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Histone Crosstalk between H2B Monoubiquitination and H3 Methylation Mediated by COMPASS

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SUMMARY

COMPASS, the yeast homolog of the mammalian MLL complex, is a histone H3 lysine 4 (H3K4) methylase consisting of Set1 (KMT2) and seven other polypeptides, including Cps35, the only essential subunit. Histone H2B monoubiquitination by Rad6/Bre1 is required for both H3K4 methylation by COMPASS, and H3K79 methylation by Dot1. However, the molecular mechanism for such histone crosstalk is poorly understood. Here, we demonstrate that histone H2B monoubiquitination controls the binding of Cps35 with COMPASS complex. Cps 35 is required for COMPASS' catalytic activity in vivo, and the addition of exogenous purified Cps35 to COMPASS purified from a *Δrad6* background results in the generation of a methylation competent COMPASS. Cps35 associates with the chromatin of COMPASS-regulated genes in a H2BK123 monoubiquitination-dependent but Set1-independent manner. Cps35 is also required for proper H3K79 trimethylation. These findings offer insight into the molecular role of Cps35 in translating the H2B monoubiquitination signal into H3 methylation.

INTRODUCTION

A large number of distinct chromosomal translocations are involved in leukemogenesis. Most often, their products are transcription factors, and thus, the consequences of their disruption are uncontrolled gene expression contributing to the etiology of cancer. The mixed lineage leukemia (MLL) protein positively regulates homeotic (*HOX*) genes, which are essential for hematopoiesis (Tenney and Shilatifard, 2005; Rowley, 1998). Chromosomal translocations involving the *MLL* gene are associated with a variety of aggressive acute leukemias in children, and are also found in therapy-induced leukemia in adults (Tenney and Shilatifard, 2005; Rowley, 1998). The *Drosophila* ortholog of MLL is *trithorax* (TRX), which activates the expression of homeotic genes. Mutations in *trx* mimic homeotic transformations caused by mutations of *homeotic* genes, such as *Antennapedia* and *Ultrabithorax*. The current model is that TRX is necessary to maintain *homeotic* gene expression (Breen and Harte, 1991; Simon, 1995; Orlando and Paro, 1995).

To begin to define the molecular and biochemical properties of both MLL and TRX, several years ago we identified the Set1 protein as their *Saccharomyces cerevisiae* homolog (Miller et al., 2001). We demonstrated that Set1 is found in a large macromolecular complex called COMPASS (*Com*plex of *P*roteins *A*ssociated with *S*et1) and that COMPASS can mono-, di- and trimethylate lysine four of histone H3 (H3K4) (Shilatifard, 2006; Miller et al., 2001; Roguev et al., 2001; Nagy et al., 2002; Krogan et al., 2002; Nislow et al., 1997) (Figure 1A). We now know that mammalian MLL, like its yeast counterpart, is found in a COMPASS-like complex that also methylates H3K4 (Hughes et al., 2004; Shilatifard, 2006).

Covalent modifications of histones and transcription factors are closely linked to the control of gene expression by RNA polymerase II (Pol II) (Workman and Kingston, 1998; Berger, 2001; Li et al., 2007; Gill, 2004; Couture and Trievel, 2006; Berger, 2007). Since COMPASS methylates H3K4 within the chromatin of actively transcribed genes (Krogan et al., 2003; Ng et al., 2003), and since this modification is highly conserved from yeast to human (Shilatifard, 2006), we aimed to define the pathway of H3K4 methylation. In so doing, we developed a global functional proteomic screen which we named Global Proteomic analysis in *S. cerevisiae* (GPS) (Schneider et al., 2004). In GPS, we test extracts of each of the nonessential yeast gene deletion mutants in different mating types for defects in histone modifications by western blotting (Schneider et al., 2004). So far, GPS has revealed that the monoubiquitination of lysine 123 of histone H2B by the Rad6/Bre1 complex (the E2/E3 enzymes) is required

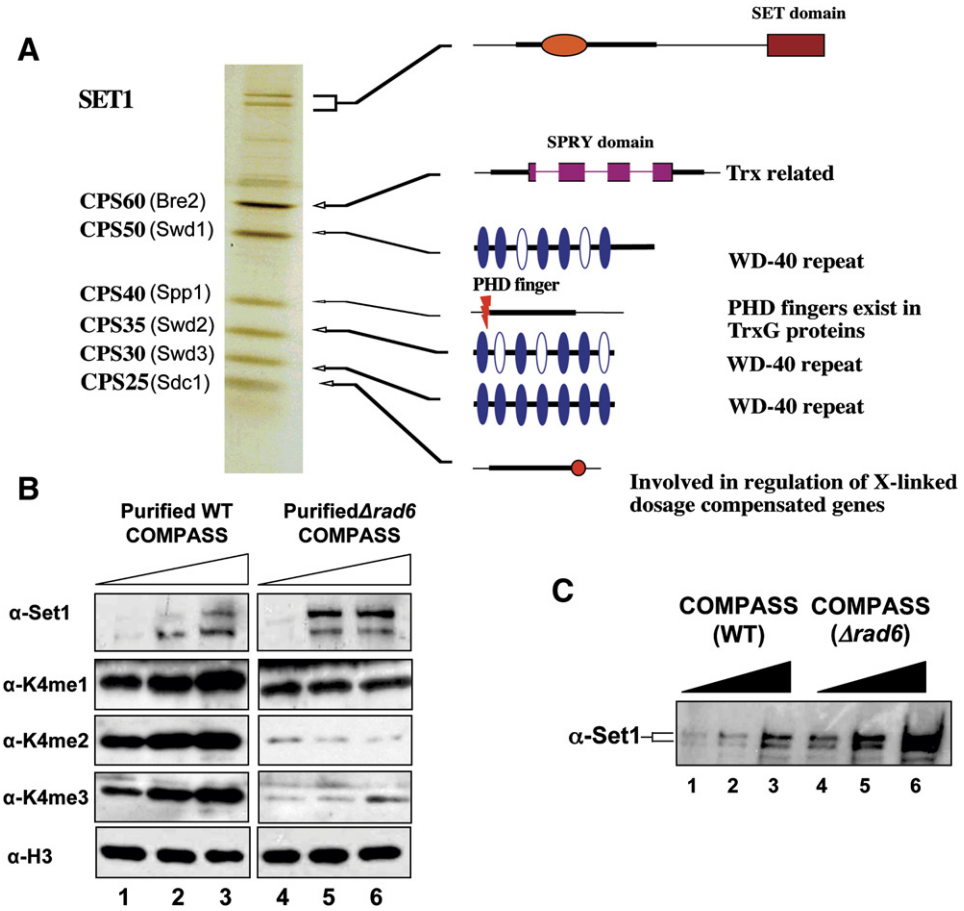

Figure 1. The Role of Rad6/Bre1 in Histone Methylation by COMPASS

(A) Set1 is purified within a macromolecular complex called COMPASS. Set1 within the purified COMPASS runs as a doublet indicating that it perhaps could be either posttranslationally modified or processed.

(B) Purified COMPASS from either wild-type (WT) strains or from *rad6Δ* strains was tested for its H3K4 methyltransferase activity in vitro. As expected, WT COMPASS is capable of mono-, di- and trimethylating H3K4. Although COMPASS purified from *rad6Δ* strains was capable of monomethylating H3K4, this complex was unable to di- or trimethylate H3K4. This observation indicates that Rad6/Bre1 and/or H2B monoubiquitination can modify COMPASS in a way that it would be H3K4 trimethylation competent.

