Current Biology, Vol. 15, 1487–1493, August 23, 2005, ©2005 Elsevier Ltd All rights reserved. DOI 10.1016/j.cub.2005.07.028

BUR Kinase Selectively Regulates H3 K4 Trimethylation and H2B Ubiquitylation through Recruitment of the PAF Elongation Complex

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Summary

Histone-lysine methylation is linked to transcriptional regulation and the control of epigenetic inheritance. Lysine residues can be mono-, di-, or trimethylated, and it has been suggested that each methylation state of a given lysine may impart a unique biological function [1, 2]. In yeast, histone H3 lysine 4 (K4) is mono-, di-, and trimethylated by the Set1 histone methyltransferase [3, 4]. Previous studies show that Set1 associates with RNA polymerase II and demarcates transcriptionally active genes with K4 trimethylation [5]. To determine whether K4 trimethylation might be selectively regulated, we screened a library of yeast deletion mutants associated with transcriptional regulation and chromatin function. We identified *BUR2*, a cyclin for the Bur1/2 (BUR) cyclin-dependent protein kinase, as a specific regulator of K4 trimethylation [6]. Surprisingly, BUR also regulated H2B monoubiquitylation, whereas other K4 methylation states and H3 lysine 79 (K79) methylation were unaffected. Synthetic genetic array (SGA) and transcription microarray analyses of a *BUR2* mutant revealed that BUR is functionally similar to the PAF, Rad6, and Set1 complexes. These data suggest that BUR acts upstream of these factors to control their function. In support, we show that recruitment of the PAF elongation complex to genes is significantly impaired in a *BUR2* deletion. Our data reveal a novel function for the BUR kinase in transcriptional regulation through the selective control of histone modifications.

Results and Discussion

In an attempt to understand how K4 trimethylation is regulated, we carried out Western analyses for K4 trimethylation on extracts from 384 different yeast strains, each containing a unique gene deletion. The product of each deleted gene is involved in some aspect of transcription or chromatin function (N.J.K. and J.F.G., unpublished data). We reasoned that factors influencing K4 trimethylation would most likely be involved in some aspect of transcription or chromatin regulation because this modification is associated with actively transcribing genes [5, 7]. As shown in Figure 1A, we identified a number of factors previously characterized as global regulators of K4 methylation, including members of the Set1 methyltransferase complex (COMPASS), the PAF elongation complex, and the Rad6/Bre1 H2B ubiquitylation complex [8–12]. Surprisingly, we also identified *bur2* deletion (*bur2*Δ) as a mutation causing significantly decreased K4 trimethylation (Figure 1A). Bur2 is a cyclin regulatory subunit for the essential Bur1 cylin-dependent kinase (cdk), and this protein kinase complex is known to regulate transcription elongation [6, 13, 14]. However, the mechanism by which the BUR kinase complex functions to regulate transcription elongation is not known.

Because histone lysine residues can be mono-, di-, or trimethylated, we next characterized whether the *bur2*Δ mutant affected any of the other known K4 methylation states. Although initial studies revealed a partial decrease in the K4 mono- and dimethylation forms based on the loading of equal amounts of WCEs, it became apparent that H3 levels were also reduced in the *bur2*Δ mutant (see Figure S1). Although it is unclear why histone levels in the *bur2*Δ are lower, this reduction does not appear to be caused by a generalized defect in transcription, as determined by microarray studies (see http://www.utoronto.ca/greenblattlab/BUR.xls), nor does it appear to be a global defect in protein translation because the protein levels of other factors examined in the *bur2*Δ mutant were not affected (Figure 2C and data not shown). We therefore normalized the amount of protein loaded in our Western analyses to the levels of H3. As shown in Figure 1B, we found that the *BUR2* deletion nearly abolished K4 trimethylation. However, this deletion did not affect the mono- or dimethylation states of K4. In addition to K4 methylation, we also examined the effects of the *bur2*Δ on other histone modifications. As shown, the loss of Bur2 did not affect the levels of K79 di- or trimethylation or other sites of histone acetylation, suggesting that the effects of *bur2*Δ are highly specific for the trimethylation state of K4 (Figure 1B and data not shown). Importantly, the loss of K4 trimethylation could be restored with ectopically expressed *BUR2* (Figure 1C). As a comparison, we examined these same modifications in a *rad6*Δ strain. We did not detect any of the K4 or K79 methyl states in this mutant (Figure 1B). These results show that the BUR kinase selectively regulates K4 trimethylation but not K4 mono- or dimethylation. These data support the idea that the K4-trimethylation state may have a unique role in transcription because its regulation can be uncoupled from the mono- and dimethylation states [5, 15, 16].

