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Chromatin signaling to kinetochores: Trans-regulation of Dam1 methylation by histone H2B ubiquitination

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Summary

Histone H3K4 trimethylation by the Set1/MLL family of proteins provides a hallmark for transcriptional activity from yeast to humans. In *S. cerevisiae*, H3K4 methylation is mediated by the Set1-containing COMPASS complex and is regulated *in trans* by prior ubiquitination of histone H2BK123. All of the events that regulate H2BK123ub and H3K4me are thought to occur at gene promoters. Here we report that this pathway is indispensable for methylation of the only other known substrate of Set1, K233 in Dam1, at kinetochores. Deletion of *RAD6*, *BRE1*, or Paf1 complex members abolishes Dam1 methylation, as does mutation of H2BK123. Our results demonstrate that Set1-mediated methylation is regulated by a general pathway regardless of substrate that is composed of transcriptional regulatory factors functioning independently of transcription. Moreover, our data identify a node of regulatory cross-talk *in trans* between a histone modification and modification on a non-histone protein, demonstrating that changing chromatin states can signal functional changes in other essential cellular proteins and machineries.

Keywords

Dam1; Set1; MLL; Ipl1; Aurora kinase; Rad6; Bre1; Paf1; histone; ubiquitination; methylation; kinetochore; cross-talk

Introduction

Histones are subject to multiple different types of post-translational modifications including methylation, acetylation, phosphorylation, and ubiquitination. Lysine (K) residues can be modified by the addition of one, two, or three methyl groups (me1, me2, and me3,

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respectively), and these different modification states are important for different physiological outcomes. Histone modifications also undergo cross-regulation. The addition of a post-translational modification to one residue can affect the subsequent modification of another residue(s) either *in cis*, on the same histone molecule, or *in trans*, between histone molecules (Latham and Dent, 2007).

Regulation of H3K4 methylation by H2B ubiquitination provides the canonical example of histone modification cross-talk *in trans*. Conserved from yeast to humans, ubiquitination of histone H2BK123 in yeast or H2BK120 in humans is required for the subsequent di- and trimethylation of H3K4 at gene promoters (Dover et al., 2002; Sun and Allis, 2002; Zhu et al., 2005). The E2 ubiquitin conjugating enzyme Rad6, together with its E3 ligase partner Bre1, ubiquitinates histone H2BK123 in yeast (Wood et al., 2003a). Both the E2 and E3 enzymes and K123 within H2B are essential for H3K4 methylation by the Set1 (Kmt2) methyltransferase complex, COMPASS (Sun and Allis, 2002; Wood et al., 2003a). Rad6 recruitment to promoters is dependent on both Bre1 and transcriptional activators (Wood et al., 2003a). A direct role for transcription in regulating H2B ubiquitination is indicated by global loss of this modification at gene promoters upon inactivation of a temperature-sensitive allele of the RNA pol II C-terminal domain (CTD) serine 5 kinase, *KIN28* (Xiao et al., 2005). Also necessary for H2BK123 ubiquitination is the Paf1 transcriptional elongation complex, including Ctr9, Rtf1, and the Paf1 protein itself (Krogan et al., 2003; Ng et al., 2003a; Wood et al., 2003b). The Paf1 complex is not required for Rad6 or Bre1 recruitment to promoters, but it is required for their catalytic activity (Wood et al., 2003b). Additionally, the Paf1 complex mediates association of Rad6-Bre1 with elongating RNA polymerase (Wood et al., 2003b).

The Paf1 complex also facilitates recruitment of the Set1-containing COMPASS complex to gene promoters (Krogan et al., 2003). The Set1 protein is catalytically inactive in the absence of the other members of the COMPASS complex. In particular, complex core members Swd1, Swd2, and Swd3 are required for all catalytic activity, whereas deletion of Sdc2 or Bre2 leads to substantial reduction in dimethylation and complete loss of trimethylation of H3K4. The Spp1 subunit is only required for H3K4 trimethylation (Dehe et al., 2006; Schneider et al., 2005). Swd2 is the sole member of this complex that is essential for viability in yeast, likely due to its involvement outside of COMPASS in transcriptional termination and RNA processing (Cheng et al., 2004; Dichtl et al., 2004). Swd2 has been proposed as the link between H2BK123ub1 and H3K4me. Ubiquitination of Swd2 on two lysines by Rad6-Bre1 is required for H3K4 trimethylation (Vitaliano-Prunier et al., 2008). Additionally, ubiquitination of H2BK123 is required for Swd2 association with COMPASS and formation of a catalytically active complex at gene promoters (Lee et al., 2007).

The H3K4 methylation pathway from yeast is well conserved in humans. The Bre1 ortholog, RNF20, together with the Rad6 orthologues hHR6A/hHR6B, ubiquitinate H2B on lysine 120 (Kim et al., 2005; Zhu et al., 2005). In addition, H2BK120 ubiquitination requires a functional hPAF complex and directly activates H3K4 methylation by hSET1 and MLL proteins during transcription (Kim et al., 2009; Zhu et al., 2005). Since MLL is subject to translocations associated with acute leukemias (Berdasco and Esteller, 2010), much research effort is directed towards defining MLL functions. Studies of Set1 in yeast have provided paradigms for understanding the enzymatic activity and regulation of MLL and other H3K4 methyltransferases in higher eukaryotes (Tenney and Shilatifard, 2005).

Only one non-histone substrate has been identified for Set1 to date, the kinetochore protein Dam1 (Zhang et al., 2005). Dam1 is a component of the ten-member Dam1 (or DASH) complex (Cheeseman et al., 2001; Janke et al., 2002; Li et al., 2002), which oligomerizes

into rings around microtubules to anchor the kinetochore to the microtubules (Miranda et al., 2005; Westermann et al., 2005). Ipl1, the sole Aurora kinase orthologue in yeast, regulates the integrity of the Dam1 complex. When improper kinetochore-microtubule attachments occur, Ipl1 phosphorylates Dam1 and other kinetochore proteins, resulting in disruption of existing protein-protein interactions so that microtubule-kinetochore interactions can be reformed correctly (Cheeseman et al., 2002). Aurora kinases are also essential for proper chromosome segregation in humans (Lampson and Cheeseman, 2011). Overexpression of the Aurora kinases is associated with aneuploidies and chromosome instabilities in several types of tumors and these enzymes are emerging therapeutic targets (Lens et al., 2010).

Our previous work established that deletion of *SET1* suppresses both the temperature sensitivity and the chromosome segregation defects of the *ipl1-2* Aurora kinase conditional allele due to changes in Dam1 methylation levels (Zhang et al., 2005). A balance in dimethylation of Dam1K233 by Set1 and phosphorylation of flanking serines by Ipl1 is important for proper Dam1 function in chromosome segregation illustrating a close functional connection between Set1 and Ipl1 (Zhang et al., 2005). This work demonstrated that ‘phospho-methyl’ switches also occur in a non-histone protein, and it raises the possibility that MLL functions might be connected to those of Aurora kinases in mammalian cells.