(C) Since purified Set1 within COMPASS behaves as a doublet on SDS/PAGE (Figure 1A), the possibility of its monoubiquitination via Rad6/Bre1 was tested. Purified COMPASS from both WT and from *rad6Δ* strains were subjected to SDS/PAGE and western analysis using polyclonal antibodies generated toward recombinant Set1. The loss of either Rad6 or other factors required for H2B monoubiquitination (such as Bre1, and Bur2) did not alter the mobility of purified Set1 on SDS/PAGE (data not shown). These data indicate that Set1 is not monoubiquitinated by Rad6/Bre1.

for histone methylation by COMPASS (Shilatifard, 2006; Wood et al., 2003a, 2003b; Dover et al., 2002; Sun and Allis, 2002). Indeed, this crosstalk pathway is highly conserved and is specifically required for proper H3K4 di- and trimethylation by COMPASS. In the absence of H2B monoubiquitination, the pattern of H3K4 monomethylation is somewhat lowered but still present, however, H3K4 di- and trimethylation are not detectable (Schneider et al., 2005; Shahbazian et al., 2005).

Although we have learned much from GPS about the factors/proteins required for H2B monoubiquitination (Dover et al., 2002; Wood et al., 2003a, 2003b, 2005a; Krogan et al., 2003; Xiao et al., 2005; Laribee et al., 2005; Shilatifard, 2006), and that this histone crosstalk pathway is highly conserved from yeast to human, the molecular mechanism for such histone crosstalk is poorly understood. In this manuscript, we have identified a role for the Cps35 (also known as Swd2) component of COMPASS in H2B monoubiquitination dependent of H3K4 and K79 methylations helping to better understand the molecular mechanism of histone crosstalk.

RESULTS

In Vitro Methylase Properties of COMPASS Purified from Wild-Type and Cells Lacking H2B Monoubiquitination

To better define the molecular role of H2B monoubiquitination in H3K4 trimethylation by COMPASS, we tested the in vitro methylase activity of COMPASS purified from either

wild-type strains or strains defective for H2B monoubiquitination (*Δrad6*) (Figure 1B). COMPASS purified from a wild-type strain is capable of mono-, di-, and trimethylating H3K4 in vitro, as anticipated (Figure 1B, lanes 1–3). Although COMPASS purified from strains lacking *RAD6* is capable of monomethylating H3K4 (although not to the same level as wild-type), this form of COMPASS is unable to di- or trimethylate histone substrates (Figure 1B, lanes 4–6). These in vitro properties of COMPASS in the absence of H2B monoubiquitination are very similar to its reported in vivo properties (Schneider et al., 2005; Shahbazian et al., 2005).

Although COMPASS purified from WT background is active toward free histone H3 or recombinant nucleosomes, COMPASS purified from strains lacking H2B monoubiquitination cannot di- or trimethylate H3. Therefore, this observation indicates to us that Rad6 and/or H2B monoubiquitination directly regulates COMPASS' methylase activity. We envision three possible modes of regulation of COMPASS: (1) Set1 could directly be posttranslationally modified by Rad6, and that such a modification could be required for proper COMPASS' catalytic activity in H3K4 di- and trimethylation; (2) other components of COMPASS, besides Set1, could be modified by Rad6 and their modification(s) could be required for proper H3K4 methylation by COMPASS; and (3) monoubiquitination of H2B could be required for the proper association of COMPASS' subunits within its complex; its stability; and proper catalytic activity. In this manuscript, we have addressed each of these possibilities.

The Role of Rad6 in the Posttranslational Modification of Set1 and COMPASS Components

When we purified COMPASS several years ago (Miller et al., 2001), we noticed that Set1 within COMPASS behaves as a doublet on SDS/PAGE (Figure 1A). To determine whether this electrophoretic mobility, characteristic of Set1 on SDS/PAGE, is due to its monoubiquitination by Rad6, we developed polyclonal antibodies toward Set1. Purified COMPASS from both wild-type (WT) and *rad6Δ* strains were tested by western analyses (Figure 1C). Our data show that the loss of Rad6 and the monoubiquitination of H2B has no effect on Set1's doublet electrophoretic mobility characteristics. This result indicates that Rad6 does not modify Set1 (please compare Figure 1C lanes 1–3 to lanes 4–6). We also tested the electrophoretic characteristics of Set1 in the absence of Bre1 and did not observe any effect (data not shown). Since we previously demonstrated that Bur1/Bur2 kinase is also required for proper H3K4 methylation, we wanted to test whether it could play a role in Set1's electrophoretic mobility. We did not observe any difference in Set1's electrophoretic properties in their absence (data not shown).

To determine whether Rad6 can modify any of the components of COMPASS, we TAP and HA tagged subunits of COMPASS in wild-type strains and in strains deleted for Rad6. Tagged COMPASS subunits from these strains were then analyzed by two-dimensional SDS/PAGE gel electrophoresis (2D-PAGE) (Figures 2A–2H). Our analyses indicate that the loss of Rad6 does not have any substantial affect on the mobility of the COMPASS subunits on 2D-PAGE. (Figures 2A–2H).

H2B Monoubiquitination Regulating COMPASS Stability

Since it does not appear that the posttranslational modifications of either of Set1 or the COMPASS subunits are altered by the loss of Rad6 or single point mutations in lysine 123 of histone H2B, it is possible that H2B monoubiquitination could regulate the stability of the components of COMPASS within the complex, and thereby regulate its methylase activity. To test this hypothesis, we initially analyzed the chromatographic properties of two components of COMPASS, Set1 and Cps60, in the presence and absence of H2B monoubiquitination. As shown in Figures 2I and 2J, both Set1 and Cps60 coelute on analytical size exclusion column at about the same fractions near the 670 kDa molecular weight marker (Figures 2I and 2J, fractions 23–27). We note that a portion of Cps60 in COMPASS, bearing a single point substitution on lysine 123 of histone H2B, elutes away from COMPASS as a free form (please compare Figures 2I and 2J). We have also observed the same properties for Cps60 in strains deleted for *RAD6* (data not shown). This observation indicated to us that the loss of H2B monoubiquitination could alter COMPASS' stability. Perhaps in the absence of H2B monoubiquitination, COMPASS is not held together as tightly. To address this hypothesis, we endogenously tagged individual components of COMPASS with either TAP, HA or Myc. We then analyzed their chromatographic properties either from WT strains or strains lacking either Rad6 or bearing a single point mutation in K123 of H2B (data not shown). From this comprehensive study, we observed that the association of Cps35 within COMPASS is severely affected in strains bearing a single point mutation on lysine 123 of histone H2B (Figure 3). Both Set1 and Cps35 from WT strains coelute from size exclusion at about the same fractions near the 670 kDa molecular weight marker (Figure 3). In wild-type extracts, we also association Cps35 on other fractions in addition to fractions containing Set1. This is to be expected as Cps35 is a component of other macromolecular complexes in addition to COMPASS. However, in strains defective for H2B monoubiquitination, either deleted for Rad6 (Figure 3B) or bearing a single point substitution on lysine 123 of histone H2B (Figure 3D), Cps 35 elutes away from COMPASS as a free form (please compare Figures 3A and 3B or 3C and 3D). To determine whether or not the observed effect is due to the HA tag on Cps35, we also generated strains where Cps35 was tagged with Myc, and observed that the loss of either Rad6 or a single point mutation in K123 of histone H2B results in the complete loss of the association of Cps35 with COMPASS (data not shown). All together, these data indicate that

