Methylation of H3 K4 and K79 is not strictly dependent on H2B K123 ubiquitylation

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Covalent modifications of histone proteins have profound consequences on chromatin structure and function. Specific modification patterns constitute a code read by effector proteins. Studies from yeast found that H3 trimethylation at K4 and K79 is dependent on ubiquitylation of H2B K123, which is termed a "trans-tail pathway." In this study, we show that a strain unable to be ubiquitylated on H2B (K123R) is still proficient for H3 trimethylation at both K4 and K79, indicating that H3 methylation status is not solely dependent on H2B ubiquitylation. However, additional mutations in H2B result in loss of H3 methylation when combined with *htb1-K123R*. Consistent with this, we find that the original strain used to identify the trans-tail pathway has a genomic mutation that, when combined with H2B K123R, results in defective H3 methylation. Finally, we show that strains lacking the ubiquitin ligase Bre1 are defective for H3 methylation, suggesting that there is an additional Bre1 substrate that in combination with H2B K123 facilitates H3 methylation.

Introduction

In eukaryotes, DNA is condensed into chromatin, the basic unit of which is the nucleosome. The DNA in a nucleosome is wrapped around an octamer of histone proteins: two each of H2A, H2B, H3, and H4. All four histones are subjected to numerous covalent modifications, including acetylation, phosphorylation, mono-, di-, and trimethylation, ubiquitylation, and sumoylation. These modifications can have profound consequences on the ability of chromatin fibers to form higher order structures. In addition, modified histones can provide binding sites for proteins and thus help to recruit and/or retain proteins at particular regions of chromatin. The combination of modifications present on a histone constitutes a code that is read by cellular proteins (Strahl and Allis, 2000; Jenuwein and Allis, 2001).

Between 1 and 10% of histone H2B in budding yeast is ubiquitylated on K123 (Robzyk et al., 2000). This is catalyzed by Rad6 and Bre1, which encode an E2 and E3 ubiquitin ligase, respectively (Robzyk et al., 2000; Hwang et al., 2003; Wood et al., 2003a). Previous studies found that disruption of *RAD6* or *BRE1* or mutation of H2B K123 results in a loss of H3 methylation at K4 and K79 (Briggs et al., 2002; Dover et al., 2002; Ng et al., 2002; Sun and Allis, 2002), and this has been termed a "trans-tail pathway" (Briggs et al., 2002; Henry et al., 2003). More recently, it appears that monomethylation of H3 K4 and K79 is unaffected by the absence of H2B ubiquitylation (Dehe et al., 2005). Although it is not clear whether dimethylation is dependent on H2B ubiquitylation, there is a consensus that this trans-tail pathway is important for trimethylation of these residues (Dehe et al., 2005; Laribee et al., 2005; Shahbazian et al., 2005; Shukla et al., 2006).

H3 K4 methylation is catalyzed by the Set1 methyltransferase and H3 K79 methylation by Dot1. In addition to effects on regulation of gene expression, these modifications contribute to DNA damage responses. *dot1* or H3 K79E mutant strains are UV sensitive (Bostelman et al., 2007). Additionally, a *dot1* mutant strain was found to be ionizing radiation sensitive in one study (Game et al., 2006) but only when combined with mutations in other methyltransferases, such as *set1*, in another (Wysocki et al., 2005). The *dot1* and H3 K79A mutant strains were found to have a defect in DNA damage checkpoints (Giannattasio et al., 2005; Wysocki et al., 2005), and genetic analysis of the strains in response to UV irradiation suggests that there is also a contribution to survival after DNA damage beyond mediating checkpoint responses (Bostelman et al., 2007). Although the involvement of Set1-mediated methylation of H3 K4 in DNA damage responses is less well characterized, Set1

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Abbreviation used in this paper: PIKK, phosphatidylinositol kinase–like kinase.

The Rockefeller University Press $30.00
J. Cell Biol. Vol. 184 No. 5 631–638
www.jcb.org/cgi/doi/10.1083/jcb.200812088

appears to contribute to the intra–S phase DNA damage checkpoint (Wysocki et al., 2005).

Consistent with a dependence on K123 ubiquitylation, *rad6* and *bre1* mutant strains are hypersensitive to ionizing radiation, function on the same pathway as *dot1* (Game et al., 2006), and, like *htb1-K123R* mutant cells, show DNA damage checkpoint defects (Giannattasio et al., 2005; Wysocki et al., 2005).

One of the most well-characterized DNA damage–dependent histone modifications is the phosphorylation of histone H2A (H2AX in higher eukaryotes) by the phosphatidylinositol kinase–like kinases (PIKKs; Mec1 and Tel1 in budding yeast; Downs et al., 2007). The phosphorylated motif is a well-defined consensus site for this family of kinases (S/TQ), and its presence in histone H2A variants is well conserved throughout eukaryotic evolution (Foster and Downs, 2005). Despite the rapid and robust phosphorylation of H2A in response to DNA damage, mutation of the site results in a very mild hypersensitivity to DNA-damaging agents (Downs et al., 2000; Redon et al., 2003). We noted the existence of an additional PIKK consensus site in the C-terminal tail of H2B and considered that it may play a redundant role in mediating cellular responses to DNA damage.

In this study, we investigate the role of the C-terminal tail of H2B in DNA damage responses. We found that the PIKK consensus site does not contribute to DNA damage responses but that other mutations in the C-terminal tail result in hypersensitivity to DNA damage. In contrast to previous studies (Briggs et al., 2002; Dover et al., 2002; Ng et al., 2002; Sun and Allis, 2002; Henry et al., 2003), we also found that a strain in which H2B cannot be ubiquitylated (*htb1-K123R*) was not defective in H3 trimethylation at K4 and K79, indicating that H3 methylation is not strictly dependent on H2B ubiquitylation. Interestingly, we found that if the K123R mutation was combined with either mutation of two serines in the H2B tail or the addition of an N-terminal Flag tag (*htb1-K123R/S125A/S126A* or Flag–*htb1-K123R*), the resulting strains were both hypersensitive to DNA damage and lost methylation at H3 K4 and K79. Moreover, we found that the original strain used to elucidate the trans-tail pathway has a mutation in the genome that, when combined with *htb1-K123R*, results in defective H3 methylation. Together, these data demonstrate that H2B ubiquitylation contributes to normal H3 methylation but is not the sole determinant of this pathway.

Consistent with previous results (Briggs et al., 2002; Dover et al., 2002; Ng et al., 2002; Sun and Allis, 2002; Henry et al., 2003), however, we found that disruption of the Bre1 ubiquitin ligase resulted in both hypersensitivity to DNA damage and loss of H3 K4 and K79 trimethylation. This suggests that Bre1 may have an additional target that is critical for H3 methylation.