The discovery of a second Set1 substrate raised the question of how Set1 function is regulated towards non-histone substrates and at non-promoter sites. Here we show that the Paf1 complex and Rad6-Bre1 mediated ubiquitination of H2BK123 are required for Dam1 methylation at the kinetochore and therefore inhibit Ipl1-mediated phosphorylation, revealing unexpected functions for these proteins in mitosis. In contrast to methylation of H3K4 at gene promoters, methylation of Dam1 is not dependent on active transcription. Our data indicate that several factors previously thought to be required solely for Set1 function at gene promoters are more generally required for the catalytic activity of the COMPASS complex regardless of substrate or cellular process. Additionally, our study demonstrate that cross-talk can occur *in trans* between modifications on a histone and a non-histone protein, indicating that chromatin states can trigger changes in non-DNA templated processes, such as the regulation of chromosome segregation by the kinetochore machinery.

Results

Set1 functions within COMPASS at the kinetochore

We previously determined that Set1 functions within COMPASS to regulate Ipl1 functions at the kinetochore using a genetic approach (Zhang et al., 2005). To determine directly whether suppression of the temperature sensitive phenotype of the mutant *ipl1-2* allele by deletion of members of the COMPASS complex reflects changes in Dam1 dimethylation levels at lysine 233 (Dam1K233me2), we examined Dam1 methylation levels by immunoprecipitation of HA-Dam1 expressed from the native Dam1 locus followed by immunoblots with antisera specific for Dam1K233me2 (Figure 1A). We compared Dam1K233me2 levels in strains deleted for *SWD1,* which binds H3K4 and is required for all H3K4 methylation, *SDC1* and *BRE2,* which form a heterodimer and whose deletion reduces the levels of H3K4 dimethylation and all trimethylation, and *SPP1,* which is only necessary for H3K4 trimethylation (Dehe et al., 2006; Schneider et al., 2005). Our previous results indicated that deletion of each of these COMPASS components except *SPP1* suppressed the *ipl1-2* kinase mutation (Zhang et al., 2005). Our immunoblots (Figure 1A) reveal a striking correspondence between loss of Dam1K233me2 and the suppression of *ipl1-2*. Dam1 K233me2 was reduced to a similar level in *swd1*Δ, *sdc1*Δ, *bre2*Δ, and *set1*Δ strains (Figure 1A), whereas deletion of *SPP1*, which did not suppress the *ipl1-2* temperature sensitive allele, had little effect on Dam1K233me2. Notably, the roles of the COMPASS

subunits in Dam1K233 dimethylation correlate with their roles in H3K4 dimethylation (Figure 1B and 1C). Swd1 and Sdc1 are required for both H3K4 and Dam1K233 dimethylation, while Spp1 does not affect either H3K4 or Dam1K233 dimethylation (Figure 1A and 1B). Others have previously published that deletion of *BRE2* or *SDC1* only partially reduces H3K4me2 (Dehe et al., 2006; Schneider et al., 2005). However, in our hands deletion of either of these COMPASS subunits completely abolishes H3K4me2 (Figure 1B). These differences might reflect strain differences or differences in the antibody used in these studies. Deletion of *SWD1* abolishes the integrity of the COMPASS complex (Dehe et al., 2006), so loss of Dam1 K233me2 in the *swd1*Δ strain indicates that a functional COMPASS complex is required for the catalytic activity of Set1 regardless of substrate.

Similarly loss of Dam1 dimethylation upon deletions of either *BRE2* or *SDC1* indicates that Set1-mediated dimethylation requires this heterodimer for either of its substrates. Our data additionally suggest that Spp1 may only be required for trimethylation at either substrate indicating conserved enzymatic functions for the Set1 complex. We were not able to directly test this idea, as we do not have an antibody specific to Dam1K233me3. Our analyses demonstrate that the catalytic activity of Set1 is dependent on a functional COMPASS complex regardless of substrate suggesting that Set1 alone is unlikely to methylate substrates *in vivo*. Finally, these results indicate that suppression of the temperature sensitive *ipl1-2* allele serves as a good predictor of a requirement of a particular gene product for Dam1K233 dimethylation, further demonstrating the strong functional connection between Ipl1-mediated phosphorylation and methylation of Dam1 (Figure 1C).

Loss of other known and putative methyltransferases does not suppress *ipl1-2*

S. cerevisiae contains six additional SET (*Su(var)3-9, Enhancer-of-zeste, Trithorax*) domain-containing proteins with known or predicted methyltransferase activity and the non-SET domain containing H3K79 methyltransferase Dot1 (Feng et al., 2002; Ng et al., 2002). To determine whether any of these known or putative lysine methyltransferases also affect Ipl1 functions similar to Set1, individual deletions of *SET2* through *SET7* or *DOT1* were combined with the *ipl1-2* allele and growth of these cells was compared to that of *ipl1-2 set1*Δ cells at permissive and non-permissive temperatures. These studies revealed that deletion of these genes had little to no affect on the temperature sensitivity of the *ipl1-2* allele (Supplemental Figure 1). At most a small, ten-fold suppression of *ipl1-2* was observed in the *ipl1-2 set3*Δ and *ipl1-2 set4*Δ strains, but this degree of suppression is far lower (100–1000X) than that observed upon deletion of *SET1*. These data suggest that Set1 is the primary lysine methyltransferase that modulates Ipl1 function.

The Paf1 complex is required for Dam1 methylation

The Paf1 transcription elongation complex is required for recruitment of Set1 and COMPASS and subsequent di- and trimethylation of H3K4 at gene promoters (Krogan et al., 2003; Ng et al., 2003b). In addition, the Paf1 complex is essential for H3K4 methylation in Drosophila and humans suggestive of a highly conserved role in Set1-mediated methylation (Adelman et al., 2006; Zhu et al., 2005). To determine whether the role of the Paf1 complex in Set1-mediated methylation is specific for methylation of H3K4 or is more generally required for Set1 functions, we asked what effect loss of these factors might have on Set1 functions at the kinetochore by evaluating the effects of deletions in these genes on the *ipl1-2* phenotype and on Dam1K233me2 levels.

We combined deletions of genes encoding the three subunits of the Paf1 complex essential for H3K4me2, *PAF1*, *CTR9*, and *RTF1* with the *ipl1-2* allele. As we previously reported, deletion of *PAF1* did not suppress *ipl1-2* under normal growth conditions (Figure 2A) (Zhang et al., 2005). Similarly, *ctr9Δ* did not suppress the *ipl1-2* temperature sensitive

phenotype (Figure 2A). In contrast, deletion of *RTF1* did suppress the *ipl1-2* temperature sensitive phenotype (Figure 2A). These findings indicate either that Rtf1 has a unique role in regulating Ipl1 functions, or that other phenotypes associated with *paf1Δ* and *ctr9Δ* loss mask the ability to score suppression of the *ipl1* mutant phenotype. In fact, loss of either *PAF1* or *CTR9* leads to very similar phenotypes including cell wall defects that result in temperature sensitivity (data not shown) (Betz et al., 2002). These defects are suppressed by the addition of an osmotic stabilizer such as sorbitol to the growth medium (Betz et al., 2002). Repeating the *ipl1-2* suppression assays on media containing sorbitol revealed that deletion of either *PAF1* or *CTR9* suppressed the *ipl1-2* temperature sensitivity to a degree similar to that observed upon deletion of *RTF1* (Figure 2A). Importantly, the addition of sorbitol did not affect the temperature sensitivity of the cells containing only the *ipl1-2* allele (Figure 2A). Collectively, these data indicate that the Paf1 complex has a previously undiscovered role outside of transcription in negatively regulates Ipl1 functions at the kinetochore, as does Set1.

