

Methylation of Histone H3 by COMPASS Requires Ubiquitination of Histone H2B by Rad6*

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The DNA of eukaryotes is wrapped around nucleosomes and packaged into chromatin. Covalent modifications of the histone proteins that comprise the nucleosome alter chromatin structure and have major effects on gene expression. Methylation of lysine 4 of histone H3 by COMPASS is required for silencing of genes located near chromosome telomeres and within the rDNA (Krogan, N. J., Dover, J., Khorrami, S., Greenblatt, J. F., Schneider, J., Johnston, M., and Shilatifard, A. (2002) *J. Biol. Chem.* 277, 10753–10755; Briggs, S. D., Bryk, M., Strahl, B. D., Cheung, W. L., Davie, J. K., Dent, S. Y., Winston, F., and Allis, C. D. (2001) *Genes. Dev.* 15, 3286–3295). To learn about the mechanism of histone methylation, we surveyed the genome of the yeast *Saccharomyces cerevisiae* for genes necessary for this process. By analyzing ~4800 mutant strains, each deleted for a different non-essential gene, we discovered that the ubiquitin-conjugating enzyme Rad6 is required for methylation of lysine 4 of histone H3. Ubiquitination of histone H2B on lysine 123 is the signal for the methylation of histone H3, which leads to silencing of genes located near telomeres.

Heritable, quasistable modifications of histone proteins, such as acetylation, phosphorylation, and methylation, play essential roles in the regulation of gene expression in eukaryotic organisms and have been proposed to be required for development and cellular commitment in metazoans (1–3). Histone acetylations on histones H3 and H4 are the best characterized covalent modifications of histones and have been demonstrated to have wide ranging effects on the regulation of

gene expression (1). Phosphorylation has been demonstrated to be an important modification of histone in transcriptional activation, condensation of chromosomes during mitosis and meiosis, and regulation of cell division.

Methylation of lysine 4 in the amino-terminal tail of histone H3, mediated by a multiprotein complex we call COMPASS¹ (complex of proteins associated with Set1) (4–7, 9), is required for silencing of expression of genes located near chromosome telomeres and within the rDNA (5, 6). We and others have demonstrated that COMPASS includes Set1, a member of the chromatin-associated proteins (the Trithorax (Trx) group) that possess a sequence motif called the SET domain, and seven other polypeptides (4–7, 9). The SET domain takes its name from the *Drosophila* proteins Su(var)3-9, Enhancer of zeste (E(z)), and Trx. Also, COMPASS contains another yeast protein related to the human Trx protein ASH2. SET domain-containing proteins have been implicated in histone methylation and regulation of transcription in several organisms (5, 8–12). Although the SET domain-containing proteins play fundamental roles in development and oncogenesis, their molecular function is poorly understood (13–16).

To better understand the mechanism of histone H3 lysine 4 methylation, we surveyed the genome of the yeast *Saccharomyces cerevisiae* for genes necessary in this process. Analysis of ~4800 mutant strains resulted in the discovery of the ubiquitin-conjugating enzyme Rad6 as a protein required for methylation of lysine 4 of histone H3. We have demonstrated that ubiquitination of histone H2B on lysine 123 is the signal for the methylation of histone H3 by COMPASS, which leads to silencing of genes located near telomeres.

MATERIALS AND METHODS

Preparation of Yeast Cell Extracts from 96-well Plates—Using a 96-well pinning device, the entire collection of 4800 yeast non-essential gene deletion mutants was inoculated from –80 °C stocks onto agar plates containing YPD (1% yeast extract, 2% proteo-peptone, 2% dextrose) + 200 µg/ml Geneticin (Invitrogen), allowed to grow for 48 h, and used to inoculate 96-tube PCR plates filled with 100 µl of YPD. After 48 h of growth at 30 °C the plates were centrifuged at 2000 × g for 10 min. Medium was removed by wrist-snap inversion and draining into absorbent towels. Plates were then covered and frozen at –80 °C for up to 1 week. Cells were thawed at room temperature, resuspended in 30 µl of lysis buffer (20 mM Tris, pH 7.5, 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1% Nonidet P-40, 1 mg/ml Zymolyase 100T), and incubated at 37 °C for 15 min. 10 µl of 4× Laemmli loading buffer were added, and the samples were vortexed briefly before heating at 100 °C for 5 min.