Results and discussion

Characterization of H2B C-terminal tail mutations in DNA damage responses

We hypothesized that the PIKK consensus site on the C-terminal tail of histone H2B might be a target of Mec1 and Tel1 and function in the cellular response to DNA damage. To investigate this possibility, we made a truncation mutation of histone H2B in which the last six amino acid residues were lost (*htb1-S125stop*) to remove the TQ motif as well as the adjacent serine residues (Fig. 1 A). We found that this strain was hypersensitive to phleomycin (Fig. 1 B), which is consistent with a potential role in DNA damage responses. To examine its relationship with the H2A C-terminal tail, we made a strain in which both tails were truncated (*hta1-S129stop/htb1-S125stop*). This strain was more sensitive to DNA damage than strains harboring either single mutant, suggesting that the two tails play separate roles (Fig. 1 B). To determine whether the TQ motif of H2B is responsible for the phenotype observed, we generated an *htb1-T128stop* mutant strain. However, this strain appeared to behave similarly to wild type (Fig. 1 C). We considered that the motif may be functioning redundantly with the H2A SQ motif and therefore combined this mutation with an *hta1-S129stop* mutant. We found that *htb1-T128stop/hta1-S129stop* is no more sensitive to DNA damage than the *hta1-S129stop* single mutant in the presence of either methylmethanesulfonate or phleomycin (Fig. 1 C), suggesting that the H2B TQ motif is not contributing to cellular survival after exposure to DNA damage.

To identify the residues in the H2B tail that are important for DNA damage responses, we made a series of point mutants in the final six amino acid residues. Curiously, no combination of point mutations resulted in detectable hypersensitivity to DNA damage (Fig. 2 A and not depicted). H2B K123 had previously been shown to be ubiquitylated (Robzyk et al., 2000), and strains lacking enzymes that mediate this event, as well as an *htb1-K123R* mutant strain, had defective DNA damage responses (Giannattasio et al., 2005; Game et al., 2006). Therefore, we considered the possibility that our truncation mutation may have affected ubiquitylation of K123 and subsequent functions in DNA damage responses.

Notably, however, we did not detect any hypersensitivity of an *htb1-K123R* mutant strain to DNA damage (Fig. 2 A). We combined this mutation with a series of point mutants in the H2B tail and found that a triple *htb1-K123R/S125A/S126A* mutant strain (referred to as KSS hereafter) showed similar levels of hypersensitivity to phleomycin as the *htb1-S125stop* mutant strain (Fig. 2 A), suggesting that all three residues contribute to survival after exposure to DNA damage.

Next, we examined the ability of these strains to be ubiquitylated using N-terminally Flag-tagged constructs. We found that the slower migrating band corresponding to ubiquitylated H2B disappears in both the K123R and KSS mutant strains as expected. Mutation of the serines alone appears to have no effect on H2B ubiquitylation (Fig. 2 B).

Because H2B ubiquitylation had been implicated in DNA damage responses (Giannattasio et al., 2005), we compared our strains with those used in previous studies (Y133 and its parent strain Y131; Robzyk et al., 2000; Kao et al., 2004; Giannattasio et al., 2005; Shahbazian et al., 2005) and found that the *htb1-K123R* mutant in the Y133 strain background shows hypersensitivity to DNA damage relative to the Y131 parent strain background (Fig. 2 C). We also noted that although our *htb1-K123R* (K) and our *htb1-S125A/S126A* (SS) mutant strains grew as well as wild type (Fig. 2 F and not depicted), our KSS triple mutant displayed a slight slow growth phenotype (Fig. 2 D).

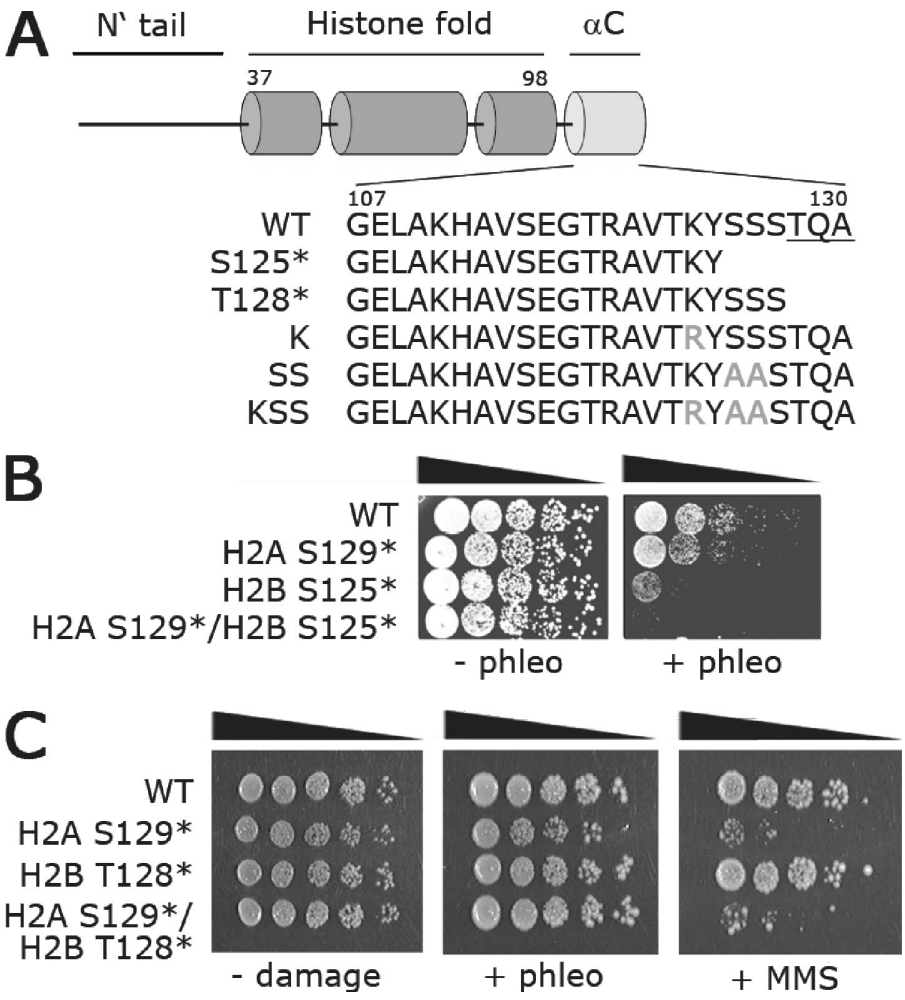

Figure 1. **The C-terminal tail of histone H2B contains a PIKK consensus site, which is not required for survival after DNA damage.** (A) A cartoon of H2B showing the amino acid sequence of the C-terminal tail, which forms an α helix (αC; Luger et al., 1997). The putative PIKK consensus site is underlined, and the sequences of mutations used in this study are listed with the mutated residues highlighted in gray. (B) Serial dilutions of midlog cultures of wild type (WT; JDY183), *htb1-S125stop* (H2B S125*; JDY184), *hta1-S129** (H2A S129*; JDY5), or cells lacking both tails (H2A S129*/H2B S125*; JDY6) were plated onto media lacking or containing phleomycin (phleo). (C) Serial dilutions of midlog cultures of *htb1-S128** (H2B T128*; JDY7) or *hta1-S129** (H2A S129*; JDY5) or lacking both tails (H2A S129*/H2B T128*; JDY8) were plated onto media in the absence or presence of phleomycin or methylmethanesulfonate (MMS).