To directly determine whether these effects reflect a requirement of the Paf1 complex for Dam1 methylation, Dam1 was immunoprecipitated from *ctr9*Δ, *rtf1*Δ, or control cells and then immunoblotted for Dam1K233me2 and H3K4me2. Deletion of *CTR9* phenocopies *paf1Δ* cells disrupting the integrity of the Paf1 complex and COMPASS recruitment to promoters (Krogan et al., 2003). Deletion of either of these Paf1 subunits resulted in loss of H3K4me2 as expected (data not shown), and loss of either *CTR9* or *RTF1* caused a significant decrease in Dam1 K233 methylation (Figure 2B). Together, these results demonstrate that the Paf1 complex is required for methylation of both known Set1 substrates, H3K4 and Dam1K233, linking a transcriptional regulatory protein to kinetochore function.

Dam1 methylation is independent of transcriptional elongation

Our finding that the Paf1 transcriptional elongation complex is essential for Dam1 methylation raises the question of whether transcription plays a role in Dam1 methylation. The H3K4 methylation pathway at active gene promoters is dependent on transcription. H2BK123 ubiquitination and Set1 recruitment to specific gene promoters requires Kin28 mediated phosphorylation of serine 5 in the C-terminal domain (CTD) of RNA polymerase (Krogan et al., 2003; Ng et al., 2003b; Xiao et al., 2005). Phosphorylation of RNA polymerase by Kin28 mediates progression of transcriptional initiation to elongation (Cismowski et al., 1995; Valay et al., 1995). To determine whether Dam1 methylation is dependent on transcription, Dam1K233 dimethylation levels were measured in the *kin28-ts16* mutant strain. Cells containing the *kin28-ts16* temperature sensitive allele are deficient in RNA polymerase II CTD serine 5 phosphorylation (data not shown) (Xiao et al., 2003). No difference in Dam1 methylation levels was observed upon inactivation of Kin28 at the restrictive temperature of 37°C (Figure 2C). We were unable to determine whether the *kin28-ts16* allele suppresses the *ipl1-2* temperature sensitive phenotype at the *kin28-ts16* restrictive temperature since the *ipl1-2* mutant is inviable at 37°C. These results demonstrate that Dam1 methylation, in contrast to H3K4 methylation, is not dependent on active transcription. Together with the role of the Paf1 complex in Dam1K233 methylation, these indicate that the Paf1 complex has function independent of transcription in regulating kinetochore function.

Methylation of Dam1 requires kinetochore association

The above results indicate that several transcription factors are required for Dam1 methylation, raising the question of whether this modification occurs at kinetochores, where Dam1 is localized (Cheeseman et al., 2001). To address this question, we asked whether mutations that disconnect the kinetochore from the centromere (in *NDC10*) or the Dam1

complex from the rest of the kinetochore (in *NDC80*) affect Dam1K233 methylation (Janke et al., 2002). At the restrictive temperature, *ncd10-1* cells exhibited clusters of cells characteristic of their inability to complete cytokinesis, and the *ndc80-1* cells were arrested in metaphase as expected (data not shown)(Bouck and Bloom, 2005; McCleland et al., 2003). Dam1 methylation was severely decreased upon inactivation of either Ndc10 or Ndc80 at 37°C (Fig 2D). As with the *kin28-ts16* mutant, we were unable to determine whether *ndc10-1* or *ndc80-1* suppress *ipl1-2* due to the high temperature required to induce the *ndc10-1* and *ndc80-1* phenotypes. Together with our results above, these findings strongly indicate that the Paf1 complex functions at the kinetochore, in the absence of active transcription, to promote methylation of Dam1 and regulation of the kinetochore.

Rad6 and Bre1 regulate Dam1 methylation

A hallmark feature of the regulation of H3K4 methylation is the necessity for prior ubiquitination of H2BK123 by Rad6 and Bre1, constituting cross-talk between histone molecules *in trans* (Shilatifard, 2006). Our data that the Paf1 complex regulates Dam1 methylation independently of transcription suggests that other members of the H3K4 methylation pathway may also contain a conserved function in Dam1 methylation. The Paf1 complex directly interacts with Rad6-Bre1 to mediate H2BK123 ubiquitination (Kim and Roeder, 2009). Therefore, we determined whether Rad6 and Bre1 regulate Dam1 methylation. Indeed, our data indicate that Rad6 or Bre1 are also required for Dam1K233me2 and Set1 function at the kinetochore. Loss of either *RAD6* or *BRE1* suppressed the *ipl1-2* temperature sensitive phenotype (Figure 3A). Suppression of *ipl1-2* by loss of *BRE1* was stronger than suppression caused by loss of *RAD6,* likely reflecting the fact that Rad6 has other functions within the cell that involve different E3 ligase partners, such as Ubr1 and Rad18 (Dohmen et al., 1991; Hoege et al., 2002). Deletion of either of these E3 ligases does not suppress *ipl1-2* demonstrating that Bre1 is the only E3 ligase for Rad6 that plays a role in regulating Ipl1 functions (Supplemental Figure 2). Immunoblots indicate that Dam1K233me2 is abolished in *rad6*Δ or *bre1*Δ cells (Figure 3B) as was H3K4 dimethylation (data not shown). These experiments indicate that Rad6 and Bre1 are both required for Dam1K233 dimethylation. Since Rad6 and Bre1 have no known function outside of ubiquitination, our results suggest a role for ubiquitination of H2B or another Rad6/Bre1 substrate in regulating Dam1 methylation.

Ubp8 mediates proper levels of Dam1 methylation

To further probe the link between ubiquitination and Dam1 methylation, we determined whether loss of specific deubiquitinating enzymes affects Dam1 K233me2 levels. The ubiquitin proteases Ubp8 and Ubp10 regulate H2BK123 ubiquitin levels. Ubp8 acts on ubiquitinated H2B at active gene promoters as a member of the SAGA histone acetyltransferase complex, and Ubp10 deubiquitinates H2B at telomeres and rDNA (Daniel et al., 2004; Emre et al., 2005; Henry et al., 2003). Immunoblots indicate that Dam1K233me2 levels in *ubp10*Δ cells are similar to those in wild-type cells, suggesting that Ubp10 does not regulate Dam1 methylation. However, deletion of *UBP8* resulted in increased Dam1K233 dimethylation levels (Figure 4). These results indicate that removal of ubiquitin from H2B or another Ubp8 substrate normally limits methylation of Dam1. Together with our discovery of Dam1 methylation modulated by Rad6-Bre1, these data strongly suggest a role for ubiquitination in regulating Dam1 methylation.