The function of Bur2 is to regulate efficient Bur1 kinase activity. Because Bur1 deletion is inviable, yet *bur2*Δ cells are viable (albeit with a slow growth phenotype), we wanted to verify that the effects on K4 trimethylation were due to downregulated kinase activity of Bur1 and not to any other potential function of Bur2. To determine this, we initially analyzed *bur2*Δ cells

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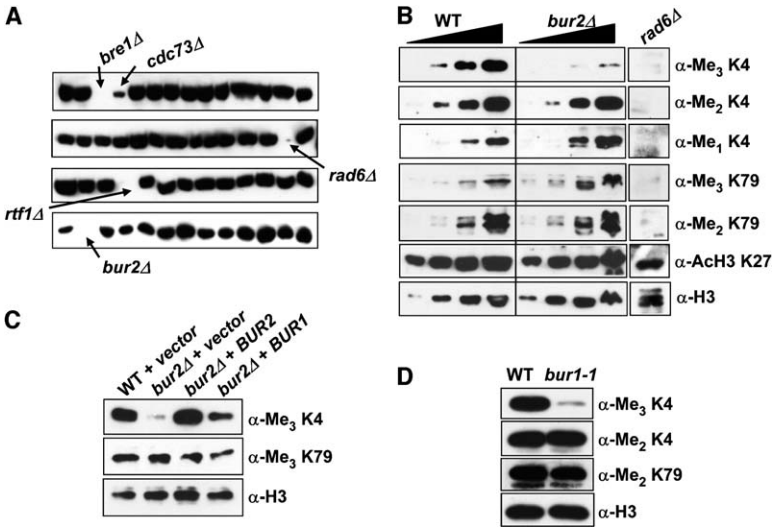

Figure 1. The BUR Kinase Complex Regulates Histone H3 K4 Trimethylation

(A) Sample of a yeast deletion screen for K4 trimethylation. Asynchronous, logarithmically growing deletion mutants were pelleted, whole-cell extracts (WCEs) prepared, and 50 μg of WCE resolved by 15% SDS-PAGE. Samples were transferred to PVDF and immunoblotted with anti-histone H3 trimethy K4 antibody. Arrows in the top three panels denote factors previously identified as regulators of global K4 methylation. The arrow in the bottom panel denotes the *bur2*Δ-specific sample.

(B) Characterization of *bur2*Δ and *rad6*Δ effects on multiple histone modifications. Increasing amounts of either wt (BY4741) or *bur2*Δ WCE, and WCE from a *rad6* mutant, were resolved by 15% SDS-PAGE and transferred to PVDF. Individual panels were immunoblotted with the specified antibody. It is notable that histone levels in *bur2*Δ cells were determined to be partially reduced as compared to wt (see Figure S1). Thus, wt and *bur2*Δ WCEs were normalized such that the histone H3 levels were equivalent.

(C) Exogenous expression of *BUR2* or *BUR1* rescues the loss of K4 trimethylation in *bur2*Δ. Normalized WCEs from wt or *bur2*Δ cells transformed with empty vector or with a *BUR2* or *BUR1* expression vector as indicated were immunoblotted as described above with the specified antibody.