We also examined the phenotypes of our Flag-tagged constructs. Although the addition of the Flag tag to wild-type H2B did not affect the ability of the strain to survive when exposed to phleomycin, the Flag–*htb1-K123R* (Flag-K) mutant strain displayed a striking hypersensitivity (Fig. 2 E). Because the *htb1-K123R* parent strain was indistinguishable from wild type in this assay (Fig. 2 E), this suggests that the combination of the two alterations to the H2B gene results in impaired DNA damage responses. Interestingly, this is very similar to the phenotype seen when the K123R mutation is combined with the S125A/S126A mutations (KSS; Fig. 2 A), and we found that the Flag-KSS mutant strain was no more sensitive than the Flag-K mutant (Fig. 2 E). Moreover, the Flag-K mutant strain displays a similar slow growth phenotype to the KSS triple mutant (Fig. 2 F). Altogether, these data suggest that the addition of the Flag epitope to H2B may impair similar functions as the *htb1-S125A/S126A* mutations. These data also indicate that H2B K123 plays a role in DNA damage responses, but this contribution is only detectable when other H2B functions are impaired.

H3 trimethylation of K4 and K79 is not dependent on H2B ubiquitylation

As previously discussed, H3 trimethylation at K4 and K79 is dependent on H2B K123 ubiquitylation in strains derived from Y131 (Dehe et al., 2005; Laribee et al., 2005; Shahbazian et al., 2005; Shukla et al., 2006). We investigated these modifications in our H2B mutant strains. Strikingly, we found that H3 trimethylation of both K4 and K79 was indistinguishable from wild type in our K123R mutant strain (Fig. 3 A), indicating that ubiquitylation of H2B is not a prerequisite for these H3 methylation events. Consistent with previous studies, however, both K4 and K79 trimethylation was abolished in the Y133 background (K123R; Fig. 3 A; Dehe et al., 2005; Laribee et al., 2005; Shahbazian et al., 2005; Shukla et al., 2006). Interestingly, in our KSS triple mutant strain, which phenocopies the Y133-based *htb1-K123R* strain, we found that the trimethylation of both K4 and K79 was lost (Fig. 3 A), whereas no change was detected in the SS mutant strain. This is consistent with a lack of hypersensitivity to DNA damage in this strain. *dot1* and *set1* mutant strains were tested in parallel to demonstrate the specificity of the antibodies (Fig. 3 A).

Because we found that the addition of the Flag epitope to the N terminus of H2B had a similar effect on DNA damage hypersensitivity as the SS mutation when combined with K123R in our strain background, this raised the possibility that H3 methylation may be affected as well. We found that Flag-*HTB1* (F-WT) and *htb1-K123R* (K) mutant strains each have normal H3 K4 and K79 trimethylation, but this is lost in the Flag-*htb1-K123R* (F-K)