H2BK123 ubiquitination is required for Dam1 methylation

The effects of loss of *RAD6*, *BRE1*, and *UBP8* on Dam1K233me2 imply that Dam1K233 methylation may be regulated through cross-regulation of post-translational modifications similar to the cross-talk that occurs *in trans* between H2BK123 ubiquitination and H3K4 methylation. The only known substrate of Rad6-Bre1 is histone H2BK123 (Wood et al.,

2003a). Our previous experiments indicated that mutation of this lysine within H2B to a residue unable to be ubiquitinated did not suppress the *ipl1-2* temperature sensitive phenotype (Zhang et al., 2005). However, others have since reported that commonly used *H2BK123R* mutant yeast strains bear additional mutations (Nakanishi et al., 2009). We subsequently discovered four additional, unexpected point mutations of lysines to arginines within histone H2A at K119, K120, K123, and K126 in the *H2BK123R* strain used in our previous studies (data not shown). Although the origin of these mutations is not clear, work by others demonstrates that mutation of these five lysines within H2A and H2B leads to pronounced mitotic and meiotic defects (Robzyk et al., 2000), compromising our ability to measure the effects of the *H2BK123R* mutation on *ipl1-2* temperature sensitivity. We therefore constructed new *ipl1-2 H2BK123R* strains by deleting both endogenous copies of H2B and inserting a plasmid containing a copy of H2B with the K123R point mutation into an *ipl1-2* strain. In this new cellular context, *H2BK123R* suppressed the *ipl1-2* temperature sensitive phenotype, indicating that this lysine affects Ipl1 functions and suggesting that ubiquitination of H2BK123 may be important for Dam1 methylation (Figure 5A). Immunoprecipitation of HA-Dam1 from strains bearing the *H2BK123R* mutation revealed that this ubiquitination site in H2B is required for Dam1K233 dimethylation (Figure 5B) similar to its requirement for H3K4 dimethylation (data not shown). As mutation of this lysine to arginine blocks ubiquitination, our results strongly argue that H2BK123 ubiquitination signals outside of chromatin to the kinetochore for Set1 to methylate Dam1.

Dam1 associates with centromeric histones

Intriguingly, histones H2A, H2B, and H4 were identified by others by mass spectroscopy of immunoprecipitates of Dam1 complexes (Janke et al., 2002). We confirmed Dam1-histone interactions using our HA-Dam1 allele. Histones H2A and H2B co-immunoprecipitated with HA-Dam1 (Figure 5C). Importantly, this association is dependent on Dam1 as immunoprecipitation with HA-conjugated beads in a strain containing untagged Dam1 or in a control strain containing the exosome Ski7 protein endogenously tagged with HA coimmunoprecipitated little to no H2A or H2B. However, histone H3 did not appear to associate with Dam1, even though all histones were present at equal levels in the immunoprecipitant inputs. In *S. cerevisiae*, centromeric sequences are incorporated into single nucleosomes that contain histones H2A, H2B, H4, and the centromere-specific H3 variant histone Cse4 (Meluh et al., 1998). The lack of association of Dam1 with H3 is consistent with the replacement of this histone by the Cse4 H3 variant at the centromere and raises the possibility that Dam1 interacts with Cse4. In fact, interaction between Dam1 and Cse4 was demonstrated by others by both a two-hybrid analysis and *in vitro* binding assays (Shang et al., 2003). We also confirmed that Cse4 and Dam1 interact as Cse4 tagged with the myc epitope at its endogenous locus coimmunoprecipitates HA-Dam1 and as was the case in a reciprocal experiment in which HA-Dam1 coimmunoprecipitates Myc-Cse4 (data not shown; Figure 5D). Together, our data demonstrate that Dam1 interacts with centromeric histones, and possibly with centromeric nucleosomes.

Lysine 123 within H2B is required for H2B and Set1 association with Dam1

The close physical interaction observed between Dam1 and H2B suggests that H2B ubiquitination may directly trigger Dam1 methylation as is the case with H3K4 methylation (Kim et al., 2009). To investigate this possibility, we first determined whether K123 within H2B was required for its association with Dam1. In the *H2BK123R* point mutant, H2B association with Dam1 is abolished suggesting that only ubiquitinated H2B associates with Dam1 (Figure 5E). Surprisingly, Dam1 interaction with H2B is similarly disrupted upon deletion of *SET1* (Figure 5E). This may reflect a requirement for an intact COMPASS complex for H2B interaction with Dam1 as deletion of *SET1* disrupts the integrity of the COMPASS complex (Dehe et al., 2006). Since H2B interaction with Dam1 is dependent on

H2BK123, we asked if Set1 association is dependent on H2BK123 as we had previously demonstrated that Set1 interacts with Dam1 (Zhang et al., 2005). In the *H2BK123R* mutant, interaction between Dam1 and Set1 is abolished (Figure 5E). These results strongly suggests that H2BK123 ubiquitination is directly required for interaction of Set1 with Dam1 and subsequent methylation of Dam1K233. Furthermore, our data suggest a mechanism by which chromatin signals through H2B ubiquitination to the kinetochore to regulate Ipl1 function through Dam1 methylation.

Discussion

Regulation of Set1-mediated methylation of H3K4 has been extensively studied over the past decade, and these studies have revealed an exquisite orchestration of upstream events required for this histone modification (Weake and Workman, 2008). This important regulatory pathway is not limited to yeast but is highly conserved in higher organisms, including humans (Weake and Workman, 2008). Since H3K4 methylation is linked to the regulation of gene expression, almost all of these events have been investigated in the context of gene promoters, and the factors involved are largely thought to function solely in gene transcription. Our findings presented here reveal unexpected roles for Rad6-Bre1, the Paf1 complex, and Ubp8 in the regulation of Dam1 methylation at the kinetochore, indicating non-transcriptional functions for these proteins. Consistent with this conclusion, our data indicate that Dam1 methylation is not dependent on transcription. Our results demonstrate that ubiquitination of histone H2B is required for Dam1 methylation, constituting a previously undescribed example of cross-talk *in trans* between a histone and a non-histone protein (Figure 6). Finally, our discoveries indicate that changes in chromatin modification states not only affect DNA-templated processes such as transcription, but also signal to and regulate other essential cellular processes including chromosome segregation.

Several orthologs of Set1 are found in human cells, including MLL1-MLL5, ASH1, SET1A, and SET1B (Allis et al., 2007; Qian and Zhou, 2006). Complexes containing these orthologs are also quite conserved and resemble the COMPASS complex in composition and in activity towards H3K4 (Dou et al., 2006; Li et al., 2007; Tenney and Shilatifard, 2005). Our data indicate that the functions of COMPASS subunits are not substrate specific, since subunits required for dimethylation of H3K4 are also required for dimethylation of Dam1K233. As additional substrates are identified for MLL and other H3K4 methyltransferases in mammalian cells, our work predicts that intact complexes will also be required for methylation of these non-histone proteins.

Like the COMPASS complex, the Paf1 complex is also highly conserved, and it is involved in multiple processes that affect gene expression, including transcription elongation and mRNA 3′ end processing (Jaehning, 2010). Additionally, the Paf1 complex is required for H3K4 methylation at active gene promoters and at silenced telomeres and rDNA (Krogan et al., 2003; Mueller et al., 2006). Our results demonstrate that the functions of the Paf1 complex extend beyond gene regulation, RNA processing, and H3K4 methylation by facilitating Dam1K233 methylation at the kinetochore. Interestingly, cells containing a deletion of the Paf1 complex member *CTR9* have chromosome segregation defects and undergo chromosome loss at a 110-fold higher rate than wild-type cells (Foreman and Davis, 1996). Our data indicate that deletion of *CTR9* suppresses the temperature sensitive phenotype of *ipl1-2* cells (Figure 2A) associated with chromosome segregation defects (Chan and Botstein, 1993; Francisco and Chan, 1994). These findings suggest that Set1-mediated regulation of Ipl1 aurora kinase functions trump the mitotic phenotype caused by *ctr9*Δ, highlighting the importance of the methylation-phosphorylation switch within Dam1 (Zhang et al., 2005). Both the human and yeast Paf1 complex are necessary for expression of cell cycle regulated genes (Moniaux et al., 2009; Porter et al., 2002). In addition, the

hPAF complex localizes to the centrosome and siRNA directed depletion of hPaf1 leads to defects in mitotic spindle formation. The hPAF complex, then, also appears to play non-transcriptional roles in mitotic functions (Moniaux et al., 2009).