(D) A *bur1* temperature-sensitive allele loses K4 trimethylation. Normalized WCEs from either wt (GY312) or *bur1-1* (GY114) grown at the semipermissive temperature 30°C were resolved as described above and immunoblotted with the indicated antibody.

transformed with a *BUR1*-containing expression vector. A previous study had shown that overexpression of a wild-type *BUR1* allele in a *bur2*Δ strain partially rescues the slow growth phenotype of the mutant, thus showing that the major, if not only, role of Bur2 is to regulate the kinase activity of Bur1 [6]. Consistent with this observation, overexpression of *BUR1* in the *bur2* deletion mutant strain resulted in a significant restoration of K4 trimethylation (Figure 1C). Independently, we analyzed the levels of K4 trimethylation in a temperature-sensitive *bur1-1* mutant strain grown at a semipermissive temperature that does not completely inactivate the kinase activity [17]. Analysis of histone modifications in this strain revealed that although still present, K4 trimethylation levels were significantly reduced as compared to its wild-type counterpart (Figure 1D). This effect again was specific for K4 trimethylation because K4 dimethylation and K79 methylation, as well as H3 acetyl K9 levels (data not shown), were not affected (Figure 1D). To confirm that the effects seen on K4 trimethylation in the *bur2*Δ strain were not attributable to deficiencies in growth, such as defects in cell-cycle progression, we analyzed logarithmically growing cells by phase-contrast microscopy. Compared to wild-type cells, the *bur2*Δ cells had no obvious defect in cell size or in the number of unbudded versus budded cells (data not shown). Furthermore, we synchronized wild-type cells with nocodazole treatment and then analyzed the levels of both K4 trimethylation and H2B ubiquitylation over time after release from the arrest. Again, we did not see any evidence that K4 trimethylation or H2B ubiquitylation were regulated in a cell-cycle-dependent fashion (data not shown), thus suggesting that the effects on K4 trimethylation in the *bur2* mutant are not due to altered cell-cycle progression. These data firmly establish that one function of the BUR kinase is to specifically regulate global K4 trimethylation levels. Importantly, this is the first factor identified outside of the COMPASS complex that selectively controls a specific K4 methyl state and separates the regulation of K4 and K79 methylation from one another [18, 19].

Histone H2B ubiquitylation is a known prerequisite for the establishment of both K4 and K79 methylation. Deletion of factors such as the ubiquitin E2 conjugating enzyme Rad6, the E3 ligase Bre1, or members of the PAF elongation complex results in the loss of H2B ubiquitylation and leads to near abolishment of both K4 and K79 methylation [8, 10–12, 20, 21]. To determine if a loss of Bur2 would affect H2B ubiquitylation, we deleted *BUR2* in a strain that carries a single copy of H2B that is N-terminally Flag epitope tagged. Surprisingly, the *bur2*Δ strain had significantly less ubiquitylated H2B as compared to its wild-type counterpart strain (Figure 2A). Furthermore, this defect in H2B ubiquitylation could be rescued by expressing exogenous *BUR2*, thus showing that the loss of H2B ubiquitylation was due solely to the loss of Bur2 (Figure 2B). We also compared the effects of *bur2*Δ with a *rad6* mutant, and, consistent with work by others, we found that *rad6*Δ completely abolished ubiquitylated H2B (data not shown) [9, 10].

We next wondered whether the loss of H2B ubiquitylation and K4 trimethylation might be due to down-regulation of the machineries that mediate these modifications. We found, however, that the loss of H2B ubiquitylation could not be explained by decreased expression of factors known to regulate H2B ubiquitylation, such as Rad6, Rtf1, or Leo1, because deletion of Bur2 in yeast strains containing these factors TAP tagged did not show any decreased protein levels (Figure 2C). We also confirmed that Bre1 protein levels and the protein levels of two members of the COMPASS