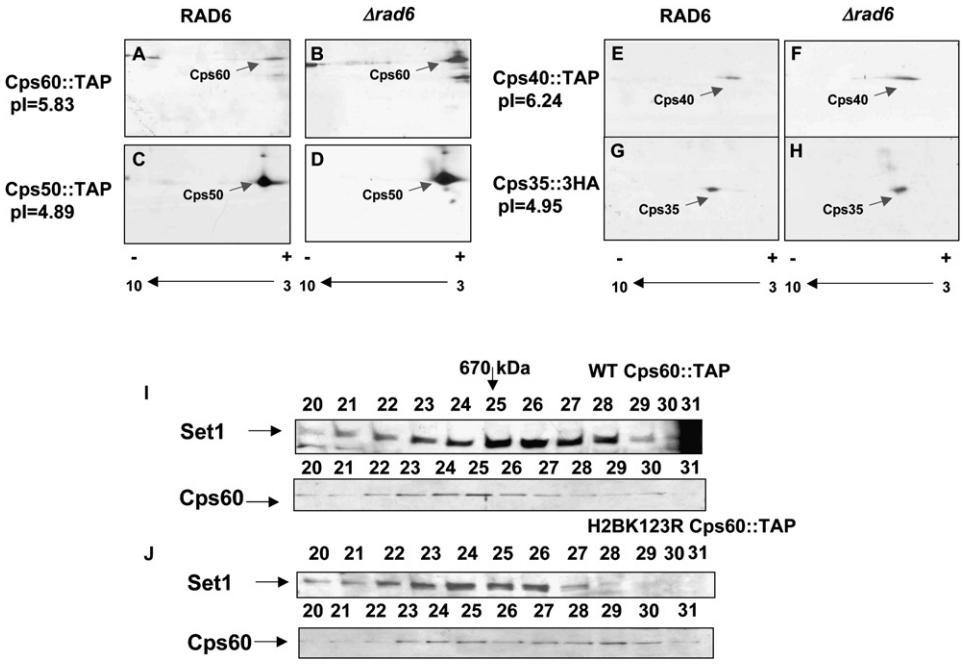

Figure 2. The Role of Rad6/Bre1 in Posttranslational Modifications of COMPASS Components

Subunits of COMPASS were C-terminally tagged under their endogenous promoter in both WT (A, C, E, and G) and *rad6Δ* strains (B, D, F, and H). COMPASS from these strains were analyzed by two-dimensional gel electrophoresis followed by western analysis using antibodies directed toward the tag. Similar 2D properties were also observed for Cps30 (data not shown). (I and J) To determine whether histone H2B monoubiquitination could alter COMPASS' stability, the chromatographic properties of Set1 and Cps60 were tested in both the presence and absence of H2B monoubiquitination. Briefly, COMPASS either from (I) wild-type strains or (J) strains bearing a K123R single point mutation in histone H2B, were subjected to size exclusion chromatography on a Superose 6 HR column (Pharmacia) operated on a SMART system. Resulting fractions were subjected to SDS/PAGE followed by western analysis using either Set1 specific polyclonal antibodies, or antibodies toward the tag on Cps60.

Cps35's interaction with COMPASS requires histone H2B monoubiquitination.

Cps35 contains several WD repeat domains, and in addition to being a subunit of COMPASS, it is also found in other complexes (Miller et al., 2001; Roguev et al., 2001; Dichtl et al., 2004; Cheng et al., 2004). Indeed, in addition to histone methylation, Cps35 is involved in 3′ end formation of specific mRNAs and snoRNAs, and transcriptional termination (Nedea et al., 2003; Dichtl et al., 2004; Cheng et al., 2004). As a matter of fact, Cps35 is the only subunit of COMPASS that is essential for viability in yeast (Miller et al., 2001; Roguev et al., 2001). However, the temperature sensitive alleles of Cps35 demonstrated a loss of H3K4 methylation indicating its importance in H3K4 trimethylation (Cheng et al., 2004).

To further examine the observation that the loss of H2B monoubiquitination results in the loss of the association of Cps35 with COMPASS, we used Multidimensional Protein Identification Technology (MudPIT) (Washburn et al., 2001) to carry out proteomic analyses of purified COMPASS in the presence and absence of H2B monoubiquitination (Figures 4A and 4B). Using spectral counts normalized against protein length (NSAF) (Zybailov et al., 2006; Florens et al., 2006; Paoletti et al., 2006), we have determined that each COMPASS contains a copy of the Set1, Cps50, Cps30 and Cps35 subunits, and multiple copies of Cps40 (2), Cps60 (2–4) and at least 6 copies of Cps25 (Schneider et al., 2005). MudPIT analyses of COMPASS purified from either WT strains or strains lacking H2B monoubiquitination (rad6 null or K123R-H2B) indicates that the loss of H2B monoubiquitination results in the loss of the association of Cps35 and Cps15 within COMPASS (Figures 4A and 4B). We do not observe any significant effects in the association of the other components of COMPASS with Set1. Cps15 is the smallest subunit of COMPASS, and its deletion has little to no effect in the H3K4 methylation pattern (Krogan et al., 2002; Roguev et al., 2001). Therefore, we did not follow up on its loss in the absence of H2B monoubiquitination. Furthermore, we did not observe the gain of any polypeptides or additional polypeptides in COMPASS when purified in the absence of H2B monoubiquitination (data not shown). Given the above observation and the fact that Cps15, in contrast to Cps35, is not required for H3K4 methylation by COMPASS, we chose to focus on the ubiquitin-dependent association of Cps35 with COMPASS.

To demonstrate that the loss of H2B monoubiquitination does not alter Cps35 transcription or message stability, we performed RT PCR analyses from strains either deleted for *RAD6* or that bear a single point mutation in K123 of

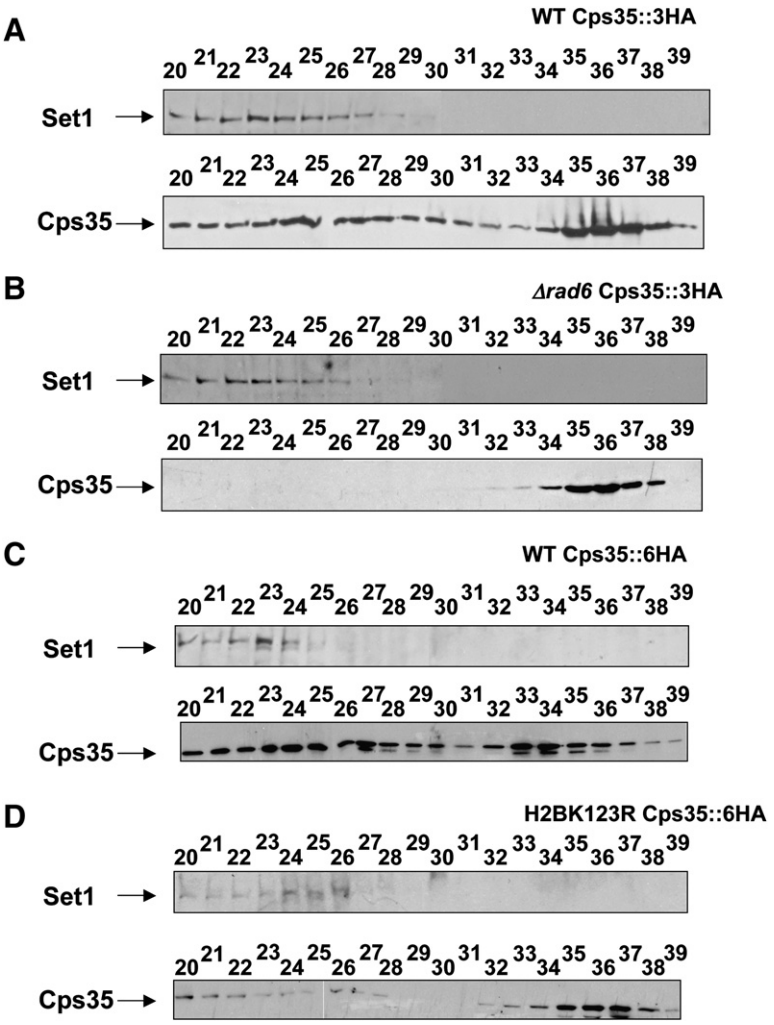

Figure 3. Histone H2B Monoubiquitination and the Association of Cps35 with COMPASS