Suppression of *ipl1-2* phenotypes in our studies correlates strongly with loss of Dam1 methylation. These findings indicate that the roles of Rad6, Bre1, and the Paf1 complex in regulating Ipl1 functions are not likely related to the transcriptional functions of these proteins. H2BK123 ubiquitination at gene promoters requires active transcription by RNA polymerase II at that locus, as both events are dependent on Kin28-mediated phosphorylation of serine 5 in the CTD of RNA polymerase (Krogan et al., 2003; Ng et al., 2003b; Xiao et al., 2005). However, our results demonstrate that although H2BK123 is required for Dam1 methylation, Kin28 is not. Together, these data indicate that Kin28 is essential for H2BK123 ubiquitination at promoters, but it is not required for H2BK123 ubiquitination at centromeres. Previous studies examining H2B ubiquitination indicate that this modification is not detectable upon inactivation of Kin28 (Xiao et al., 2005). It should be noted, however, that only ~10% of total H2B is ubiquitinated (Robzyk et al., 2000), and since only one nucleosome is present at each centromere in yeast, it would be very difficult to detect the small amount of ubiquitinated H2B remaining at the centromere in *kin28-ts16* cells by immunoblot. Precedence for this exists in that H2BK123 ubiquitination is undetectable in the *rad6-149* mutant, yet the cells retain H3K4 methylation and avoid growth defects associated with this mutation indicating that low levels of H2BK123 ubiquitination persist (Robzyk et al., 2000; Sun and Allis, 2002).

Our data clearly indicate that Dam1 methylation is dependent on association of Dam1 with the kinetochore through Ndc10 and Ndc80. These data further demonstrate that Rad6, Bre1, and the Paf1 complex act independently of gene promoters to activate COMPASS for Dam1 methylation and that this event occurs at kinetochores. Others have shown that deletion of *RAD6* or mutation of H2BK123 to arginine has no effect on *SET1* expression (Sun and Allis, 2002), indicating that these proteins influence methylation of Dam1K233 (and H3K4) through regulation of Set1 catalytic activity.

Interestingly, parallels between *S. cerevisiae* CEN sequences, which nucleate kinetochores, and gene promoters were noted several years ago due to the shared functions of Cbf1 at both the Met16 promoter and centromeres (Hemmerich et al., 2000). In addition, several factors that influence CEN function also regulate transcription, including Spt4 and H2A.Z (HTZ) (Basrai et al., 1996; Crotti and Basrai, 2004; Mizuguchi et al., 2007). However, no transcripts have been reported for CEN sequences in *S. cerevisiae,* and CEN sequences provide a strong barrier to transcription initiated from external promoters (Doheny et al., 1993). In fact, the yeast centromeric nucleosome induces positive DNA supercoils making transcription through this region by RNA polymerase topologically infeasible (Furuyama and Henikoff, 2009).

Aurora kinase functions are essential for normal chromosome segregation, and overexpression of these enzymes occurs in many types of human cancers (Lampson and Cheeseman, 2011; Lens et al., 2010). Methylation of Dam1 by Set1 inhibits phosphorylation of flanking serines by the Ipl1 aurora kinase (Zhang et al., 2005). While Dam1 seems to be specific to the Saccharomyces genus as other eukaryotes lack an orthologue based on sequence homology, several likely functional orthologues have been identified that are regulated by Aurora B (Andrews et al., 2004; Emanuele and Stukenberg, 2007; Hanisch et al., 2006; Lan et al., 2004). Whether histone modifications signal changes in the functions of these proteins to regulate chromosome segregation is a question of much interest for the future.

In addition to the connection of the Set1 ortholog MLL proteins to leukemias (Tenney and Shilatifard, 2005), orthologs of several of the Set1/MLL regulators examined here have also been linked to human cancers. The human ortholog of Ubp8, USP22, is part of an eleven gene signature in malignant cancers that marks poor patient prognosis (Glinsky, 2006). Additionally, Rad6 and Bre1 are associated with tumorigenesis in breast cancers, and members of the human Paf1 complex are disregulated in pancreatic cancers (Chaudhary et al., 2007; Liu et al., 2009; Lyakhovich and Shekhar, 2004; Shekhar et al., 2002). The hPAF complex directly recruits MLL1 translocation fusion proteins to the *Hoxa9* locus in leukemia cells, further illustrating the close functional connections between these proteins (Milne et al., 2010; Muntean et al., 2010). Our work raises the possibility that all of these proteins are involved in non-transcriptional processes, such as chromosome segregation, which may also contribute to tumorigenesis, thereby highlighting the importance of defining the full range of functions of these proteins.

Experimental Procedures

Yeast strains, plasmids, and growth media

All *S. cerevisiae* strains used in this study are listed in Supplemental Table 1. All yeast strain growth conditions and construction methods are described in the supplemental experimental methods.

10-fold serial dilution growth assays

10-fold serial dilution growth assays with *ipl1-2* temperature sensitive allele were performed as previously described (Zhang et al., 2005).