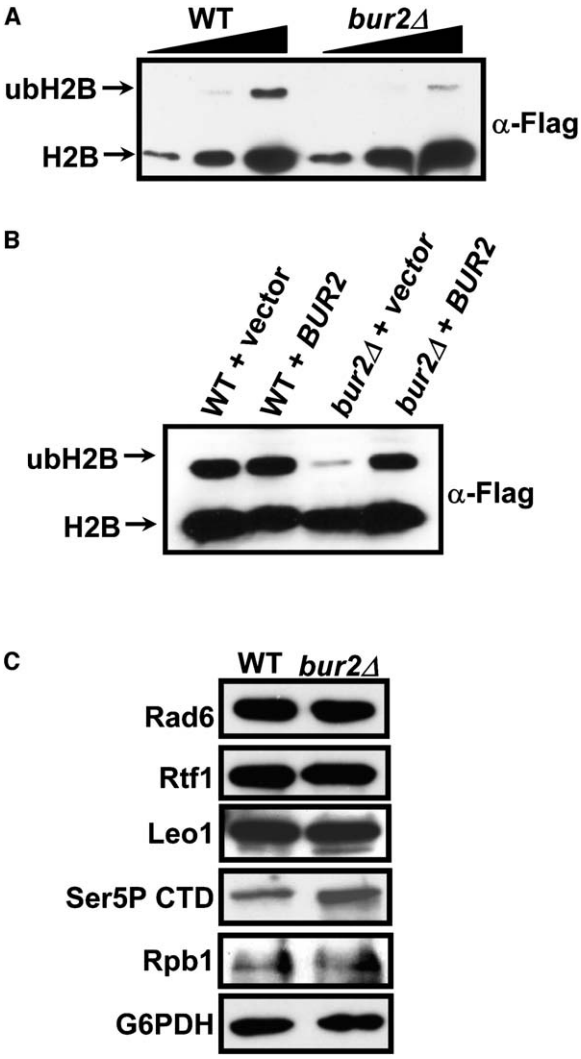

Figure 2. BUR Regulates Rad6-Mediated H2B Monoubiquitylation

(A) Increasing cell numbers of asynchronous, logarithmically growing wt (YZS276) or *bur2*-deleted cells (YNL001) were pelleted, resuspended in SDS-PAGE loading buffer, and boiled. Samples were resolved on a 15% SDS-PAGE, transferred to PVDF membrane, and immunoblotted with anti-Flag antibody. The upper arrow (ubH2B) denotes the monoubiquitylated form of H2B, whereas the lower arrow (H2B) shows the Flag-H2B levels.

(B) *BUR2* expression rescues the H2B ubiquitylation defect in *bur2*Δ. WCEs from wt (YZS276) or *bur2*-deleted cells (YNL001), transformed with either an empty vector or *BUR2* expression vector, were resolved on an SDS-PAGE gel and immunoblotted with the anti-Flag antibody.

(C) Factors that regulate H2B ubiquitylation are not downregulated. 50 μg of WCE from Rad6-TAP or Rtf1-TAP strains containing a *bur2* deletion were resolved by 10% SDS-PAGE, transferred to PVDF, and immunoblotted with the indicated antibodies. The anti-G6PDH and anti-Pol II (Santa Cruz, sc8952) panels are representative immunoblots taken from extracts of the Rad6-TAP background. In addition to the above, we found that the *BUR2* deletion did not change the protein levels of Set1 complex members that were TAP tagged (not shown).

complex, Cps40 and Cps60, were not affected in *bur2*Δ (data not shown). Furthermore, microarray analysis of gene expression in a *bur2* deletion mutant revealed that the mRNA of factors known to affect H2B ubiquitylation and K4 methylation, such as components of the Rad6, PAF, and Set1 complexes, were unaffected (see http://www.utoronto.ca/greenblattlab/BUR.xls). These results are surprising because to date, factors that have been shown to regulate H2B ubiquitylation also affect the levels of both K4 and K79 methylation. Thus, the data show the ability to uncouple the absolute levels of H2B ubiquitylation from the individual methyl states of K4 and K79.

Recent studies have shown that the PAF complex controls H2B ubiquitylation, at least in part, through its ability to recruit the E2-ubiquitin-conjugating enzyme Rad6, and its E3 ligase, Bre1, to Pol II [11, 21]. To determine whether *bur2*Δ affected PAF-complex recruitment to genes, we performed chromatin immunoprecipitation (ChIP) to monitor Rtf1 association with the *PMA1* gene by using yeast strains carrying *RTF1* TAP tagged or a derivative strain also deleted for *BUR2*. Although we detected significant enrichment of Rtf1 on the promoter and entire open reading frame (ORF) of *PMA1*, this enrichment was dramatically reduced in a *bur2*Δ strain (Figure 3B). The loss of Rtf1 recruitment was not due to an effect on the amount of Rtf1 protein in the *bur2*Δ (see Figure 2C) nor was it specific for only Rtf1. Strains carrying Leo1 TAP tagged or a derivative strain with *BUR2* deleted also showed similar results (data not shown). We also deleted *CTK1*, as a control, in the Rtf1-TAP strain and monitored PAF recruitment. Whereas with *bur2*Δ we observed a significant reduction in Rtf1 recruitment to *PMA1*, *ctk1*Δ had either no affect (in the promoter) or marginally reduced Rtf1 recruitment (within the ORF) (Figure 3B), which is consistent with work by others [22]. Similar to results shown for the *PMA1* gene, we found PAF reduction on other genes we examined in *bur2*Δ (Figure S2 and data not shown). These results demonstrate that the decreased PAF recruitment to genes in the *bur2* deletion mutant is specific to BUR function and is not shared with another kinase complex (i.e., the Ctk1-containing CTDK-I complex) that also regulates transcription elongation.