To determine whether histone H2B monoubiquitination is required for COMPASS to maintain its stability, the chromatographic properties of Set1, and the Cps35 component of COMPASS were tested in the presence and absence of H2B monoubiquitination. Briefly, COMPASS either from (A and C) WT, (B) *rad6Δ*, or (D) strains bearing a K123R single point mutation in histone H2B, were subjected to size exclusion chromatography on a Superose 6 HR column (Pharmacia) operated on a SMART system under the similar conditions used in Figures 2I and 2J. The resulting fractions were subjected to SDS/PAGE followed by western analysis using either Set1 specific polyclonal antibodies, or antibodies toward the tag on Cps35.

H2B (Figure 4C). Furthermore, our western analysis also indicates that Cps35 protein stability is not altered in the absence of H2B monoubiquitination (Figure 4D). Together, our data so far indicates that the loss of H2B monoubiquitination alters the interaction properties of Cps35 with COMPASS without altering Cps35's message or protein stability.

Cps35 Interacts with Chromatin in a H2B Monoubiquitination-Dependent Manner

Next, we wanted to determine whether Cps35's association with COMPASS-regulated genes is depended on H2B monoubiquitination status. Components of Set1/COMPASS associate with the early elongating form of Pol II (Figure 5A). We, therefore, performed chromatin immunoprecipitation (ChIP) to determine the association of both Cps60 and Cps35 at *GAL1* in the presence and absence of H2B monoubiquitination (Figures 5B and 5C). Our data indicates that the loss of either Rad6 or a mutation at the monoubiquitination site of H2B has little to no affect in Cps60 s localization to this gene (Figures 5B and 5C). However, Cps35's presence at *GAL1* is reduced by ~90% in the strains lacking *rad6* or bearing the *H2BK123R* single point mutation (Figures 5B and 5C). We also confirmed our ChIP studies by the direct immunoprecipitation of Cps35 from WT and *rad6* deleted background, and probed for the presence of histone H3 to determine the association of Cps35 with chromatin in the presence and absence of H2B monoubiquitination (data not shown). To further confirm that the H2B monoubiquitination-depndent interaction of Cps35 also occurs on other genes, we performed similar ChIP studies on two constitutively active genes, *ADH1* and *PHO84* (Figures 5D and 5E). Overall, the above study (Figure 5) indicates that Cps35's association with chromatin is dependent on H2B's monoubiquitination status.

Cps35 and H3K79 Methylation

As discussed above, monoubiquitination of histone H2B not only regulates the pattern of histone H3K4 methylation by COMPASS, but is also required for H3K79 methylation by Dot1. Dot1 and its H3K79 methylation function is highly

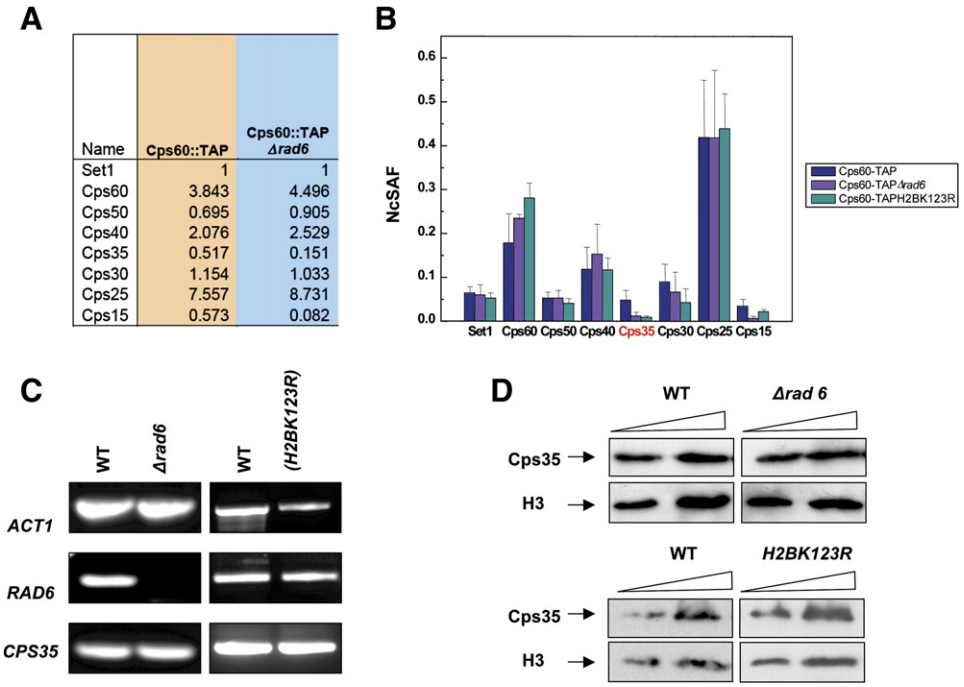

Figure 4. Cps35 Interacts with COMPASS in a Histone H2B Monoubiquitination-Dependent Manner

(A and B) Using spectral counts (Paoletti et al., 2006), we have determined COMPASS' composition purified from either WT strains, *Δrad6* or strains bearing a single point mutation in K123 of histone H2B. Purified COMPASS from these strains were subjected to Multidimensional Protein Identification Technology (MudPIT) (Washburn et al., 2001) to carry out proteomic analyses of COMPASS. Spectral counts normalized against protein length (NSAF) were determined for purified COMPASS in the presence and absence of H2B monoubiquitination. MudPIT analyses demonstrated that H2B monoubiquitination is required for the association of Cps35 and Cps15 within COMPASS. Furthermore, MudPIT analyses indicated that the loss of H2B monoubiquitination does not result in the association of any other factors with COMPASS (data not shown). To confirm that the status of the monoubiquitination of H2B does not alter (C) Cps35 expression levels and message stability, or (D) protein stability, Cps35 levels in wild-type and strains defective for H2B monoubiquitination were measured by RT-PCR and western blot analysis respectively.

conserved from yeast to human (Lacoste et al., 2002; van Leeuwen et al., 2002; Ng et al., 2002; Okada et al., 2005; Shilatifard, 2006). Dot1 is a very low abundant protein, and due to this reason, its macromolecular complex has not been purified to homogeneity from endogenous sources. Therefore, we do not know whether Cps35 interact with Dot1. Since Cps35 is an essential gene in yeast, synthetic genetic analyses have not detected its gene product as a genetic interactor with Dot1 or other factors required for H2B monoubiquitination. We first sought to determine whether Dot1 could physically interact with Cps35. To this end, we generated strains coexpressing Dot1::9 myc and Cps35::Flag tagged proteins. We then determined whether Dot1-9myc could be coimmunoprecipitated with Cps35-Flag. As shown in Figure 6A, Dot1 and Cps35 can physically interact. Given the fact that *CPS35* is an essential gene in yeast, we used *Cps35*ts alleles (*swd2-1* and *swd2-3*, a gift from Dr. Moore's laboratory) to determine whether its loss has any affect on H3K79 methylation pattern. Previously, it was demonstrated that the loss of Cps35 could affect the pattern of H3K4 trimethylation (Cheng et al., 2004). Our studies show not only is Cps35 required for the H3K4 methylation pattern (Figure 6B upper panel), but also that its partial loss can result in the reduction of H3K79 trimethylation levels in vivo (Figure 6B lower panel). It is not clear at this time whether the physical interactions between Dot1 and Cps35 require H2B monoubiquitination. Future studies addressing this possibility and determining if Cps35 and Dot1 are part of a macromolecular complex or whether their interaction is transient through chromatin should shed further light on this subject.