Preparation of Total Yeast Cell Extracts and Analysis for Histone H3 Lys-4 Methylation—Yeast cells were grown to mid-log phase in YPD medium, pelleted, washed with distilled water, pelleted, and resuspended in lysis buffer (20 mM Tris, pH 7.5, 50 mM KCl, 1 mM EDTA, 0.1% Nonidet P-40, 1 mM dithiothreitol, and fresh protease and phosphatase inhibitors (1 µg/ml aprotinin, leupeptin, and pepstatin A, 1 mM phenylmethylsulfonyl fluoride, 1 µM microcystin-LR, 2 mM *p*-chloromercuriphenylsulfonic acid). Cells were then disrupted by vortexing with glass beads (0.5 mm, Biospec Products) for 15 min at 4 °C. The bottoms of the microcentrifuge tubes were punctured, and cell extracts were recovered into a larger tube by brief centrifugation in a microcentrifuge. The lysate was clarified by centrifugation at 20,000 × g for 30 min, subjected to SDS-PAGE, transferred to nitrocellulose membrane, and probed with anti-methylhistone antiserum at 1:1000 dilution followed

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¹ The abbreviations used are: COMPASS, complex of proteins associated with Set1; Trx, Trithorax; E2, ubiquitin-conjugating enzyme; E3, ubiquitin-protein isopeptide ligase.

by detection of the bound antibody with horseradish peroxidase-conjugated anti-rabbit IgG secondary antibodies (1:10,000 dilution).

RESULTS AND DISCUSSION

Development of a Biochemical Screen in 96-well Plates to Identify Genes Essential for Lys-4 Methylation of Histone H3—To learn more about the mechanism of histone methylation by COMPASS, we sought to identify all the (non-essential) proteins in *S. cerevisiae* necessary for methylation of Lys-4 of histone H3. As described under "Materials and Methods," we developed a method to survey the genome of *S. cerevisiae* for genes required for this histone modification by testing extracts of each of the ~4800 viable mutants for the presence of Lys-4-methylated histone H3. Mutants missing a component of COMPASS are defective in this histone H3 modification. As shown in Fig. 1A, cells deleted for *set1* are defective for the methylation of Lys-4 of histone H3. When this assay was performed in a 96-well plate, the mutant cell missing *Cps50/Yar003* was

found to be defective for the methylation of Lys-4 of histone H3. However, all of the other 95 mutants in this microtiter plate possess this modification (Fig. 1B).

Rad6 Is Essential for Lys-4 Methylation of Histone H3—Screening of all 52 microtiter plates containing the 4827 mutants we tested revealed that the *rad6* deletion mutant is defective in histone H3 Lys-4 methylation (Fig. 2A). This was confirmed by testing histone H3 Lys-4 methylation in two independently generated *rad6* mutants and the homozygous diploid generated from them (Fig. 2, B and C). Also, the H3 Lys-4 methylation-deficient phenotype of the *rad6Δ* cells was complemented by a plasmid containing *RAD6* (Fig. 2D).

Lysine 123 of Rad6 Is Essential for Lys-4 Methylation of Histone H3—Rad6 is a ubiquitin-conjugating enzyme (E2) (17) involved in DNA repair (17, 18), DNA damage-induced mutagenesis (19, 20), meiosis (21), transposition of retrotransposons (22), and gene silencing (23). Rad6 catalyzes ubiquitination of Lys-123 of histone H2B (24). It seemed possible that this modification of histone H2B is responsible for the requirement of Rad6 for methylation of histone H3. Indeed, histone H3 is not Lys-4-methylated in a strain that is unable to attach ubiquitin to Lys-123 of histone H2B (due to a change of Lys-123 to Arg) (Fig. 3). We conclude that ubiquitination of H2B at Lys-123 is required for methylation of Lys-4 of histone H3. This is consistent with the observation that *RAD6* is required for telomeric gene silencing (23) since methylation of histone H3 Lys-4 is required for telomeric and rDNA silencing (5, 6).