The BUR kinase complex plays a critical but as yet undefined role in the regulation of transcription elongation. Studies have found that the BUR complex can phosphorylate the C-terminal domain (CTD) of the largest subunit of Pol II in vitro with little substrate preference for either the serine 2 or serine 5 position within the heptapeptad repeat sequence [13, 14]. However, in vivo BUR does not appear to be a source of CTD kinase activity [13]. Because PAF complex is known to travel with the elongating Pol II complex, we determined whether the Pol II distribution on the *PMA1* gene was altered between wild-type and *bur2*Δ cells as a means for explaining the decreased PAF recruitment. Using ChIP to monitor Pol II localization, we found no change between wild-type and *bur2*Δ cells in the density or distribution of Pol II along the entire *PMA1* gene (Figure 3C). We also analyzed Pol II distribution in a *ctk1*Δ and found deleting this kinase also does not affect the distribution of Pol II on genes, which is consistent with other studies (Figure 3C) [22, 23]. To confirm that *bur2*Δ did not affect the Pol II CTD serine 5 phosphorylation state, we immunoblotted wild-type and *bur2*Δ whole-cell extracts with an antibody that speci-

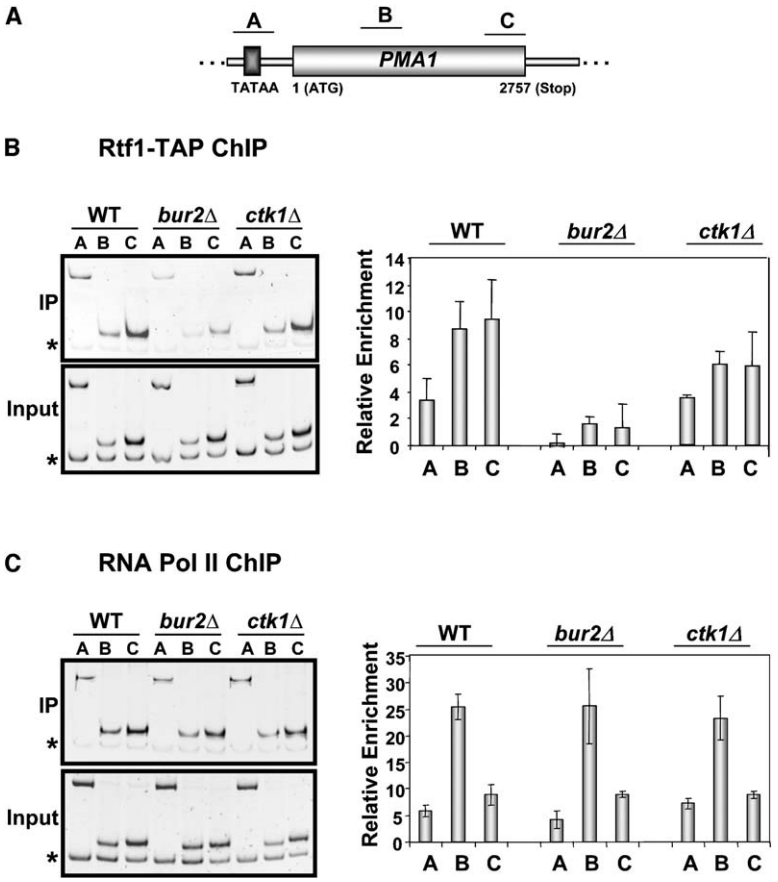

Figure 3. BUR Regulates PAF-Elongation-Complex Association to Genes

(A) Schematic of the *PMA1* locus and the relative location of PCR primers used in this study.