Cps35-Dependent Complementation of H3K4 Methylation by COMPASS Purified from a *Δrad6* Background

Our studies so far have demonstrated that COMPASS purified from strains lacking H2B monoubiquitination is devoid of Cps35, and is incapable of di- or trimethylating H3K4. We set out to determine whether the addition of exogenous Cps35 to such purified COMPASS could complement H3K4 di- and trimethylation in vitro. Indeed COMPASS purified from a *Δrad6* background is incapable of methylating H3K4 (Figure 6C, lane 2). However, the addition of exogenous Cps35 (purified from a delta Set1 background) to this form of COMPASS, can complement H3K4 di- and trimethylation in vitro (Figure 6C, lanes 3 and 4). To determine that purified Cps35 alone does not have

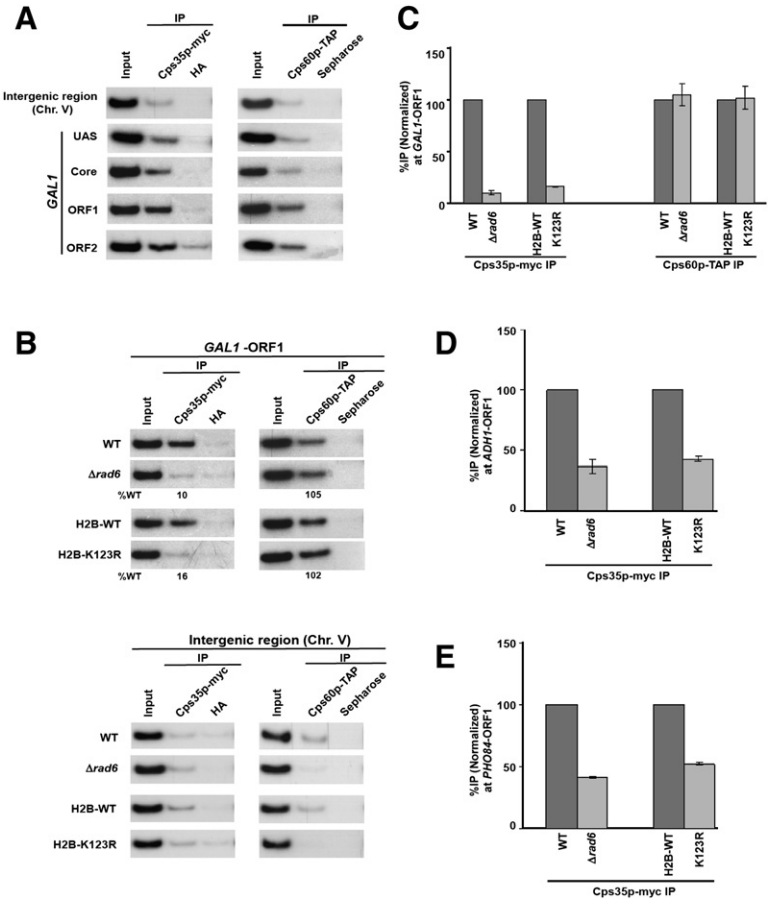

Figure 5. Monoubiquitination-Dependent Interactions of Cps35 with Chromatin

(A) Both Cps35p and Cps60p are recruited to the coding sequence of the active *GAL1* gene. The wild-type yeast strains expressing myc epitope-tagged Cps35 or TAP-tagged Cps60 were grown in a raffinose-containing growth medium up to OD_{600} of 0.9, and then switched to a galactose-containing growth medium for 90 min prior to formaldehyde-based in vivo crosslinking. Immunoprecipitations were performed following a modified ChIP protocol (Shukla et al., 2006). The anti-myc antibody and IgG-sepharose were used against myc-epitope and TAP, respectively, in the ChIP assay. An anti-HA was used as a nonspecific antibody control. The primer-pairs located at the UAS, core promoter and two regions (toward 5′ and 3′ ends) in the ORF (ORF1 and ORF2) of *GAL1,* were used in the PCR analysis of the immunoprecipitated DNA samples. An intergenic control primer-pair on chromosome V was used for background determination.

(B) (Upper panel) Recruitment of Cps35 to chromatin is significantly impaired in the Δ*rad6* and H2B-K123R mutant strains. The wild-type and mutant strains were grown, cross-linked and immunoprecipitated as in panel A. DNA immunoprecipitated relative to wild-type (WT) is presented as %WT. (lower panel) An intergenic control primer-pair on chromosome V was used for background determination.

(C) The data in panel (B) are represented in the form of a histogram. The normalized %IPs of Cps35 and Cps60 at the *GAL1* coding sequence in the wild-type and mutant strains are presented in the form of a histogram. The ratio of immunoprecipitated (IP) DNA over the input is represented as %IP.

(D) The normalized %IPs of Cps35 at the 5′end of the *ADH1* coding sequence in the Δ*rad6* and H2B-K123R mutant strains and their isogenic wild-type equivalents are presented in the form of a histogram. The yeast cells expressing myc epitope-tagged Cps35 in the wild-type and mutant strains were grown in a dextrose-containing growth medium up to an OD_{600} of 1.0 followed by formaldehyde-based in vivo cross-linking. Immunoprecipitations were performed as in panel A. The primer-pair located at the 5′end of the *ADH1* ORF (*ADH1*-ORF1) was used in the PCR analysis of the immunoprecipitated DNA samples.

(E) The normalized %IPs of Cps35p at the 5′end of the *PHO84* coding sequence in the Δ*rad6* and H2B-K123R mutant strains and their isogenic wild-type equivalents are presented in the form of a histogram. The yeast strains were grown, crosslinked and immunoprecipitated as in panel D. The primer-pair located at the 5′end of the *PHO84* ORF (*PHO84*-ORF1) was used in the PCR analysis of the immunoprecipitated DNA samples. The error bars in this figure represent standard deviation.

any HMTase activity, we demonstrated that in the absence *Δrad6* purified COMPASS, purified Cps35 cannot methylate H3K4 (Figure 6C, lane 5).

Interaction of Cps35 with Chromatin in a Set1-Independent Manner

Our studies have demonstrated that Set1 is essential for COMPASS formation, COMPASS subunit stability and H3K4 methylation (Steward et al., 2006). Therefore, we wanted to determine whether Cps35 is capable of associating with chromatin in the absence of COMPASS. As demonstrated before, chromatin is devoid of H3K4 methylation in the absence of Set1 (Shilatifard, 2006) (Figure 6D). However, it appears that in the absence of Set1, Cps35 stability is not altered and it is still capable of interacting with chromatin (Figures 6E and 6F). Together, our data indicate that Cps35's interactions with chromatin in vivo is independent of Set1 and requires monoubiquitinated H2B.