Involvement of Other Rad6-interacting Proteins in Lys-4 Methylation of Histone H3—Several proteins are involved with Rad6 in diverse cellular processes (17–22). However, our genome-wide survey of *S. cerevisiae* revealed that none of the mutants missing these (non-essential) Rad6-interacting proteins, including those that act with Rad6 in DNA damage repair (Rad18 and Rex4) and the N-end rule-dependent protein degradation pathway (Ubr1 and Ubr2), are involved in methylation of histone H3 Lys-4 (Fig. 3C). This indicates that Rad6 functions in the regulation of gene expression by controlling methylation of histone H3 independently of the above proteins.

To define the biochemical characteristics of Rad6 protein, we used Rad6-specific polyclonal antibody to determine whether Rad6 exists in a large macromolecular complex. Our analysis suggests that at least a portion of Rad6 protein is found in a large complex in yeast extracts (Fig. 3D). Perhaps this complex

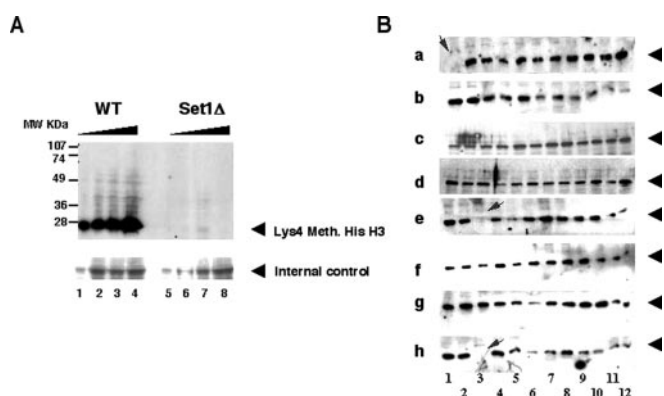
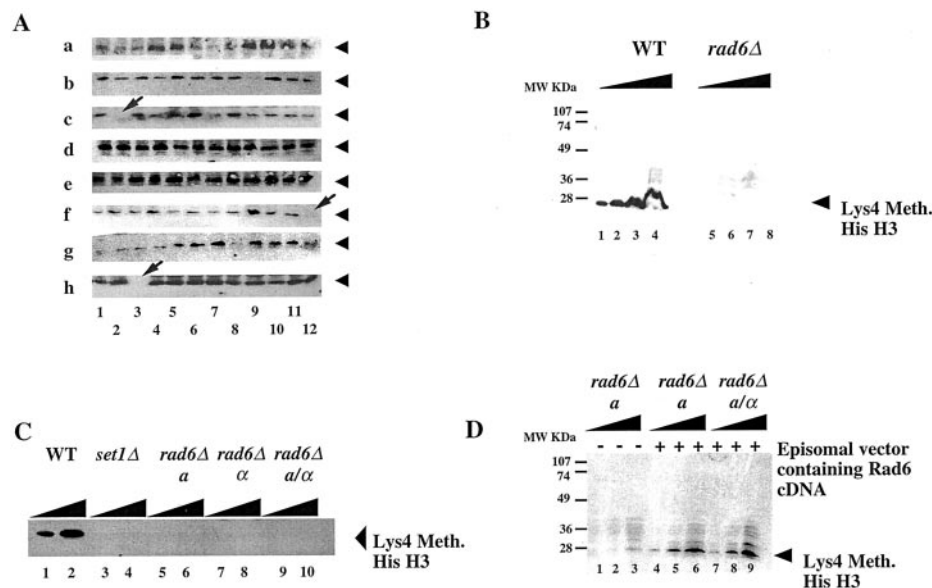


FIG. 1. Surveying the *S. cerevisiae* genome for genes required for methylation of lysine 4 of histone H3. A, whole cell extracts from wild-type *S. cerevisiae* or a *set1* deletion mutant were tested for the presence of Lys-4-methylated histone H3. Cell extracts were subjected to 16% SDS-PAGE, blotted to nitrocellulose membrane, and probed with affinity-purified polyclonal antiserum (obtained from Abcam) specific for Lys-4 of histone H3 as described previously (4 and 5). B, whole cell extracts from a microtiter plate containing 96 different yeast strains, each missing a different (non-essential) gene, one of which (row e, lane 3) is the *cps50/yar003w* mutant missing a subunit of COMPASS that is essential for histone H3 Lys-4 methylation, were analyzed for this histone modification as described above. Arrows at position a1 and h3 indicates empty wells as plate markers. WT, wild type; Meth., methylated.

