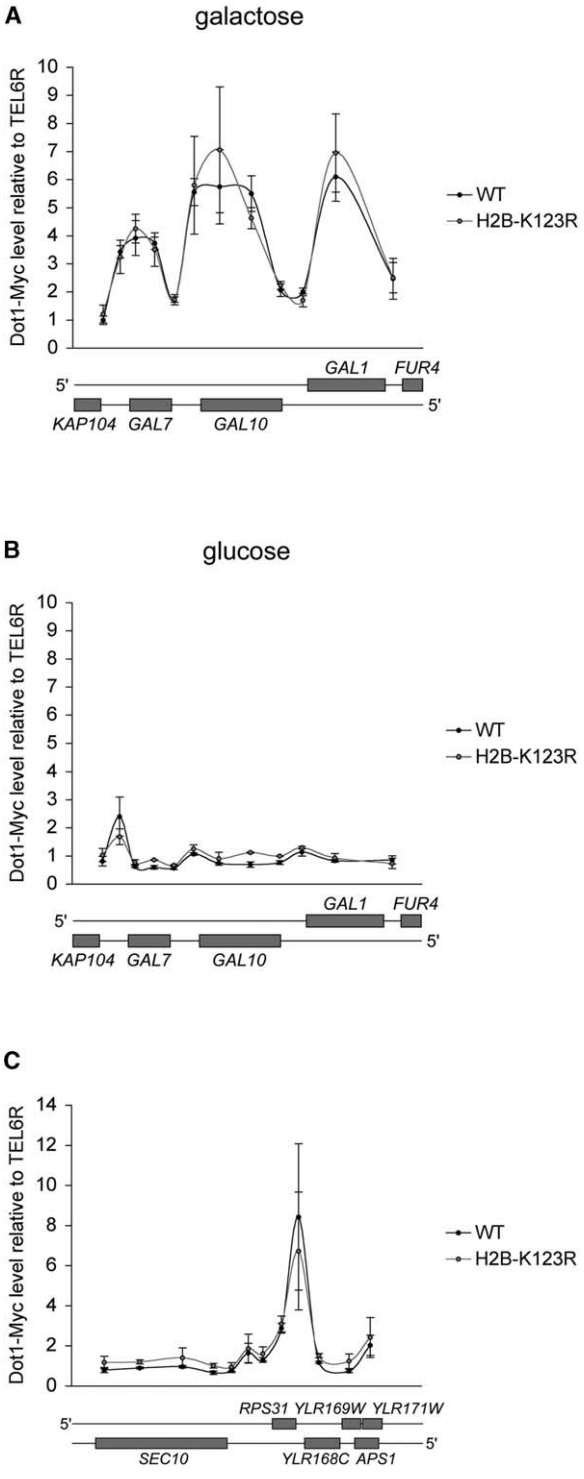

Figure 4. ChIP of Myc-Tagged Dot1 around the *GAL* Genes and the *RPS31* Gene in Wild-Type and H2B-K123R Strains

(A) Myc-tagged Dot1 is immunoprecipitated strongly within the coding regions of the *GAL* genes in galactose in both the wild-type and H2B-K123R strains.

(B) Myc-tagged Dot1 is not detectable at the GAL genes under repressive conditions in either the wild-type or H2B-K123R strains.

(C) Dot1 is present at the coding region of *RPS31* at similar levels in wild-type and H2B-K123R strains. All data points are plotted as the mean value from three to four independent ChIPs, and error bars represent the standard error.

substrates, when altered by a F281Y mutation, is able to only mono- and dimethylate its substrates (Zhang et al., 2003).

The characteristic channel of histone methyltransferases is thought to allow exchange of cofactor while the enzyme remains bound to its substrate, thus enabling multiple rounds of methylation to the same substrate (Patnaik et al., 2004; Tamaru et al., 2003). In certain cases, an accessory factor is additionally required for the enzyme to carry out multiple reactions (Wang et al., 2003). This appears to be the case for Dot1 and Set1, which alone can monomethylate their substrates but require ubiquitylated H2B to stimulate subsequent rounds of methylation. Whether it is the ubiquitin moiety itself or another factor that is required for successive methylation is unknown. Molecular modeling with the crystal structure of Dot1 and the nucleosome has revealed that a region of Dot1 is likely to reside near lysine 123 of histone H2B (Min et al., 2003). Furthermore, a 60 amino acids-long region of the Dot1 protein is predicted to form a helical structure similar to two different ubiquitin binding domains (Sawada et al., 2004). These findings indicate that a ubiquitin molecule attached to histone H2B may interact directly with Dot1, altering the conformation of the interior channel. Such an allosteric change could allow methylated substrates to be accommodated, permitting the processivity of Dot1 and perhaps Set1.