Immunoprecipitation and Immunoblotting

Immunoprecipitation of Dam1 and immunoblotting with the Dam1K233me2 specific antibody was performed as previously described (Zhang et al., 2005) with the following changes. 500mL or 1L cultures were grown to an OD_{600} of 1.0. After preclearing with protein A agarose beads, whole cell extracts were incubated with HA affinity beads (3F10, Roche) overnight. For the coimmunoprecipitation of HA-Dam1 with either histones or Set1, lysates were incubated with 100 μg/mL ethidium bromide on ice for 30 minutes before preclearing. Immunoblotting with the Dam1K233me2 antibody was done at a concentration of 1:2500 in 5% milk/TBST overnight. Immunoprecipitated HA-Dam1 and immunoprecipitation inputs were immunoblotted with yeast specific histone H2A (Active Motif), yeast specific H2B (Active Motif), H3 (Active Motif), Set1 (a gift from Peter Nagy) antibodies at 1:2000 overnight. Immunoprecipitation of Cse4 was performed the same as immunoprecipitation of Dam1 except whole cell extracts were incubated with myc affinity beads (9B11, Cell Signaling) overnight. The myc immunoprecipitates were then immunoblotted with either a myc antibody (9B11, Cell Signaling) for 2 hours at 1:10,000 or HA (3F10, Roche) antibody 1:5000 overnight. For immunoblotting from total protein extracts, yeast cultures of indicated strains were grown to 1.5 OD_{600}, harvested, and then pellets were frozen with liquid nitrogen. The pellets were then lysed with NaOH and proteins were precipitated with TCA as previously described (Ooi et al., 1996). Total protein extracts were immunoblotted with H3K4me2 and H3 antibodies (Active Motif) 1:5000 overnight. Quantification of immunoblots was performed using ImageJ (http://rsbweb.nih.gov/ij/).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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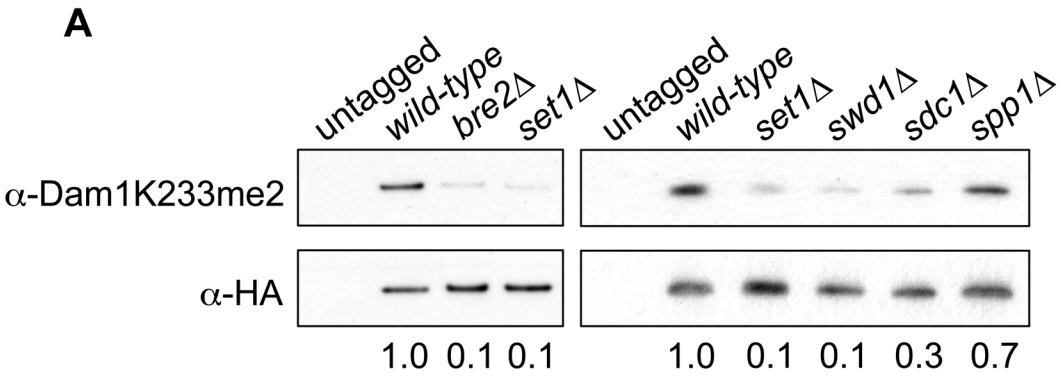
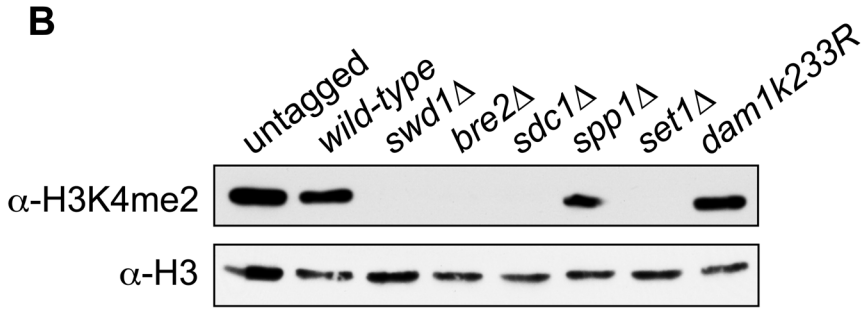
Highlights

- Methylation of the kinetochore protein Dam1 is regulated by transcription factors
- H2BK123 ubiquitination is required *in trans* for Dam1 methylation by Set1
- Cross-talk occurs between modifications in histone and non-histone proteins
- Histone modification states can regulate non-DNA templated processes





C

	ipl1-2 suppression*	Dam1K233me2	H3K4me2
***set1*Δ**	+	-	-
***swd1*Δ**	+	-	-
***bre2*Δ**	+	-	-
***sdc1*Δ**	+	-	-
***spp1*Δ**	-	+	+

Figure 1. The COMPASS complex mediates Dam1 methylation

(A) Endogenously tagged HA-Dam1 was immunoprecipitated from wild-type or COMPASS deletion strains. Immunoblots were then probed with either a HA or Dam1K233me2 specific antibody. The numbers below the blots in this and subsequent figures indicate ratios of signals quantitated for methylated Dam1 relative to total HA-Dam1.

(B) Total protein extracts were immunoblotted for either H3K4me2 or H3.

(C) Summary of COMPASS complex requirements for Dam1K233me2, *ipl1-2* suppression, and H3K4me2. **ipl1-2* suppression summarized from (Zhang et al., 2005). See also Figure S1 for an assessment of regulation of Ipl1 function by other known and putative methyltransferases.

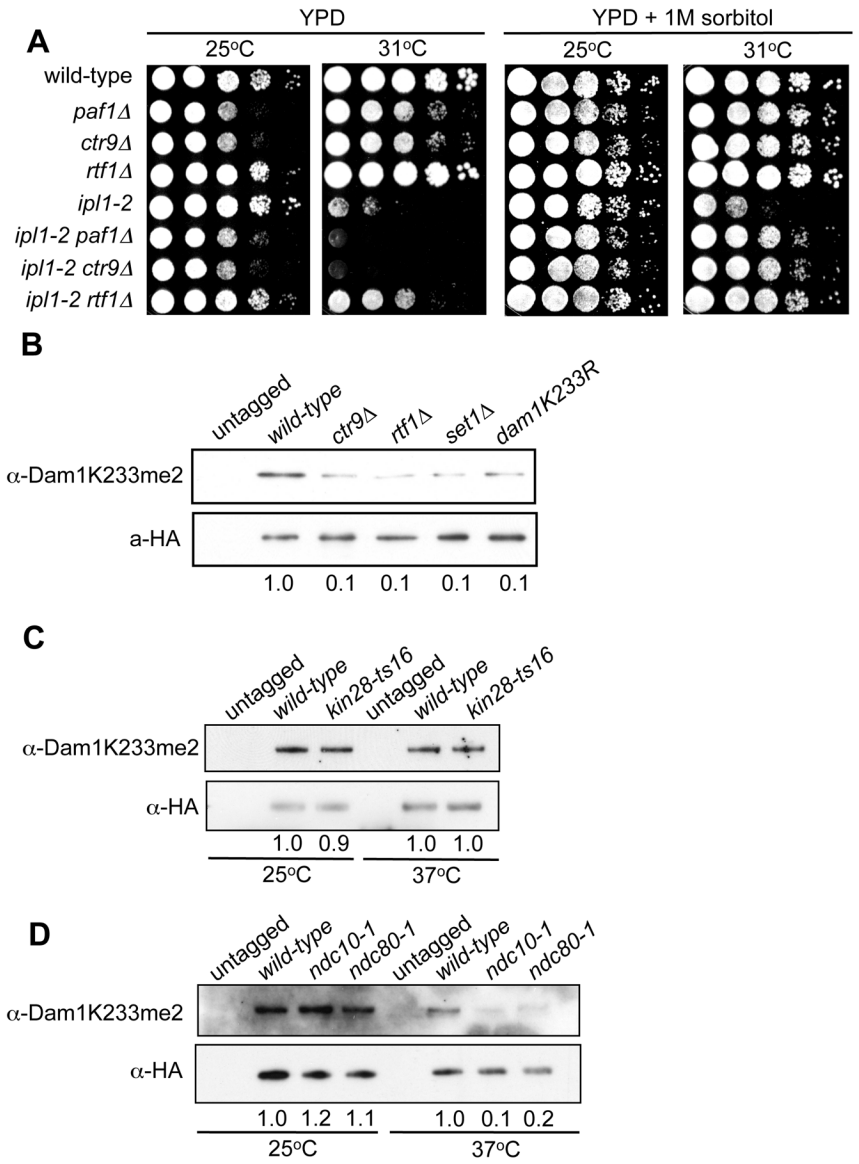


Figure 2. The Paf1 complex is required for Dam1 methylation at the kinetochore independently of transcription

(A) The indicated yeast strains were serially diluted 10-fold, spotted on rich media with and without 1M sorbitol, and grown at the indicated temperatures for 3 days.

(B) HA-Dam1 was immunoprecipitated from wild-type or Paf1 complex deletion strains then probed with either a HA or Dam1K233me2 specific antibody.