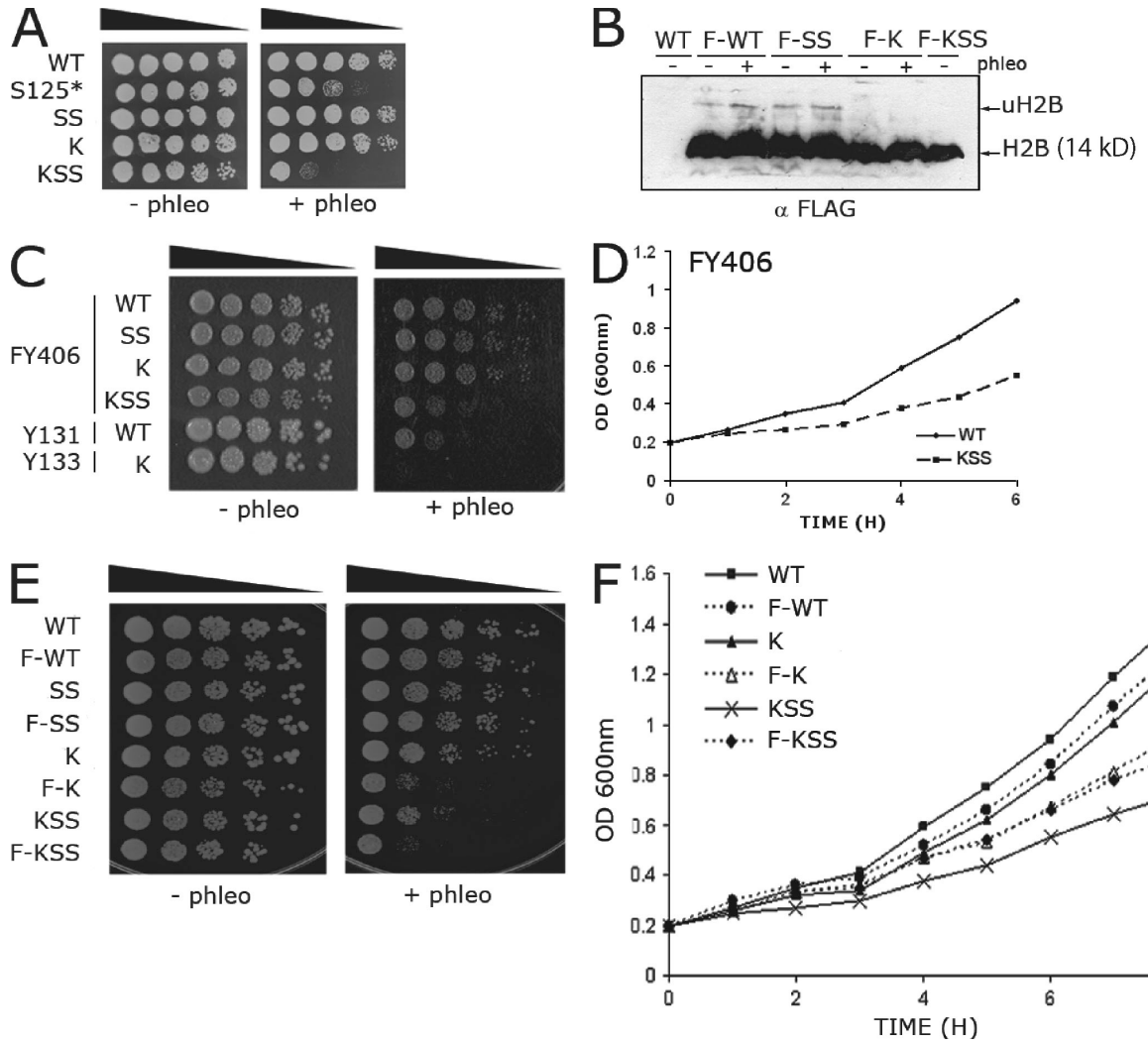

Figure 2. **Genetic analysis of the C-terminal tail of histone H2B.** (A) Serial dilutions of midlog cultures of wild-type (WT; JDY183), *htb1-S125stop* (S125*; JDY184), *htb1-S125A/S126A* (SS; JDY185), *htb1-K123R* (K; JDY186), and *htb1-K123R/S125A/S126A* (KSS; JDY187) mutant strains were plated onto media in the absence or the presence of phleomycin (phleo). (B) Western blot analysis of extracts from strains containing untagged H2B (WT; JDY183), Flag-tagged H2B (F-WT; JDY630), Flag-tagged *htb1-S125A/S126A* (F-SS; JDY206), Flag-tagged *htb1-K123R* (F-K; JDY251), and Flag-tagged *htb1-K1233R/S125A/S126A* (F-KSS; JDY253) using anti-Flag antiserum. (C) Serial dilutions of midlog cultures of FY406-based strains as in A and Y131-based strains; wild type (YM5037) and *htb1-K123R* in Y133 (YM5038) mutant strains were plated onto media in the absence or the presence of phleomycin. (D) Growth curve of wild type (JDY183) and the *htb1-K123R/S125A/S126A* (KSS; JDY187) mutant strain. (E) Serial dilutions of midlog cultures of untagged and Flag-tagged H2B-containing strains were plated onto media in the absence or presence of phleomycin. The strains used were untagged H2B (WT; JDY183), Flag-tagged H2B (F-WT; JDY630), untagged *htb1-S125A/S126A* (SS; JDY185), Flag-tagged *htb1-S125A/S126A* (F-SS; JDY206), untagged *htb1-K123R* (K; JDY186), Flag-tagged *htb1-K123R* (F-K; JDY251), untagged *htb1-K123R/S125A/S126A* (KSS; JDY187), and Flag-tagged *htb1-K123R/S125A/S126A* (F-KSS; JDY253). (F) Growth curve of untagged and tagged H2B-containing strains from E.

mutant strain (Fig. 3 B). These data indicate that although H2B ubiquitylation alone is not required for H3 trimethylation, additional alterations to the H2B coding sequence in combination with *htb1-K123R* result in defective H3 trimethylation.

Next, we investigated the loss of H3 trimethylation in the Y133 (*htb1-K123R*) mutant strain background. We considered that perhaps differences in the H2B coding sequences present on the plasmids used in Y133 compared with FY406 accounted for the different phenotypes. We shuffled our *htb1-K123R*–encoding plasmid into the Y133 strain background, replacing the original *htb1-K123R* plasmid in this strain, and found that the resulting strain was still hypersensitive to phleomycin and still had defective H3 methylation (Fig. 3, C and D). Interestingly, however, when we shuffled our *htb1-K123R* mutant construct into the Y131-based parent strain, we found that similar to our strain background, this Y131-based *htb1-K123R* mutant strain was not hypersensitive to DNA damage and had normal H3 methylation (Fig. 3, C and D).

Because Y133 was created from Y131, once the H2B-encoding plasmids were replaced with our *htb1-K123R* construct, the resulting strains should be genetically identical. Therefore, one explanation for these results is that Y133 sustained a mutation in the genome that, when combined with the *htb1-K123R* mutation (regardless of which plasmid construct is used), results in the phenotypes seen here.

To test this, we performed a genetic cross between the Y131- and Y133-based strains. Tetrads were dissected, and the *htb1-K123R* mutant construct was shuffled into the haploid