(B) Chromatin immunoprecipitation (ChIP) monitoring the localization of the Rtf1-TAP subunit of the PAF complex in wt, *bur2*Δ, or *ckt1*Δ backgrounds. Asterisks denote the location of an internal control band amplified by PCR primers specific to a region of chromosome-V-lacking open-reading frames. Data were normalized as described in the Supplemental Experimental Procedures. Error bars represent the SEM of three independent replicates.

(C) Same as in (B) except strains used were wt, *bur2*Δ, or *ctk1*Δ in the BY4741 background. RNA polymerase II was monitored with the 8WG16 antibody; similar results were obtained with an anti-N-terminal Rpb1 antibody (Santa Cruz, sc25758) (not shown). Error bars are the SEM of three independent replicates.

fically recognizes the Pol II CTD phosphorylated at the serine 5 position. *bur2*Δ did not affect the global levels of CTD serine 5 phosphorylation, nor did it affect the amount or distribution of serine 5 phosphorylated Pol II on the *PMA1* gene as determined by ChIP (Figure 2C and data not shown). Because Ctk1 is the sole serine 2 CTD kinase in yeast, these data reveal that the loss of PAF complex recruitment is not due to generalized defects in Pol II transcription elongation or Pol II CTD serine 2 or serine 5 phosphorylation [24]. These data therefore indicate that at least one function of the BUR complex in transcription elongation is to regulate recruitment of the PAF complex to genes.

Genes encoding proteins in the same functionally distinct pathway should have similar effects on gene expression and similar sets of synthetic genetic interactions. To further demonstrate that BUR kinase is functionally linked to histone H2B ubiquitylation and K4 trimethylation, we first used automated synthetic genetic array (SGA) analysis. Nat^R strains harboring individual gene deletions of nonessential components of the Rad6 (*RAD6*, *BRE1*), Paf1 (*RTF1*, *PAF1*), COMPASS (*SWD1*, *SWD3*), and BUR (*BUR2*) complexes were generated and crossed to a set of viable deletion strains selected for their involvement in gene expression, and the growth of resulting double mutant strains was analyzed (data not shown). Similar sets of genetic interactions were obtained for *rad6*Δ, *bre1*Δ, *rtf1*Δ, *paf1*Δ, *swd1*Δ, *swd3*Δ, and *bur2*Δ, a result consistent with all four complexes being involved in the same functional pathway (Figure 4A). These included genetic interactions with the transcriptional elongation factors *DST1*, *SPT4*, Elongator (*ELP1*, *ELP2*, *ELP3*, *ELP4*, *ELP6*), as well as with nonessential components of the 26S proteasome (*RPN4*, *RPN10*, *PRE9*, *SEM1*, *UBP6*), which has been recently implicated in histone H2B ubiquitylation by Rad6 and H3 methylation via COMPASS [25–31]. Synthetic growth defects were also observed with *RTT103*, a component of the transcriptional termination complex, Torpedo, and the CTDK-I kinase [32–34]. The fact that the Rad6, Paf1, and COMPASS complexes share similar sets of genetic interactions with *BUR2* strongly supports the notion that all are working to regulate a similar process in vivo.

To further characterize the relationship between BUR and these other complexes, we used DNA microarrays to analyze gene expression in strains containing deletions of components of the Rad6 (*RAD6*, *BRE1*, *LGE1*), COMPASS (*SDC1*), and PAF1 (*CTR9*, *CDC73*) complexes as well as *bur2*Δ (raw microarray data at http://www.utoronto.ca/greenblattlab/BUR.xls). Pearson correlation coefficients were calculated for each pair of deletions, and the strains organized by 2D hierarchical clustering according to the similarities of their effects on gene expression (Figure 4B). As a control, we also analyzed gene expression data generated from deletions of the Swr1 chromatin remodeling complex (*SWR1*, *SWC2*, *SWC3*, *SWC5*) as well as from deletion