(C) HA-Dam1 was immunoprecipitated from wild-type or *kin28-ts16* cells incubated at either 25°C or cells heat shocked at 37°C for 3 hours. The immunoprecipitated HA-Dam1 was then probed with either a HA or Dam1K233me2 specific antibody.

(D) HA-Dam1 was immunoprecipitated from wild-type, *ndc10-1*, or *ndc80-1* cells incubated at either 25°C or heat shocked at 37°C for 3 hours. The immunoprecipitated HA-Dam1 was then probed with either a HA or Dam1K233me2 specific antibody.

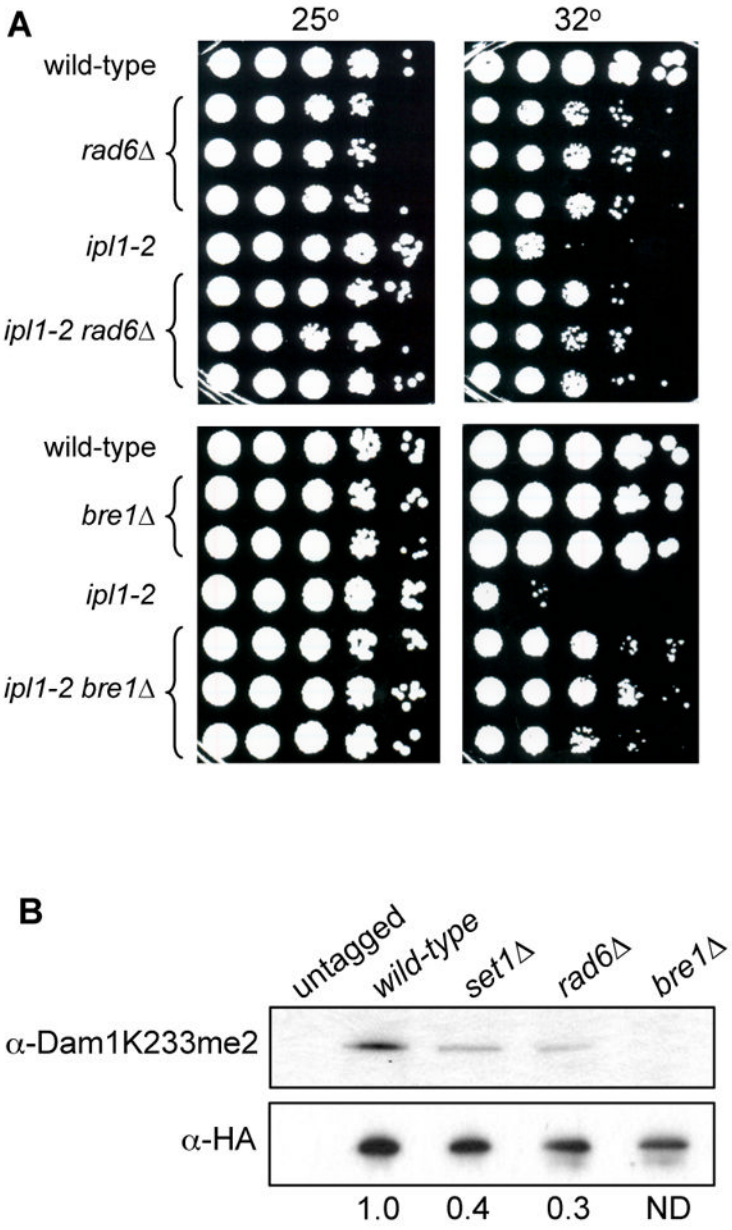


Figure 3. Rad6-Bre1 are required for Dam1 methylation

(A) The individual replicates of yeast were serially diluted 10-fold, spotted on rich media and grown at the indicated temperature for 3 days (See also Figure S1).

(B) HA-Dam1 was immunoprecipitated from wild-type, *rad6*Δ, or *bre1*Δ strains then immunoblotted with either a HA or Dam1K233me2 specific antibody. ND indicates that the Dam1K233me2 was not detectable by quantitation.

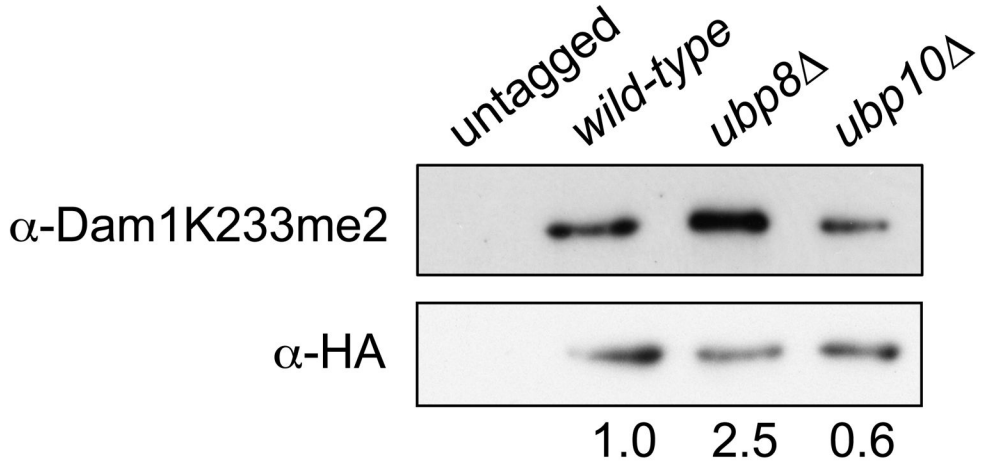


Figure 4. Ubp8 modulates the level of Dam1 methylation

HA-Dam1 was immunoprecipitated from wild-type, *ubp8*Δ, or *ubp10*Δ strains then immunoblotted with either a HA or Dam1K233me2 specific antibody.