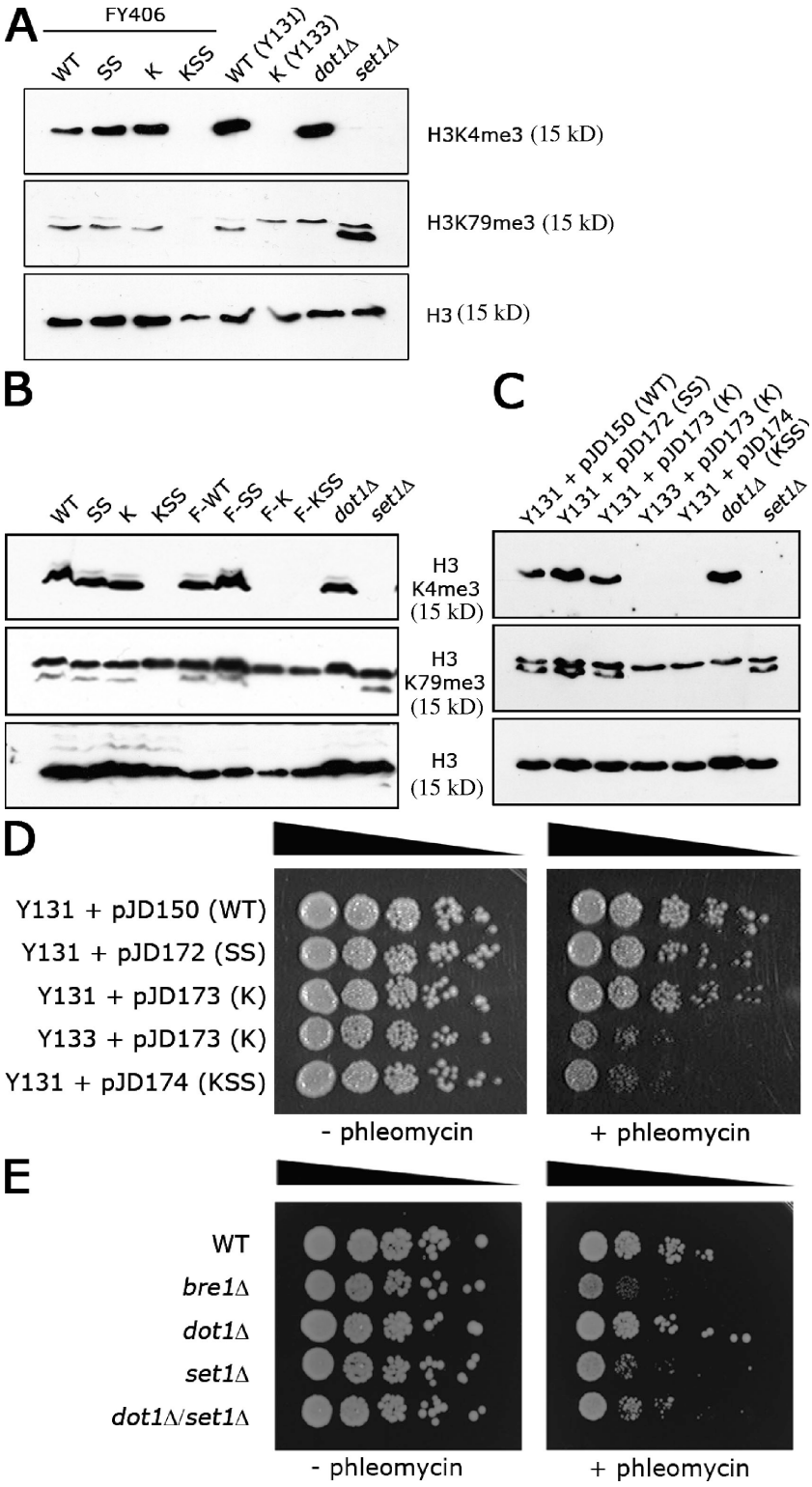

Figure 3. **Histone H3 trimethylation on K4 and K79 is not solely dependent on ubiquitylation of H2B K123.** (A) Western blot analysis of the indicated strains using an antibody raised against trimethylated H3 K4 (H3K4me3), trimethylated H3 K79 (H3K79me3; the top band is a nonspecific cross-reacting species), or H3. FY406-based H2B mutant strains or Y131-based H2B mutant strains as in Fig. 2 C were compared. Strains lacking Dot1 (*dot1Δ*) or Set1 (*set1Δ*) were used as a control for antibody specificity. (B) Western blot analysis as in A of cell extracts from untagged (first, second, third, and fourth lanes) or Flag-tagged (fifth, sixth, seventh, and eighth lanes) H2B-expressing strains. (C) Western blot analysis of the strains in C using an antibody raised against trimethylated H3 K4 (H3K4me3), trimethylated H3 K79 (H3K79me3), or H3. (D) DNA damage hypersensitivity of Y131- or Y133-based strains in which the H2B-encoding plasmids were replaced with the constructs used in the FY406 strain background (Fig. 2 C). Serial dilutions of midlog cultures were plated onto media in the absence or the presence of phleomycin. (E) Serial dilutions of midlog cultures of wild-type (WT; W303α), *bre1* (*bre1Δ*; JDY286), *dot1* (*dot1Δ*; SKY2849), *set1* (*set1Δ*; SKY2856), and *dot1/set1* (*dot1Δ/set1Δ*; SKY2859) mutant strains were plated onto media in the absence or the presence of phleomycin.

daughter strains. Hypersensitivity to phleomycin and defective H3 methylation segregated 2:2 (Fig. 4 and not depicted). In contrast, spores derived from Y131 crossed with itself as a control consistently showed no hypersensitivity to phleomycin and had normal H3 methylation when carrying the *htb1-K123R* mutation (Fig. 4 and not depicted). These data demonstrate that the defect in H3 trimethylation in Y133 is a product of the *htb1-K123R* mutation in combination with a mutation in the genome.

In this study, we show that H3 can be trimethylated at K4 and K79 in strains that cannot be ubiquitylated on H2B K123. However, this is not entirely contradictory with previous studies (Briggs et al., 2002; Dover et al., 2002; Ng et al., 2002; Sun and Allis, 2002; Henry et al., 2003; Dehe et al., 2005; Laribee et al., 2005;

Shahbazian et al., 2005; Shukla et al., 2006) because we find that ubiquitylation of H2B K123 clearly contributes to this trans-tail pathway, and this is consistent with recent studies in which H2B ubiquitylation was found to stimulate methylation in vitro (Frederiks et al., 2008; McGinty et al., 2008). Importantly, however, it is not the sole determinant, and it is the loss of H2B ubiquitylation in combination with a second event that results in the inability of cells to trimethylate H3 K4 and K79 in vivo.

We identified three different genetic changes that, in combination with *htb1-K123R*, result in defective H3 trimethylation. Serine to alanine substitutions of H2B S125 and S126 or the addition of an N-terminal Flag epitope can result in the loss of H3 trimethylation when combined with the *htb1-K123R* mutation. Although we do not know the change in Y133 responsible for the loss of H3 trimethylation, we can rule out changes in H2B because swapping the sole source of H2B makes no difference (Fig. 3, compare A [fifth and sixth lanes] with C [third and fourth lanes]). We are currently working to identify the nature of the mutation in Y133, as this will shed light on the mechanism of the trans-tail pathway. It is also worth emphasizing that the addition of an N-terminal Flag epitope is not a neutral event, and studies using strains with this construct must be interpreted with caution.

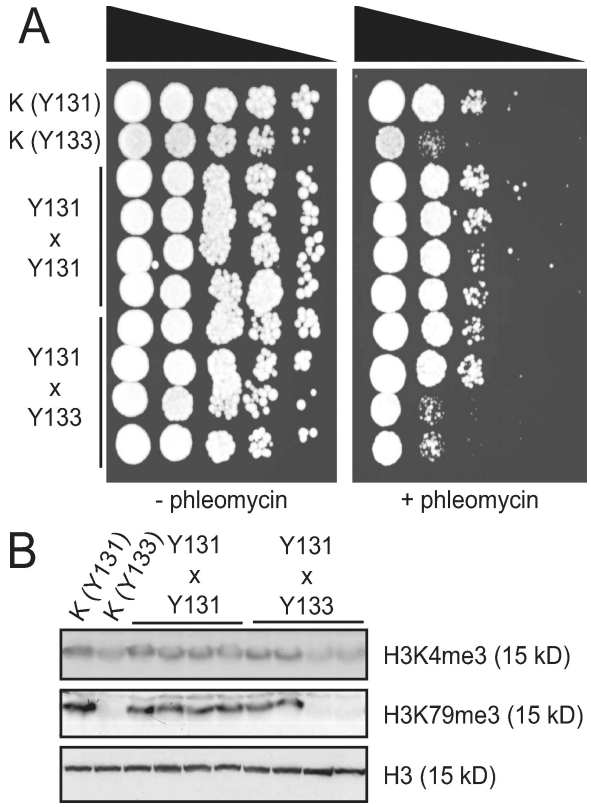

Figure 4. **The Y133 strain carries a mutation in the genome that, in combination with *htb1-K123R*, results in hypersensitivity to phleomycin and loss of H3 trimethylation.** (A) Serial dilutions of midlog cultures of Y131- or Y133-based strains and four independent segregants from the Y131 × Y131 cross or the Y131 × Y133 cross were plated onto media in the absence or the presence of phleomycin. All strains contain pJD173 (*HTA1-htb1-K123R*) as the sole source of H2B. (B) Western blot analysis of the strains in A using an antibody raised against trimethylated H3 K4 (H3K4me3), trimethylated H3 K79 (H3K79me3), or H3.

H3 trimethylation of K4 and K79 is dependent on the Bre1 ubiquitin ligase

Notably, multiple groups using different strain backgrounds found that strains lacking genes required for H2B ubiquitylation such as *RAD6* and *BRE1* are hypersensitive to DNA damage and lose H3 K4 and K79 methylation (Dover et al., 2002; Sun and Allis, 2002; Wood et al., 2003b; Laribee et al., 2005; Game et al., 2006). We created a *bre1* mutant strain in W303 and found that, consistent with previous studies (Dover et al., 2002; Sun and Allis, 2002; Wood et al., 2003b; Laribee et al., 2005; Game et al., 2006), H3 K4 and K79 trimethylation is absent in this strain (Fig. 5 A).