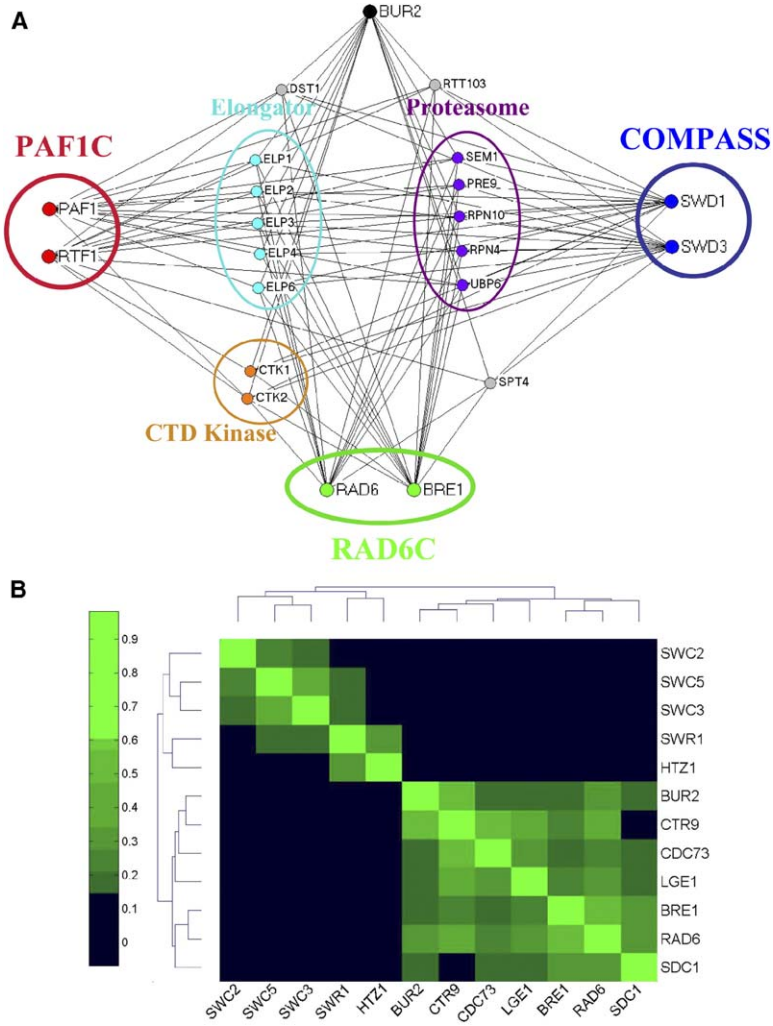

Figure 4. Synthetic Genetic Interactions for *PAF1*, *RTF1*, *BUR2*, *SWD1*, *SWD3*, *RAD6*, and *BRE1*

(A) SGA technology was used to cross Nat^r strains harboring individual deletions of genes encoding Paf1, Rtf1, Bur2, Swd1, Swd3, Rad6, and Bre1 with a transcription-targeted array of deletion strains to create sets of Nat^r Kan^r haploid double mutants. Growth rates were assessed by automated image analysis of colony size. Lines connect genes with synthetic genetic interactions. The lengths of lines and proximity of boxes in this diagram are unrelated to the strengths of the indicated synthetic genetic interactions.

(B) Microarray analysis of gene expression was performed for the indicated deletion strains. Pearson correlation coefficients were then calculated for each pair of deletions, and the deletions were organized by 2D hierarchical clustering.

of the histone H2A variant *HTZ1*. Swr1 is a member of the Snf2 family of ATPases, and the Swr1 complex incorporates the histone H2A variant, Htz1, into chromatin [35–37]. As was observed with our genetic analyses, the gene expression profiles of strains containing deletions of components of the Rad6, Paf1, and COMPASS complexes were very similar to each other as well as with the profile generated from a *BUR2* deletion (Figure 4B). In contrast, *HTZ1*, *SWR1*, *SWC2*, *SWC3*, and *SWC5* clustered next to each other and were distinct from *BUR2*, *CTR9*, *CDC73*, *LGE1*, *BRE1*, *RAD6*, and *SDC1* in this gene-expression analysis. Microarray experiments also have been conducted on over 400 other strains containing individual deletions of genes implicated in some aspect of transcription or chromatin function (N.J.K., T.R.H., and J.F.G., unpublished data). The clustering of this larger data set (not shown) also reflects the data represented in Figure 4B, in which *BUR2* clustered next to components of the Rad6, Paf1, and COMPASS complexes. Therefore, both the genetic and the gene-expression data suggest that all four complexes are functionally similar, and these data would argue strongly that the BUR, Rad6, PAF, and COMPASS complexes are in the same genetic pathway.