FIG. 2. Rad6 is essential for the methylation of lysine 4 of histone H3.

A, extracts of *S. cerevisiae* mutants missing one of the ~4800 non-essential genes were tested for the presence of Lys-4-methylated histone H3. One of the mutants lacking this histone modification is *rad6* (row f, lane 12). Arrows at position a1 and h3 indicates empty wells as plate markers. B and C, extracts of wild-type or two independently generated mutant strains (MATa and MATα, and the homozygous diploid generated from them) were tested for the presence of Lys-4-methylated histone H3. D, the methylation-deficient phenotype of the *rad6Δ* cells was complemented by an episomal vector containing Rad6 cDNA both in *rad6Δ* α strain and also *rad6Δ* homozygous diploid strain. WT, wild type; Meth., methylated.



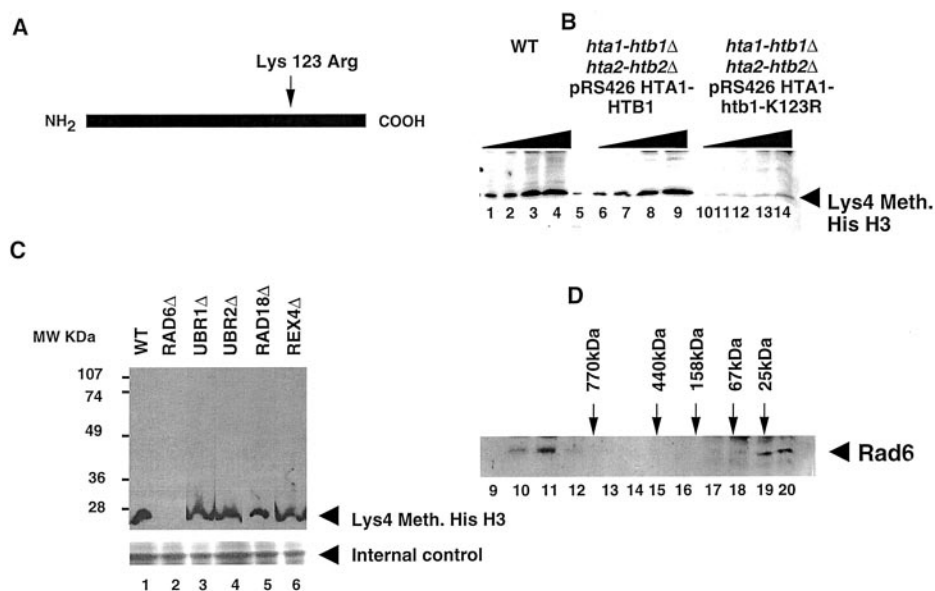


FIG. 3. Ubiquitination of lysine 123 of histone H2B is essential for Lys-4 methylation of histone H3. *A*, schematic representation of histone H2B and its lysine 123 that is ubiquitinated by Rad6 (24). *B*, cell extracts from haploid strains missing *htb1-1* and *htb2-1* and containing a CEN-URA3 plasmid with *HTA1* and either wild-type *HTB1* or *htb1* with lysine 123 converted to arginine (K123R, generously provided by Mary Anne Osley, Ref. 24) were analyzed for the presence of Lys-4-methylated histone H3. *C*, the presence of methylation of Lys-4 of histone H3 in yeast strains missing genes encoding Rad6, Ubr1, Ubr2, Rad18, and Rex4 was determined by subjecting 2000 ng of whole cell extracts from cells deleted for these genes to 16% SDS-PAGE followed by immunoblotting as described above. *D*, the presence of Rad6-containing macromolecular complexes was determined by the application of wild-type yeast extract on a Superose-6 PC size exclusion column. 100- μ l fractions were collected, and 25 μ l were subjected to SDS-PAGE, then transferred to nylon membranes, and probed with a Rad6-specific polyclonal antibody. WT, wild type; Meth., methylated.