Next, we examined whether this was related to H2B serines 125 and 126 by making a *bre1*/KSS mutant strain. First, we found that *bre1* mutant cells are hypersensitive to phleomycin, again suggesting that the loss of H3 methylation results in hypersensitivity to DNA damage. Importantly, we also found that the *bre1*/KSS mutant strain is no more sensitive to phleomycin than either the KSS or the *bre1* mutant strains (Fig. 5 B). This raises the possibility that Bre1, H2B K123, and S125/S126 function in the same pathway to regulate H3 methylation status.

Because trimethylation is lost in the absence of Bre1, but not in the *htb1-K123R* mutant strain, this suggests that Bre1 has

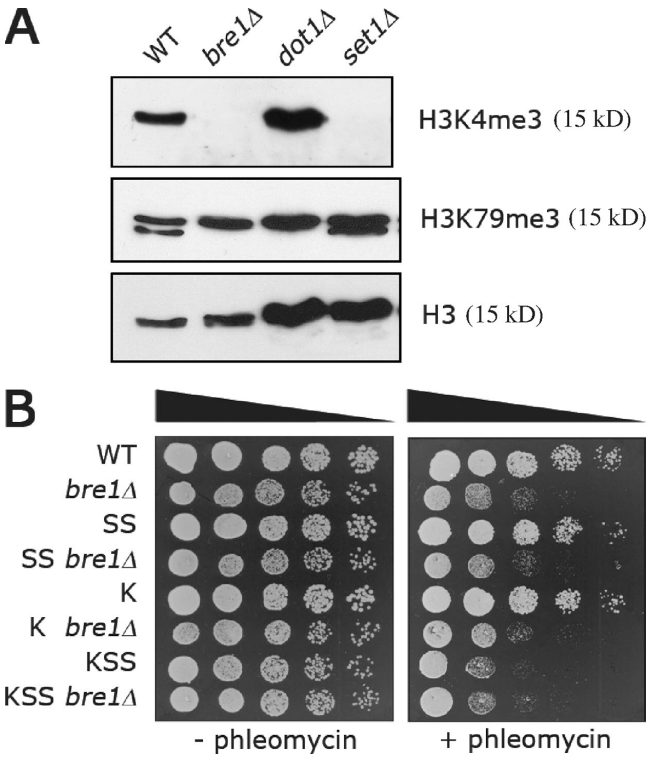

Figure 5. **The E3 ubiquitin ligase Bre1 is required for H3 K4 and K79 trimethylation.** (A) Western blot analysis of wild-type (WT; JDY183), *bre1* (*bre1Δ*; JDY193), *dot1* (*dot1Δ*; SKY2849), and *set1* (*set1Δ*; SKY2856) mutant yeast strains using an antibody raised against either trimethylated H3 K4 (H3K4me3), trimethylated H3 K79 (H3K79me3), or H3. (B) DNA damage hypersensitivity of strains lacking *BRE1* combined with mutations in H2B. The strains used were wild type (WT; JDY183), *bre1* (*bre1Δ*; JDY193), *htb1-S125A/S126A* (SS; JDY185), *htb1-S125A/S126A/bre1* (SS *bre1Δ*; JDY195), *htb1-K123R* (K; JDY186), *htb1-K123R/bre1* (K *bre1Δ*), and *htb1-K123R/S125A/S126A/bre1* (KSS *bre1Δ*; JDY197).

a contribution to the regulation of H3 methylation beyond the ubiquitylation of H2B K123. Notably, the absence of ubiquitin from a reconstituted in vitro transcription system results in the loss of H3 trimethylation of K4 (Pavri et al., 2006), suggesting that the putative second target is either another residue in the nucleosome or the methyltransferase itself. This is consistent with a recent study in which Bre1 was found to ubiquitylate the Swd2 subunit of COMPASS (complex proteins associated with Set1) to facilitate H3 K4 trimethylation (Vitaliano-Prunier et al., 2008). Additionally, H2B was recently found to be ubiquitylated at other sites, which may be important for H3 methylation (Geng and Tansey, 2008). Although the trans-tail pathway exists to regulate the methylation status of histone H3, it is clearly more complex than originally thought and requires activities beyond ubiquitylation of histone H2B at K123.

Materials and methods

Strains and plasmids

Yeast strains and plasmids used in this study are listed in Table I. F. Winston (Harvard Medical School, Boston, MA) provided the strain FY406. M. Grunstein (University of California, Los Angeles, Los Angeles, CA) provided the strains YM5037 and YM5038. M.A. Osley (University of New Mexico, Albuquerque, NM) provided the strains Y131 and Y133. S. Kron (University of Chicago, Chicago, IL) provided the strains SKY2849, SKY2856, and SKY2859.

DNA damage sensitivity assays

Midlog cultures were diluted to a density corresponding to an absorbance of 0.3 at 600 nm, and fivefold serial dilutions were plated onto medium containing the indicated DNA damage–inducing agents. Phleomycin was