In this study, we have defined a novel function for the BUR kinase complex via its ability to selectively regulate PAF recruitment to genes, histone K4 trimethylation, and H2B ubiquitylation. Furthermore, we show that BUR uncouples the regulation of K4 trimethylation and H2B ubiquitylation from the control of mono- and dimethylation at K4 and also from K79 methylation. To our knowledge, this is the first demonstration that the different methyl states of a single histone lysine residue can be independently regulated by factors existing outside of the target lysine's HMT complex. These data also suggest that the K79 methylation levels in yeast are not coupled directly to the absolute levels of histone H2B ubiquitylation per se because in the *bur2*Δ, in which there is a significant decrease in H2B ubiquitylation, no effect on K79 di- and trimethylation was observed. Such data may seem difficult to reconcile given the known requirement of Rtf1 and H2B ubiquitylation for K79 methylation; however, recent reports have shown that the recruitment of Dot1 to coding regions of genes is largely independent of Rtf1, whereas Set1 recruitment is dependent on Rtf1 [7, 8, 20]. Such findings suggest that these two HMTs are regulated by independent mechanisms and, therefore, might be af-

fected differently by the reduction of PAF and H2B ubiquitylation on genes.

Our interpretation of the data presented here is that low levels of H2B ubiquitylation are sufficient to trigger wild-type levels of K4 mono- and dimethylation and also K79 di- and trimethylation. This may be explained by the proposed "wedge" model, which states that H2B ubiquitylation would unfold chromatin to make condensed regions of chromatin accessible to the Set1 and Dot1 enzymes [38]. However, additional events must occur for Set1 to mediate K4 trimethylation. Because Set1 recruitment to genes is dependent on PAF, one explanation could be that PAF recruits a specific form of COMPASS that selectively trimethylates. In this scenario, an alternate form of the Set1 complex gains access, in a H2B ubiquitylation-dependent manner, to genes and mediates K4 mono- and dimethylation. A second possibility could be that a COMPASS member directly interacts with ubiquitylated H2B and this interaction triggers the K4 trimethylating activity. Recent reports show that the Spp1 subunit of COMPASS is required for K4 trimethylation [18, 19]. Thus, Spp1 may directly interact with the ubiquitylated H2B tail, and as a consequence, decreased H2B ubiquitylation would be reflected in a selective loss of K4 trimethylation. Finally, the Bur1 kinase may directly phosphorylate a COMPASS subunit to trigger K4 trimethylation. It is important to distinguish that in scenarios one and three, the wild-type levels of H2B ubiquitylation are coincident with K4 trimethylation but are not the cause. It will be intriguing to determine in future studies which, in any, of these proposed models are correct.

Supplemental Data

Supplemental Data include two figures and Supplemental Experimental Procedures and can be found with this article online at http://www.current-biology.com/cgi/content/full/15/16/1487/DC1/.

Acknowledgments

We would like to thank Greg Prelich for the generous gifts of plasmids and yeast strains. We also would like to thank S. Briggs, J. Cook, C.J. Ingles, J. Lieb, Z.W. Sun, and members of the Strahl laboratory for discussions and helpful comments. We also thank N.J. Thompson and T. Punna for help with figures. This work is supported by National Institutes of Health postdoctoral training grant (CA 09156) and postdoctoral fellowship (1 F32 GM071106-01A1) to R.N.L. and by National Institutes of Health (grant GM68088) and American Heart Association (grant 0465506U) to B.D.S. This work was also supported by funds from the Canadian Institutes of Health Research, Ontario Genomics Institute, National Cancer Institute of Canada, and Canadian Cancer Society to J.F.G. N.J.K. is supported by a Doctoral Fellowship from Canadian Institutes of Health Research. B.D.S. is a Pew Scholar in the Biomedical Sciences.

Received: May 27, 2005
Revised: July 4, 2005
Accepted: July 6, 2005
Published online: July 21, 2005

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