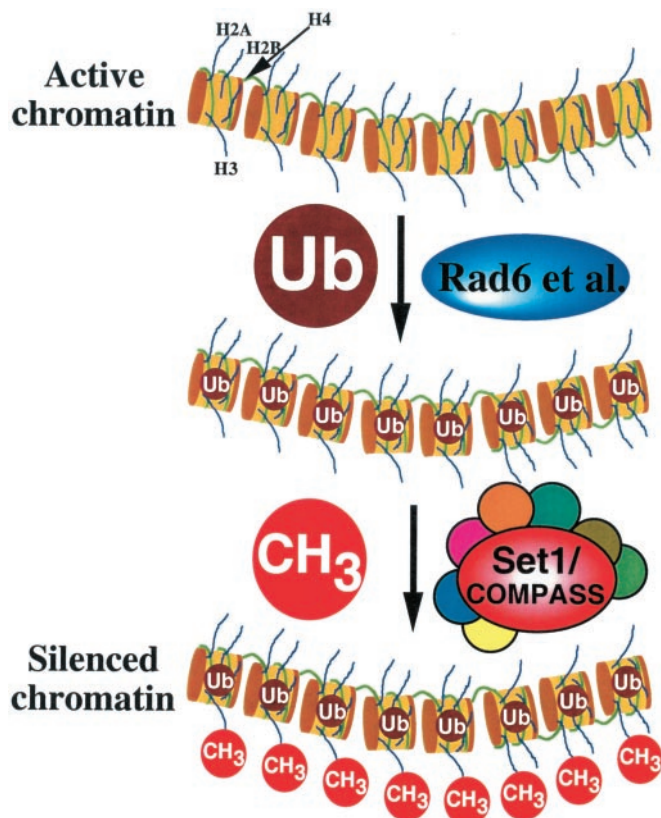


FIG. 4. Model of the role of Rad6 and COMPASS in regulating chromatin structure and gene silencing. Histone H2B within the active region of chromosomal DNA is recognized by the Rad6 protein complex and ubiquitinated on its lysine 123. This ubiquitination of histone H2B serves as a direct or indirect recognition signal and/or activation signal for COMPASS, which catalyzes methylation of lysine 4 of histone H3 and results in silencing of that region of chromosomal DNA. Ub, ubiquitination; CH₃, methylation.

includes an E3 ligase required for the recognition of histone H2B as specific substrate.

Our understanding of the role of the Trx class of proteins in regulation of gene expression and development is rudimentary. The Set1 protein of yeast is similar to the *Drosophila* and human trithorax proteins (Trx and MLL, respectively). The Trx protein may function as a DNA-binding protein and appears to be a regulator of gene expression. Mutations affecting MLL, the human homologue of Trx, result in the development of hematological malignancies. Our molecular and biochemical characterization of the Set1-containing protein complex we call COMPASS is a first step toward understanding the function of SET domain-containing proteins in regulation of gene expression. We and others have now provided evidence that COMPASS is a histone methyltransferase that catalyzes methylation of Lys-4 of histone H3 (4–7, 9).

Our analysis of ~4800 mutant strains of the yeast *S. cerevisiae* for a defect in histone H3 Lys-4 methylation resulted in the discovery of the ubiquitin-conjugating enzyme Rad6 as a protein required for this process (Fig. 4). We have shown that lysine 123 of histone H2B, which is ubiquitinated by Rad6 (24), is essential for the methylation of histone H3 by COMPASS, which leads to silencing of genes located near telomeres. Our results presented here (Fig. 4) suggest that histone H2B in nucleosomes in “active” regions of chromatin is recognized by the Rad6 complex and ubiquitinated on Lys-123. The ubiquitination of histone H2B provides a signal for either direct or indirect recruitment and/or activation of COMPASS. COMPASS can then catalyze the methylation of lysine 4 of histone H3, which leads to silencing of that region of chromosomal DNA (5, 6). An important, unanswered question is whether ubiquitination and/or methylation of histones is limited to certain (silent) regions of chromosomes or whether these modifications occur throughout chromosomes. Ubiquitination has recently been demonstrated to be involved in the regulation of gene expression in eukaryotic cells (25). Although the work presented here cannot at this time rule out a role for protea-

somes in this process, our results suggest a mechanism by which ubiquitination of histone H2B can control transcription by regulation of methylation on histone H3 via COMPASS.

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