Table I. ***Saccharomyces cerevisiae* strains used in this study**

Strain	Genotype	Source
FY406	MATa *his3Δ200 leu2Δ1 lys2-128Δ trp1Δ63 ura3-52 (hta1-htb1)Δ::LEU2 (hta2-htb2)Δ::TRP1*, <pAB6 [*URA3*, *HTA1-HTB1*]>	Hirschhorn et al., 1995
JDY183	As FY406 except pJD150 [*HTA1-HTB1*, *HIS3*] replaces pAB6	Downs et al., 2000
JDY184	As FY406 except pJD157 [*HTA1-htb1-S125stop*, *HIS3*] replaces pAB6	This study
JDY5	As FY406 except pJD149 [*hta1-S129stop-HTB1*] replaces pAB6	This study
JDY6	As FY406 except pJD167 [*hta1-S129stop-htb1-S125stop*] replaces pAB6	This study
JDY7	As FY406 except pJD168 [*HTA1-htb1-T128stop*, *HIS3*] replaces pAB6	This study
JDY8	As FY406 except pJD180 [*hta1-S129stop-htb1-T128stop*, *HIS3*] replaces pAB6	This study
JDY185	As FY406 except pJD172 [*HTA1-htb1-S125A/S126A*, *HIS3*] replaces pAB6	This study
JDY186	As FY406 except pJD173 [*HTA1-htb1-K123R*, *HIS3*] replaces pAB6	This study
JDY187	As FY406 except pJD174 [*HTA1-htb1-K123R/S125A/S126A*, *HIS3*] replaces pAB6	This study
JDY630	As FY406 except pJD183 [*HTA1*-Flag-*HTB1*, *HIS3*] replaces pAB6	This study
JDY206	As FY406 except pJD287 [*HTA1*-Flag-*htb1-S125A/S126A*, *HIS3*] replaces pAB6	This study
JDY251	As FY406 except pJD288 [*HTA1*-Flag-*htb1-K123R*, *HIS3*] replaces pAB6	This study
JDY253	As FY406 except pJD253 [*HTA1*-Flag-*htb1-K123R/S125A/S126A*, *HIS3*] replaces pAB6	This study
Y131	*MATa ura3-1 leu2-3,-112 ade2-1 trp1-1 his3-11,15 can1-100 hta1-htb1::LEU2, hta2-htb2Δ* <pRS426-*HTA1-HTB1*[*URA3*]>	Kao et al., 2004; Robzyk et al., 2000
Y133	*MATa ura3-1 leu2-3,-112 ade2-1 trp1-1 his3-11,15 can1-100 hta1-htb1::LEU2, hta2-htb2Δ* <pRS426-HTA1-htb1-K123R[URA3]>	Kao et al., 2004; Robzyk et al., 2000
YM5037	As Y131 except *DOT1-13Myc::KANMX*	Shahbazian et al., 2005
YM5038	As Y133 except *DOT1-13Myc::KANMX*	Shahbazian et al., 2005
JDY631	As YM5037 except pJD150 [*HTA1-HTB1*, *HIS3*]	This study
JDY632	As YM5037 except pJD172 [*HTA1-htb1-S125A/S126A*, *HIS3*]	This study
JDY633	As YM5037 except pJD173 [*HTA1-htb1-K123R*, *HIS3*]	This study
JDY634	As YM5038 except pJD173 [*HTA1-htb1-K123R*, *HIS3*]	This study
JDY635	As YM5037 except pJD174 [*HTA1-htb1-K123R/S125A/S126A*, *HIS3*]	This study
W303α	Matα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 rad5-535	R. Rothstein[a]
JDY286	*bre1Δ::KANMX* in W303α	This study
SKY2849	*MATa ade2-1 can1-100 ura3-1 leu2-3,112 his3-11,15 trp1-1 dot1Δ::TRP1*	Wysocki et al., 2005
SKY2856	*MATa ade2-1 can1-100 ura3-1 leu2-3,112 his3-11,15 trp1-1 set1Δ::HIS3MX6*	Wysocki et al., 2005
SKY2859	*MATa ade2-1 can1-100 ura3-1 leu2-3,112 his3-11,15 trp1-1 dot1Δ::TRP1 set1Δ::HIS3MX6*	Wysocki et al., 2005
JDY193	*bre1Δ::KANMX* and <pJD150[*HTA1-HTB1*, *HIS3*]> in FY406	This study
JDY195	As JDY193 except <pJD172 [*HTA1-htb1-S125A/S126A, HIS3*]>	This study
JDY196	As JDY193 except <pJD173 [*HTA1-htb1-K123R, HIS3*]>	This study
JDY197	As JDY193 except <pJD174 [*HTA1-htb1-K123R/S125A/S126A*]>	This study
JDY771	MATa/MATα *ura3-1/ura3-1 leu2-3,-112/leu2-3,-112 ade2-1/ade2-1 trp1-1/trp1-1 his3-11,15/his3-11,15 can1-100/can1-100 DOT1-13Myc::KANMX/ DOT1-13Myc::KANMX hta1-htb1::LEU2/ hta1-htb1::LEU2, hta2-htb2Δ/hta2-htb2Δ <pRS426-HTA1-HTB1[URA3]> <pJD150 [HTA-HTB1, HIS3]>*	This study
JDY772	MATa/MATα *ura3-1/ura3-1 leu2-3,-112/leu2-3,-112 ade2-1/ade2-1 trp1-1/trp1-1 his3-11,15/his3-11,15 can1-100/can1-100 DOT1-13Myc::KANMX/ DOT1-13Myc::KANMX hta1-htb1::LEU2/ hta1-htb1::LEU2, hta2-htb2Δ/hta2-htb2Δ <pRS426-HTA1-htb1-K123R[URA3]> <pJD150 [HTA1-HTB1, HIS3]>*	This study

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used in plates at concentrations between 0.1 and 20 µg/ml. Typically, plates with 0.5 µg/ml phleomycin are shown. The plates were incubated at 30°C for 2–3 d.

Western blot analyses

Midlog cultures were treated with or without 20 µg/ml phleomycin for 2 h unless otherwise indicated. Cell lysates were prepared using standard glass bead disruption into 20% TCA. Samples were electrophoresed on 15% SDS-PAGE, transferred to nitrocellulose, and analyzed with the following antibodies: 1:500 anti–Flag M2 (Sigma-Aldrich), 1:1,000 anti-H3 (Abcam), 1:2,000 anti-H3K4me3 (Millipore), 1:500 anti-H3K79me3 (Abcam), and 1:2,000 anti-H2B (generated against recombinant H2B). HRP-conjugated anti–mouse (Abcam) or anti–rabbit (Abcam) secondary antibodies were used, and bands were visualized by chemiluminescence (Thermo Fisher Scientific).

Genetic crosses

The Y131- and Y133-based strains (YM5037 and YM5038) were transformed with pJD150 (*HTA1-HTB1, HIS3*) and were 5-fluoroorotic acid selected. The mating type of YM5037 was switched, and this strain was crossed with YM5037 or YM5038 to create the diploid strains JDY771 and JDY772, respectively. The strains were grown in rich media, and single colonies were screened for Ura+ His− phenotypes, reflecting the sole presence of the *URA3*-based plasmid encoding wild-type H2B. These were sporulated, tetrads were dissected, and sets of four segregants were transformed with pJD173 (*HTA1-htb1-K123R*, *HIS3*) and were 5-fluoroorotic acid selected.

We thank Michael Grunstein, Ali Shilatifard, Mary Ann Osley, Steve Kron, and Fred Winston for strains and reagents. We thank members of the J.A. Downs laboratory for helpful discussions, Caroline Earp and Laurence Toime for valuable input, Eva Hoffmann for assistance with tetrad dissection, and Steve Jackson for support during the early stages of this project.

E.R. Foster was supported by a studentship from the Biotechnology and Biological Sciences Research Council, and J.A. Downs was a Jenner Fellow of the Lister Institute.

Submitted: 15 December 2008
Accepted: 2 February 2009

References

Bostelman, L.J., A.M. Keller, A.M. Albrecht, A. Arat, and J.S. Thompson. 2007. Methylation of histone H3 lysine-79 by Dot1p plays multiple roles in the response to UV damage in *Saccharomyces cerevisiae*. *DNA Repair (Amst.)*. 6:383–395.

Briggs, S.D., T. Xiao, Z.-W. Sun, J.A. Caldwell, J. Shabanowitz, D.F. Hunt, C.D. Allis, and B.D. Strahl. 2002. Gene silencing: trans-histone regulatory pathway in chromatin. *Nature*. 418:498.

Dehe, P.M., M. Pamblanco, P. Luciano, R. Lebrun, D. Moinier, R. Sendra, A. Verreault, V. Tordera, and V. Geli. 2005. Histone H3 lysine 4 monomethylation does not require ubiquitination of histone H2B. *J. Mol. Biol.* 353:477–484.