DISCUSSION

Posttranslational modifications of histones and their interacting proteins play a fundamental role in the regulation of development and differentiation (Marfella and Imbalzano, 2007; Shilatifard, 2006; Zhu et al., 2005; Vakoc et al., 2006, 2005; Margueron et al., 2005; Kim et al., 2005; Su et al., 2004; Carrozza et al., 2003). The histone methyltransferase COMPASS and its mammalian homologs, the MLL complexes, can methylate lysine four of histone H3

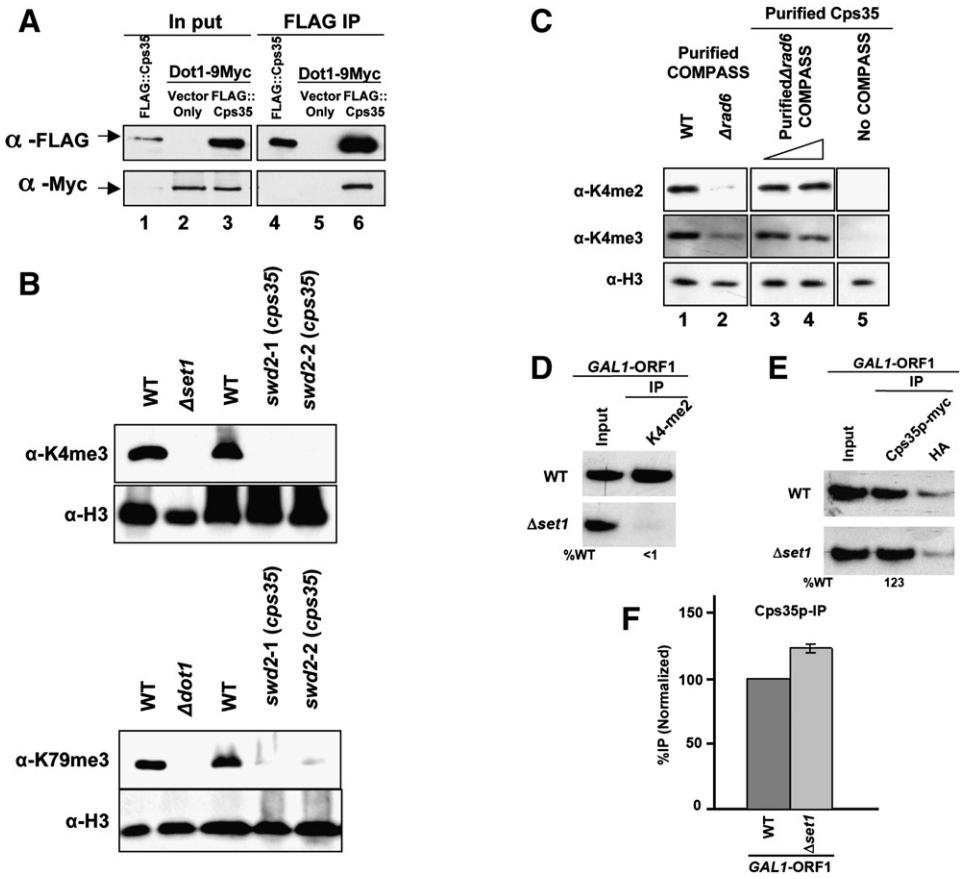

Figure 6. Cps35 and Histone H3 Methylation

(A) Cps35's interaction with Dot1 was determined in strains bearing FLAG::Cps35 tag and Dot1::9Myc tag. Extracts from strains either carrying FLAG::Cps35 (1 and 4) or vector only (2 and 5), or FLAG::Cps35 and Dot1-9Myc tag (3 and 6) were purified on a FLAG affinity column and were eluted with a Flag peptide. Resulting IP fractions were analyzed by SDS/PAGE, followed by western analysis using both anti-FLAG and anti-Myc antibodies for the recognition of Cps35 and Dot1, respectively.

(B) Since Cps35 interacts with both COMPASS and Dot1, the possibility of its requirement in H3K4 and K79 methylations were tested. Wild-type and two ts alleles of Cps35 were grown overnight at 30°C. Extracts from each strain following a temperature shift to 37°C were tested by SDS/PAGE and western analysis for H3K4 (upper panel) and H3K79 trimethylation (lower panel).

(C) Cps35 can complement H3K4 trimethylation activity of COMPASS purified from a *Δrad6* background in vitro. As before (Figure 1B) COMPASS purified from a *Δrad6* background is incapable of di- or trimethylating H3H4 in vitro (lane 2). Briefly, FLAG::Cps35 was purified from *set1Δ* strains by using anti-FLAG affinity resin. Purified Cps35 was then added into the histone methyltransferase assay in the presence and the absence of COMPASS purified from *rad6Δ* strains. The levels of methylated H3K4 were tested by the application of the reaction mixture to SDS-PAGE followed by western analysis with appropriate antibodies (lanes 4 and 5). Purified Cps35 alone was also tested for possible HMTase activity (lane 5).

(D–F) Cps35 interacts with chromatin independently of COMPASS, (D) Histone H3K4-dimethylation at the *GAL1* coding sequence is lost in Δ*set1*. Both the wild-type and *SET1* deletion mutant strains were grown and crosslinked, as in Figure 5A. Immunoprecipitations were performed using the antibody against H3-dimethyl K4 (Upstate Biotechnology, Inc.). The primer-pair located at the 5′end of the *GAL1* coding sequence (*GAL1*-ORF1) was used in the PCR analysis of the immunoprecipitated DNA samples. (E) Recruitment of Cps35p to the *GAL1* coding sequence is not altered in Δ*set1*. The yeast strains were grown, crosslinked and immunoprecipitated as in Figure 5A. An intergenic control primer-pair on chromosome V was used for background determination (data not shown) (F). The results in panel (E) are represented in the form of a histogram. The error bars in this figure represent standard deviation.

(Shilatifard, 2006; Miller et al., 2001; Roguev et al., 2001; Krogan et al., 2002). It was first discovered in yeast *S. cerevisiae* that H3K4 methylation by COMPASS requires monoubiquitination of histone H2B by the Rad6/Bre1 complex (Shilatifard, 2006; Dover et al., 2002; Wood et al., 2003a; Sun and Allis, 2002). This crosstalk pathway is highly conserved from yeast to human (Parvi et al., 2006; Kim et al., 2005; Zhu et al., 2005; Wood et al., 2005b). Not only is H2B monoubiquitination required for COMPASS' activity, it also regulates proper H3K79 methylation by Dot1 (Shilatifard, 2006; Ng et al., 2003). Despite the fact that we have learned much about the factors required for the proper regulation of H2B monoubiquitination by Rad6/Bre1 (Dover et al., 2002; Wood et al., 2003a, 2003b, 2005a, 2005b; Krogan et al., 2003; Xiao et al., 2005; Laribee et al., 2005; Shilatifard, 2006), very little is

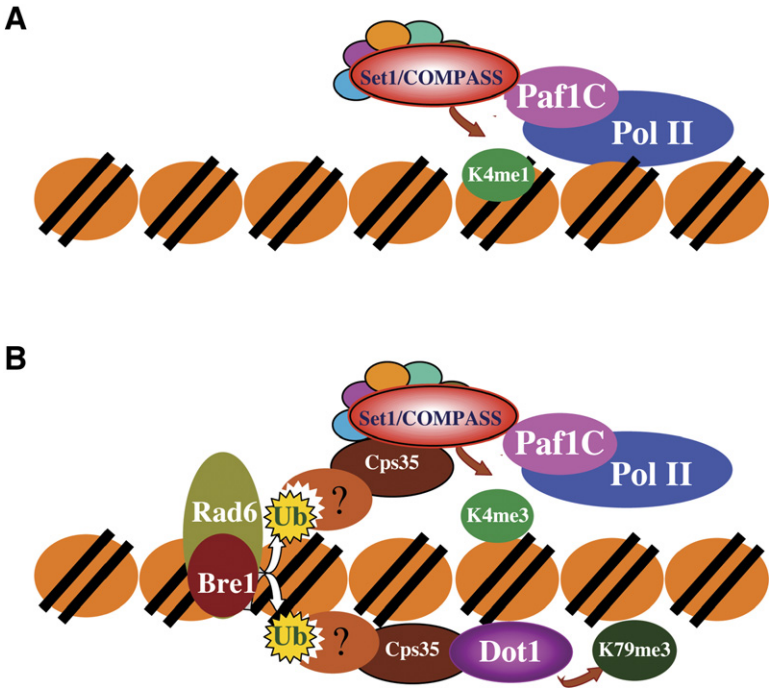

Figure 7. Cps35 Is Required for Translating Histone Crosstalk between H2B Monoubiquitination and H3 Methylation by COMPASS