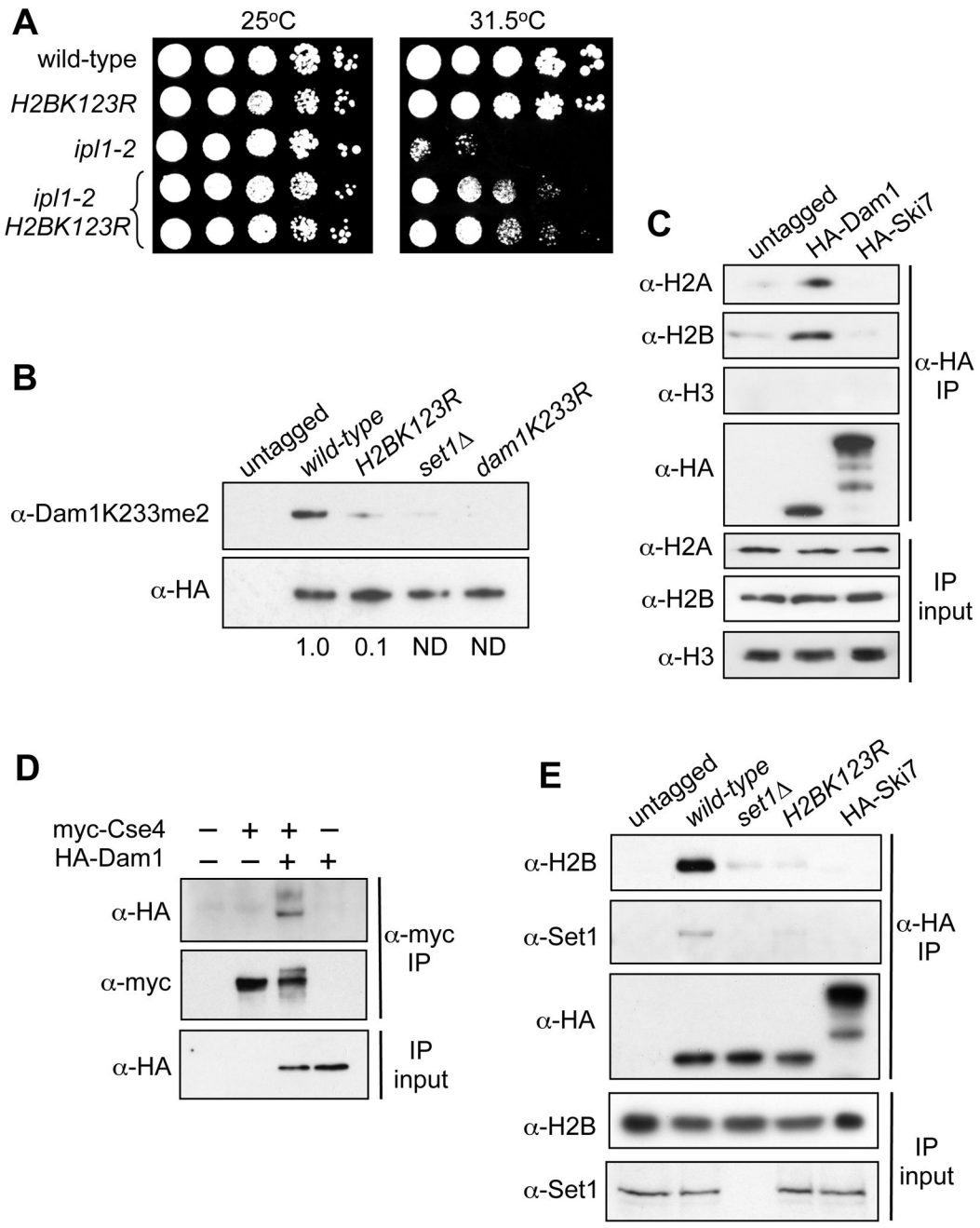


Figure 5. Lysine 123 within H2B is essential for Dam1 methylation

(A) The individual replicates of yeast were serially diluted 10-fold, spotted on rich media and grown at the indicated temperature for 3 days.

(B) HA-Dam1 was immunoprecipitated from wild-type or *H2BK123R* strains then immunoblotted with either a HA or Dam1K233me2 specific antibody. ND indicates that the Dam1K233me2 was not detectable by quantitation.

(C) HA-Dam1 or HA-Ski7 were immunoprecipitated and then immunoblotted for HA, with antibodies specific to yeast histones H2A or H2B, and with general H3 or H4 antibodies. The immunoprecipitant inputs were also immunoblotted for histones to confirm their presents at equal levels in all strains.

(D) Endogenously tagged myc-Cse4 under its own promoter was immunoprecipitated from either wild-type or HA-Dam1 cells and then probed with an antibody specific to either the myc or HA tags. The immunoprecipitant inputs were immunoblotted for HA to confirm their presents at equal levels in all strains.
(E) HA-Dam1 was immunoprecipitated from wild-type, *set1Δ*, or *H2BK123R* cells while HA-Ski7 was immunoprecipitated from wild-type cells. The immunoprecipitants were immunoblotted with antibodies specific to either H2B, Set1, of HA. The immunoprecipitant inputs were immunoblotted for H2B and Set1 to confirm their presents at equal levels in all strains.

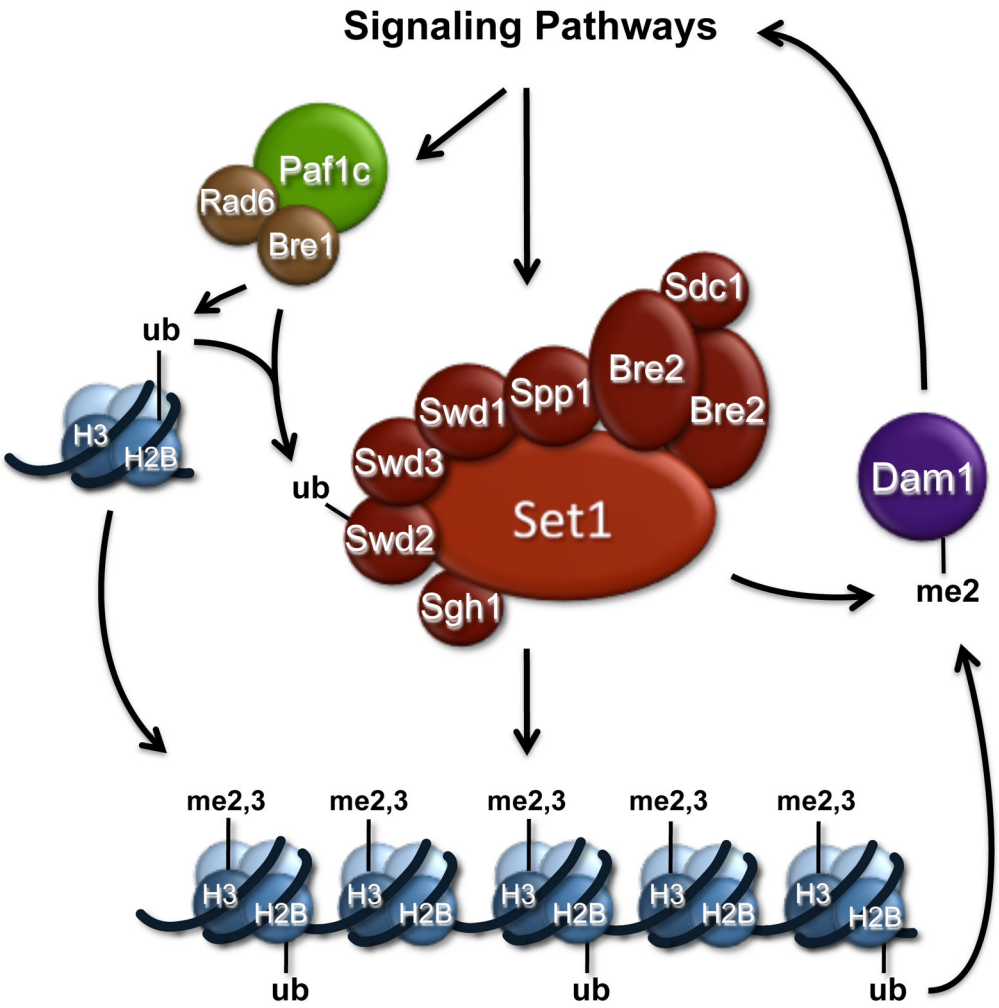


Figure 6. Model of regulation of Set1-mediated methylation

Multiple signaling pathways control Set1-mediated methylation. Methylation of H3K4 is dependent on H2BK123 ubiquitination by Rad6-Bre1 and the Paf1 complex which also ubiquitinated the COMPASS subunit Swd2. We have shown that H2BK123 ubiquitination also signals changes in methylation at least one non-histone protein, Dam1 on lysine 233, providing a mechanisms for connecting changes in chromatin structures to cellular processes independent of gene transcription.