Dover, J., J. Schneider, M.A. Tawiah-Boateng, A. Wood, K. Dean, M. Johnston, and A. Shilatifard. 2002. Methylation of histone H3 by COMPASS requires ubiquitination of histone H2B by Rad6. *J. Biol. Chem.* 277:28368–28371.

Downs, J.A., N.F. Lowndes, and S.P. Jackson. 2000. A role for *Saccharomyces cerevisiae* histone H2A in DNA repair. *Nature*. 408:1001–1004.

Downs, J.A., M.C. Nussenzweig, and A. Nussenzweig. 2007. Chromatin dynamics and the preservation of genetic information. *Nature*. 447:951–958.

Foster, E.R., and J.A. Downs. 2005. Histone H2A phosphorylation in DNA double-strand break repair. *FEBS J.* 272:3231–3240.

Frederiks, F., M. Tzouros, G. Oudgenoeg, T. van Welsem, M. Fornerod, J. Krijgsveld, and F. van Leeuwen. 2008. Nonprocessive methylation by Dot1 leads to functional redundancy of histone H3K79 methylation states. *Nat. Struct. Mol. Biol.* 15:550–557.

Game, J.C., M.S. Williamson, T. Spicakova, and J.M. Brown. 2006. The RAD6/BRE1 histone modification pathway in *Saccharomyces* confers radiation resistance through a RAD51-dependent process that is independent of RAD18. *Genetics*. 173:1951–1968.

Geng, F., and W.P. Tansey. 2008. Polyubiquitylation of histone H2B. *Mol. Biol. Cell*. 19:3616–3624.

Giannattasio, M., F. Lazzaro, P. Plevani, and M. Muzi-Falconi. 2005. The DNA damage checkpoint response requires histone H2B ubiquitination by Rad6-Bre1 and H3 methylation by Dot1. *J. Biol. Chem.* 280:9879–9886.

Henry, K.W., A. Wyce, W.-S. Lo, L.J. Duggan, N.C. Emre, C.-F. Kao, L. Pillus, A. Shilatifard, M.A. Osley, and S.L. Berger. 2003. Transcriptional activation via sequential histone H2B ubiquitylation and deubiquitylation, mediated by SAGA-associated Ubp8. *Genes Dev.* 17:2648–2663.

Hirschhorn, J.N., A.L. Bortvin, S.L. Ricupero-Hovasse, and F. Winston. 1995. A new class of histone H2A mutations in *Saccharomyces cerevisiae* causes specific transcriptional defects in vivo. *Mol. Cell. Biol.* 15:1999–2009.

Hwang, W.W., S. Venkatasubrahmanyam, A.G. Ianculescu, A. Tong, C. Boone, and H.D. Madhani. 2003. A conserved RING finger protein required for histone H2B monoubiquitination and cell size control. *Mol. Cell*. 11:261–266.

Jenuwein, T., and C.D. Allis. 2001. Translating the histone code. *Science*. 293:1074–1080.

Kao, C.-F., C. Hillyer, T. Tsukuda, K. Henry, S.L. Berger, and M.A. Osley. 2004. Rad6 plays a role in transcriptional activation through ubiquitylation of histone H2B. *Genes Dev.* 18:184–195.

Laribee, R.N., N.J. Krogan, T. Xiao, Y. Shibata, T.R. Hughes, J.F. Greenblatt, and B.D. Strahl. 2005. BUR kinase selectively regulates H3 K4 trimethylation and H2B ubiquitylation through recruitment of the PAF elongation complex. *Curr. Biol.* 15:1487–1493.

Luger, K., A.W. Mäder, R.K. Richmond, D.F. Sargent, and T.J. Richmond. 1997. Crystal structure of the nucleosome core particle at 2.8 A resolution. *Nature*. 389:251–260.

McGinty, R.K., J. Kim, C. Chatterjee, R.G. Roeder, and T.W. Muir. 2008. Chemically ubiquitylated histone H2B stimulates hDot1L-mediated intranucleosomal methylation. *Nature*. 453:812–816.

Ng, H.H., R.M. Xu, Y. Zhang, and K. Struhl. 2002. Ubiquitination of histone H2B by Rad6 is required for efficient Dot1-mediated methylation of histone H3 lysine 79. *J. Biol. Chem.* 277:34655–34657.

Pavri, R., B. Zhu, G. Li, P. Trojer, S. Mandal, A. Shilatifard, and D. Reinberg. 2006. Histone H2B monoubiquitination functions cooperatively with FACT to regulate elongation by RNA polymerase II. *Cell*. 125:703–717.

Redon, C., D.R. Pilch, E. Rogakou, A.H. Orr, N.F. Lowndes, and W.M. Bonner. 2003. Yeast histone 2A serine 129 is essential for the efficient repair of checkpoint-blind DNA damage. *EMBO Rep.* 4:678–684.

Robzyk, K., J. Recht, and M.A. Osley. 2000. Rad6-dependent ubiquitination of histone H2B in yeast. *Science*. 287:501–504.

Shahbazian, M.D., K. Zhang, and M. Grunstein. 2005. Histone H2B ubiquitylation controls processive methylation but not monomethylation by Dot1 and Set1. *Mol. Cell*. 19:271–277.

Shukla, A., N. Stanojevic, Z. Duan, T. Shadle, and S.R. Bhaumik. 2006. Functional analysis of H2B-Lys-123 ubiquitination in regulation of H3-Lys-4 methylation and recruitment of RNA polymerase II at the coding sequences of several active genes in vivo. *J. Biol. Chem.* 281:19045–19054.

Strahl, B.D., and C.D. Allis. 2000. The language of covalent histone modifications. *Nature*. 403:41–45.

Sun, Z.-W., and C.D. Allis. 2002. Ubiquitination of histone H2B regulates H3 methylation and gene silencing in yeast. *Nature*. 418:104–108.

Vitaliano-Prunier, A., A. Menant, M. Hobeika, V. Geli, C. Gwizdek, and C. Dargemont. 2008. Ubiquitylation of the COMPASS component Swd2 links H2B ubiquitylation to H3K4 trimethylation. *Nat. Cell Biol.* 10:1365–1371.

Wood, A., N.J. Krogan, J. Dover, J. Schneider, J. Heidt, M.A. Boateng, K. Dean, A. Golshani, Y. Zhang, J.F. Greenblatt, et al. 2003a. Bre1, an E3 Ubiquitin ligase required for recruitment and substrate selection of Rad6 at a promoter. *Mol. Cell*. 11:267–274.

Wood, A., J. Schneider, J. Dover, M. Johnston, and A. Shilatifard. 2003b. The Paf1 complex is essential for histone monoubiquitination by the Rad6-Bre1 complex, which signals for histone methylation by COMPASS and Dot1p. *J. Biol. Chem.* 278:34739–34742.

Wysocki, R., A. Javaheri, S. Allard, F. Sha, J. Cote, and S.J. Kron. 2005. Role of Dot1-dependent histone H3 methylation in G1 and S phase DNA damage checkpoint functions of Rad9. *Mol. Cell. Biol.* 25:8430–8443.