Our previous studies indicated that COMPASS interacts with the elongating form of Pol II via its interaction with the Paf1 complex (Wood et al., 2003b; Krogan et al., 2003). Therefore, (A) in the absence of H2B monoubiquitination, although Cps35 cannot interact with COMPASS, COMPASS can still interact with the transcribing Pol II via the Paf1 complex and monomethylate H3K4. (B) In the presence of proper H2B monoubiquitination, Cps35 is recruited to chromatin either directly or indirectly in a COMPASS independent manner (Figures 6D–6F). The association of Cps35 with chromatin in an H2B monoubiquitination-dependent manner facilitates the interaction between COMPASS and Cps35, resulting in the generation of a H3K4 trimethylation competent COMPASS. Based on the observation that Cps35 interacts with Dot1 and that it is required for proper H3K79 trimethylation (Figures 6A and 6B), we have also included in this model the possibility that H2B monoubiquitination could also facilitate the interaction between Dot1 and Cps35 and therefore regulate monoubiquitination-dependent H3K79 methylation. However, it is not clear at this time how Cps35 would regulate Dot1 s catalytic activity. Future biochemical and enzymological studies in this regard should shed further light on this subject.

known about the molecular mechanism of how H2B monoubiquitination regulates H3 methylation.

In this manuscript, we have identified the Cps35 subunit of COMPASS as a factor that is required to translate the crosstalk between H2B monoubiquitination and H3 methylation by COMPASS. We have demonstrated: (1) COMPASS purified from strains lacking H2B monoubiquitination is incapable of di- and trimethylating histone H3K4; (2) COMPASS purified from a H2B monoubiquitination-deficient background has reduced levels of Cps35; (3) the presence of Cps35 within COMPASS is required for the formation of a methylation competent COMPASS both in vivo and in vitro; (4) Cps35 interacts with chromatin in a H2B monoubiquitination dependent manner; (5) the addition of exogenous Cps35 to COMPASS purified from H2B monoubiquitination deficient strains can complement H3K4 methylation; (6) Cps35 is also capable of interacting with Dot1, and that its temperature sensitive alleles have lower levels of H3K79 trimethylation; and (7) Cps35 interacts with chromatin in a COMPASS independent manner.

Given the above observations, we propose a model for the role of Cps35 in translating the H2B monoubiquitination signal in H3 methylation by COMPASS (Figure 7). It was previously demonstrated that the loss of H2B monoubiquitination results in the loss of H3K4 di- and trimethylation with reductions in H3K4 monomethylation levels (Schneider et al., 2005; Shahbazian et al., 2005). Therefore, cells defective for H2B monoubiquitination can still monomethylate H3K4 but not di- or trimethylate. As shown in Figure 7A, in the absence of H2B monoubiquitination, COMPASS can still interact with Pol II through its interaction with the Paf1 complex (Krogan et al., 2003; Wood et al., 2003b) and monomethylate H3K4. Cps35 can interact with chromatin when H2B is monoubiquitinated by the Rad6/Bre1 complex (Figure 7B). It is not clear at this time whether this interaction is directly between Cps35 and monoubiquitinated H2B or is mediated through other factors. However, it is clear that the interaction of Cps35 with chromatin requires H2B monoubiquitination (Figure 5) and that this interaction is independent of COMPASS (Figures 6D–6F). In this model, the H2B monoubiquitination-dependent association of Cps35 with chromatin can facilitate the interaction between Pol II associated COMPASS and Cps35, resulting in the generation of an H3K4 methylation-competent COMPASS on chromatin, and hence, H3K4 di- and trimethylation. The observation that Cps35 is required for the generation of a methylation competent COMPASS is in line with our in vitro studies (Figure 6C). The in vitro studies show that the addition of exogenous Cps35 to COMPASS purified from a *Δrad6* background can complement the in vitro methyltransferase activity of COMPASS. Furthermore, our previously performed kinetic studies in which COMPASS was purified from wild-type cells demonstrated that this form of COMPASS is capable of methylating H3K4 on both free H3 and nucleosomes

lacking H2B monoubiquitination (Wood et al., 2007). These studies suggest that wild-type COMPASS can methylate H3K4 in vitro in the absence of H2B monoubiquitination. Based on our studies presented in this manuscript, we now know that H2B monoubiquitination is required for the Cps35 interaction chromatin and with COMPASS.

Several possible mechanisms for H2B monoubiquitination's role in the interaction of Cps35 with COMPASS can be proposed. First, either a direct or indirect interaction of Cps35 with monoubiquitinated H2B can change Cps35's conformation so that it can easily associate with COMPASS for the generation of a methylation competent COMPASS. Second, since Cps35 interacts with chromatin in an H2B monoubiquitination-dependent manner, its recruitment to chromatin via H2B monoubiquitination can bring Cps35 in close proximity to COMPASS (interacting with Pol II through the Paf1 complex), resulting in their physical interactions, and therefore, H3K4 di- and trimethylation. Third, it would be feasible to consider that the association of Cps35 with chromatin in a H2B monoubiquitination dependent manner can result in a posttranslational modification(s) of Cps35, and that this modification could facilitate the interaction of Cps35 with COMPASS. Although our two-dimensional gel electrophoresis studies (Figure 2) did not demonstrate substantial changes in the charge or electrophoretic mobility of Cps35 in the presence or absence of Rad6, such proposed modifications (e.g., lysine methylation) could be very difficult to detect in two-dimensional gel electrophoresis.

Since H2B monoubiquitination is required for both H3K4 and H3K79 methylation, we find the observation of interaction between Cps35 and Dot1, and the requirement of Cps35 in H3K79 trimethylation quite fascinating. Based on this preliminary observation (Figures 6A and 6B), we have included in our model (Figure 7B), the possibility that H2B monoubiquitination could also facilitate the interaction between Dot1 and Cps35, which could also regulate monoubiquitination-dependent H3K79 methylation. It is not clear to us at this time whether Cps35's interaction with Dot1 is a stable or transient interaction. However, the observation that a partial loss of Cps35 can result in a reduction in H3K79 trimethylation levels serves as an intriguing preliminary finding for further investigations. Future studies defining the molecular role of Cps35 in this process will further our understanding of the diverse molecular roles of histone modifications and their crosstalk in development and differentiation.

EXPERIMENTAL PROCEDURES

Purification of COMPASS from Wild-Type Strains and Strains Deleted for Rad6 or Bearing a Single Point Mutation in K123 of H2B

The Cps60 component of COMPASS was TAP tagged in WT strains, *rad6Δ* strains or strains bearing a single point mutation in K123 of histone H2B. The strains were inoculated into 6 L of YPD and grown to an OD600 of about 2.0. TAP purification was performed according to Rigaut et al., 1999, with several modifications. Briefly, cell extracts containing 200 mM NaCl were incubated with IgG-sepharose (GE-healthcare) at 4°C for 3 hr. The bound fractions were eluted with a TEV protease (Invitrogen) treatment. A TEV eluted fraction was incubated with calmodulin-sepharose (GE-healthcare) at 4°C for 4 hr and eluted with 2 mM EGTA.