Experimental Procedures

S. cerevisiae Strains

The yeast strains Y131 (*hta1-htb1Δ::LEU2, hta2-htb2Δ::TRP1, HTA1-HTB1*) and Y133 (*hta1-htb1Δ::LEU2, hta2-htb2Δ::TRP1, HTA1-htb1-K123R*) were described previously (Robzyk et al., 2000). The *DOT1* gene was tagged at the 3′ end with 13 copies of the Myc epitope in strains Y131 and Y133 by using an amplified sequence from the plasmid pFA6a-13Myc-kanMX6 (Longtine et al., 1998) to produce strains YMS037 (*dot1-13Myc::kanMX6, hta1-htb1Δ::LEU2, hta2-htb2Δ::TRP1, HTA1-HTB1*) and YMS038 (*dot1-13Myc::kanMX6, hta1-htb1Δ::LEU2, hta2-htb2Δ::TRP1, HTA1-htb1-K123R*).

Chromatin Immunoprecipitation

For analysis of H3-K79 methylation and Dot1 binding at the *GAL* genes, yeast cells were grown in YEP (1% yeast extract, 2% peptone) with 2% raffinose to an OD_{600} of 0.5, centrifuged at 1500 × g for 3 min, resuspended in YEP with either 2% glucose or 2% galactose, and grown for an additional hr to an OD_{600} of ~0.8. For analysis of the *RPS31* region, cells were grown to an OD_{600} of ~0.8 in 50 ml YEP medium with 2% glucose. Cells were crosslinked, and chromatin was immunoprecipitated as previously described (Hecht and Grunstein, 1999). For our experiments, we used 50 μl of whole-cell extract with the following amounts of antibodies: 2 μl of α-K79me1 (Abcam Ltd., Cambridge, UK), 2 μl of α-K79me2, 5 μl of α-K79me3, 1 μl of antibody directed against the C terminus of histone H3 (generously provided by Alain Verreault), and 0.5 μl of α-Myc (Upstate Biotechnology, Charlottesville, VA). Polymerase chain reaction (PCR) was performed with 1/100th of the immunoprecipitated DNA and 1/20,000th of the input DNA in multiplex reactions with 0.08 μCi/μl [α-^{32}P] dATP (specific activity 3000 Ci/mmol). A sequence residing ~500 base pairs from the end of the right arm of chromosome VI (TEL6R) was used as an internal control for the amplification. Band intensities were quantified with a phosphorimager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA). To measure the enrichment at a particular genomic location, we calculated the value of the compound fraction (A/B) ÷ (C/D) in which A is the intensity of the PCR product for the queried region from the immunoprecipitated DNA; B is the intensity of the TEL6R control PCR from the same reaction as A; C is the intensity of the PCR product for the queried region from the input DNA; and D is the intensity of the TEL6R control PCR from the same reaction as C. Data were analyzed and plotted with Microsoft Excel.

Histone Isolation and Mass Spectrometry

Core histones were isolated from asynchronously growing cultures according to published methods (Edmondson et al., 1996) and separated by reverse-phase C4 HPLC as described previously (Zhang et al., 2002). Purified histone H3 was then digested by endoproteinase Arg-C (Roche, Indianapolis, IN) in 25 mM ammonium bicarbonate overnight. Arg-C hydrolyzes the carboxy peptide bond of arginine, leaving the lysine residues (with and without modification) intact. Peptides from the digestion were either further purified by a reverse-phase HPLC for MALDI-TOF analysis or applied directly to ESI-LC/MS (and LC/MS/MS) analysis as described (Zhang et al., 2004). A 0.5 μl aliquot of the peptide solution from HPLC fractions was mixed with 0.5 μl of α-cyano-4-hydroxycinnamic acid matrix (Sigma, St. Louis, MO) and subjected to MALDI analysis on a Voyager DE-STR Biospectrometry Workstation (Applied Biosystems, Foster City, CA). The LC/MS (and LC/MS/MS) was run on a quadrupole time-of-flight (Q-TOF) mass spectrometer (Micromass, Manchester, UK), which was coupled with an HP 1100 capillary HPLC. Detailed running conditions for both MALDI-TOF and ESI-LC/MS (and LC/MSMS) have been described previously (Zhang et al., 2004).

MALDI-TOF or ESI-MS spectra provided the intensities of the four monoisotopic peaks (singly charged in MALDI and doubly charged in ESI) corresponding to peptides sharing the same amino acid sequence but containing either un-, mono-, di-, or trimethylated lysines. Because of the direct correlation of the mass spectrometric peak intensities with the concentrations of peptides in the solution, the methylation level for a specific site was calculated by using the fraction of the intensity of one peak corresponding to mono-, di-, or trimethylated peptide over the sum of the four peaks. Although the measured methylation levels might vary slightly from the absolute numbers due to potential ionization differences between peptides containing varying degrees of methylation, it is reasonable to compare the relative methylation levels (Zhang et al., 2004).

Supplemental Data

Supplemental Data including three figures and Supplemental Experimental Procedures are available online with this article at http://www.molecule.org/cgi/content/full/19/2/271/DC1/.

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