In Vitro Methyltransferase Assay for COMPASS

The methyltransferase activity of purified COMPASS was tested toward recombinant histone H3 or recombinant nucleosomes as described previously (Krogan et al., 2002 and Schneider et al., 2005; Wood et al., 2007). Purified COMPASS was incubated with recombinant histone H3 in the HMTase reaction buffer with cold SAM. The methylation of histone H3 was tested by the application of the reaction mixture to SDS/PAGE and western analysis with antibodies to different modified forms of H3K4.

Multidimensional Protein Identification Technology for the Analysis of the Components of COMPASS

TCA-precipitated protein mixtures from WT and mutant COMPASS purifications were digested with endoproteinase Lys-C and trypsin (Roche) as previously described (Florens et al., 2006). Peptide mixtures were loaded onto triphasic 100 μm fused silica microcapillary columns, as described previously (McDonald et al., 2002). Loaded microcapillary columns were placed in-line with a Quaternary Agilent 1100 series HPLC pump and a Deca-XP ion trap mass spectrometer (ThermoFinnigan) equipped with a nano-LC electrospray ionization source. Fully automated Multidimensional Protein Identification Technology (MudPIT) runs were carried out on the electrosprayed peptides, as described (Florens et al., 2006). Tandem mass (MS/MS) spectra were interpreted using SEQUEST (Eng et al., 1994) against a database containing *Saccharomyces cerevisiae* protein sequences downloaded from the National Center for Biotechnology Information complemented with 177 sequences from usual contaminants such as human keratins, IgGs, and proteolytic enzymes. In addition, to estimate false discovery rates, each sequence was randomized (keeping the same amino acid composition and length), and the resulting "shuffled" sequences were added to the "normal" yeast database and searched at the same time. Peptide/spectrum matches were sorted and selected using DTASelect (Tabb et al., 2002) keeping false discovery rates at 2% or less, and peptide hits from multiple runs were compared using CONTRAST (Tabb et al., 2002) and contrast-report (Florens et al., 2006). To estimate protein levels, spectral counts of nonredundant proteins were normalized (Zybailov et al., 2006) using an in-house developed script (*contrast-reportadd-nsaf*): for each protein detected in a particular MudPIT analysis.

Size-Exclusion Chromatography

Yeast cells were grown at OD600 = 1.0. Cell extracts were prepared with resuspending the cells in a lysis buffer (50 mM HEPES, [pH 7.5], 150 mM NaCl, 0.5 mM EDTA and 0.1% NP-40), and disrupting them by minibead beater (BioSpec Products, Inc). This crude extract was clarified by centrifugation at 15,000 g for 20 min. Cell extracts were separated by size exclusion using a Superose6 HR 10/30 Column operated on SMART system (Amersham Biosciences). Fractions obtained from the size-exclusion chromatography were analyzed by SDS/PAGE followed by western blotting with indicated antibodies.

Protein Purification and Two-Dimensional Gel Analysis

Yeast cells containing Cps60-TAP, Cps50-TAP, Cps40-TAP, Cps35-3HA, and Cps30-TAP in WT strains and strains deleted for Rad6 were inoculated into 1.5 L of YPD and grown to an OD600 of about 1.2. Tagged proteins were purified as described before (Wood et al., 2005b). For 2D analyses, purified proteins were precipitated with acetone and resuspended with a 2D lysis buffer (9.5 M urea, 2% (w/v) CHAPS, 0.8% (w/v) Pharmalyte (pH 3–10), 1% (w/v) dithiothreitol (DTT) and 5 mM Pefabloc). The lysis buffer-resuspended pellets

were loaded onto the first dimension IEF strips using 7 cm long precast IPG strips with a pH range from 3–10 (GE healthcare). IEF were performed at 20°C with the following parameters: 50 V for 11 hr, 250 V gradient for 1 hr, 500 V gradient for 1 hr, 1000 V gradient for 1 hr, 8000 V gradient for 2 hr, then 8000 V for a total 48 000 V hr (protocol provided by the manufacturer, Amersham Bioscieces). IPG strips were incubated in 10 ml of an equilibration buffer (6 M urea, 2% SDS, 50 mM Tris, [pH 8.8], 20% glycerol) for 15 min at room temperature with shaking, transferred to another 10 ml of equilibration for 15 min with shaking, rinsed, and loaded onto 12% polyacrylamide gels in a Tris-glycine buffer (25 mM Tris-HCl, 192 mM glycine, 0.1% SDS, [pH 8.3]). Electrophoresis was performed at 20 mA for 2 hr. The isolated proteins by 2D electrophoresis were analyzed by western analysis with anti-IgG antibody for TAP-tagged proteins, and anti-HA antibody for HA-tagged proteins, respectively.

FLAG-Purification

Strains bearing Dot1::9Myc containing pRS315-GAL1p-FLAG or pRS315-GAL1p-FLAG-Cps35 were grown in SD-Leu to OD600 = 1.0 and transferred into YPGal (1% yeast extract, 2% peptone, 2% glactose) for 4 hr at 30°C. Cells were spun down, resuspended in 4 folded cell volumes of a lysis buffer (50 mM Tris-Hcl, [pH 7.5], 150 mM NaCl, 1 mM EDTA and 1% Triton X-100) containing 50 μl of protease inhibitor cocktail (1 tablet Roche complete, mini, EDTA-free, dissolved in 1.5 ml lysis buffer), and were then disrupted with a minibead beater for 5 min at 4°C. The supernatant fraction was mixed with a 100-μl bed volume of anti-FLAG M2 affinity gel (Sigma-Aldrich) for 1 hr at 4°C and eluted with 0.1 mg/mL FLAG peptide solution.

ChIP Assay

The ChIP assays were performed as described previously (Bhaumik and Green, 2003; Shukla et al., 2006). Primer-pairs used for PCR analysis were as follows: *GAL1-UAS*: 5′-CGCTTAACTGCTCATTGCT ATATTG-3′ and 5′-TTGTTCGGAGCAGTGCGGCGC-3′ *GAL1*-Core: 5′-ATAGGATGATAATGCGATTAGTTTTTTAGCCTT-3′ and 5′-GAAAA TGTTGAAAGTATTAGTTAAAGTGGTTATGCA-3′ *GAL1*-ORF1: 5′-CAG TGGATTGTCTTCTTCGGCCGC-3′ and 5′-GGCAGCCTGATCCATAC CGCCATT-3′ *GAL1*-ORF2: 5′-CAGAGGGCTAAGCATGTGTATTCT-3′ and 5′-GTCAATCTCTGGACAAGAACATTC-3′ *ADH1*-ORF1: 5′-CTGG TTACACCCACGACGGTTCTT-3′ and 5′-GCAGACTTCAAAGCCTTGT AGACG-3′ *PHO84*-ORF1: 5′-TAGCTGATATTGTTGGTCGTAAGAG-3′ and 5′-TACCAATACCCATGACAAAACGGTA-3′. Autoradiograms were scanned and quantitated by the National Institutes of Health image 1.62 program. The ratio of immunoprecipitated (IP) DNA over the input was presented as %IP. DNA immunoprecipitated relative to wild-type (WT) is presented as %WT. These studies were performed from multiple chromatin preparations for error bar determinations.

We also used an intergenic control primer-pair on chromosome V for background determination. Forward intergenic primer: 5′ - GGC TGT CAG AAT ATG GGG CCG TAG TA - 3′. Reverse intergenic primer: 5′ - CAC CCC GAA GCT GCT TTC ACA ATA C - 3′.

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