Regulation of histone modification and cryptic transcription by the Bur1 and Paf1 complexes

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The Bur1–Bur2 and Paf1 complexes function during transcription elongation and affect histone modifications. Here we describe new roles for Bur1–Bur2 and the Paf1 complex. We find that histone H3 K36 tri-methylation requires specific components of the Paf1 complex and that K36 tri-methylation is more strongly affected at the 5' ends of genes in *paf1Δ* and *bur2Δ* strains in parallel with increased acetylation of histones H3 and H4. Interestingly, the 5' increase in histone acetylation is independent of K36 methylation, and therefore is mechanistically distinct from the methylation-driven deacetylation that occurs at the 3' ends of genes. Finally, Bur1–Bur2 and the Paf1 complex have a second methylation-independent function, since *bur2Δ set2Δ* and *paf1Δ set2Δ* double mutants display enhanced histone acetylation at the 3' ends of genes and increased cryptic transcription initiation. These findings identify new functions for the Paf1 and Bur1–Bur2 complexes, provide evidence that histone modifications at the 5' and 3' ends of coding regions are regulated by distinct mechanisms, and reveal that the Bur1–Bur2 and Paf1 complexes repress cryptic transcription through a Set2-independent pathway.

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Introduction

RNA polymerase II (pol II)-directed transcription consists of distinct initiation, elongation, and termination steps, all of which occur in the context of a nucleosomal template (Hartzog *et al*, 2002; Li *et al*, 2007). Nucleosomes can block initiation and elongation by pol II *in vitro* (Li *et al*, 2007), but eukaryotes have evolved elaborate mechanisms to overcome

nucleosome-mediated repression, including ATP-dependent nucleosome remodeling (Zofall *et al*, 2006) and post-translational histone modifications (Kouzarides, 2007; Li *et al*, 2007). We are still in the early stage of identifying and understanding the functional significance of histone modifications that occur as pol II progresses through a template.

Methylation of histone H3 on lysine 36 (K36) is a conserved modification in eukaryotes that is catalyzed by Set2 (Strahl *et al*, 2002). Set2 associates with the coding regions of transcribed genes through direct interaction with the elongating hyperphosphorylated form of pol II (Li *et al*, 2002; Xiao *et al*, 2003; Krogan *et al*, 2003b; Kizer *et al*, 2005). Consistent with the localization pattern of Set2, di-methylated and tri-methylated K36 are enriched throughout the coding regions of transcriptionally active genes (Xiao *et al*, 2003; Bannister *et al*, 2005; Morillon *et al*, 2005; Pokholok *et al*, 2005; Rao *et al*, 2005). Moreover, *set2Δ* causes a growth defect when combined with mutations in genes that encode elongation factors (Li *et al*, 2003; Krogan *et al*, 2003b). Current evidence indicates that Set2 and H3 K36 methylation perform inhibitory roles in transcription regulation: LexA–Set2 can repress transcription of a *lacZ* reporter that contains a LexA-binding site (Strahl *et al*, 2002), *set2Δ* or a histone H3 K36R substitution increases the expression of a UAS-less *GAL4* mutant promoter (Landry *et al*, 2003) and restores the binding of pol II and TBP to promoters in strains lacking yeast FACT (Biswas *et al*, 2006), and *SET2* represses cryptic transcription initiation within coding regions (Carrozza *et al*, 2005).

Histone acetylation affects transcription by leading to the recruitment of factors that further modify histones (Kouzarides, 2007) or by changing the electrostatic charge on histones and weakening histone–DNA interactions (Hong *et al*, 1993; Workman and Kingston, 1998). Histone acetylation across the *Saccharomyces cerevisiae* genome is not uniform, with promoter regions typically being acetylated more than the associated coding regions (Reid *et al*, 2004; Joshi and Struhl, 2005; Pokholok *et al*, 2005). The low level of acetylation in coding regions is maintained by a dynamic regulation between acetylation and deacetylation. Interestingly, at least part of this regulation is mediated by histone H3 K36 methylation, which can recruit the Rpd3S histone deacetylase (HDAC) complex through its chromodomain-containing Eaf3 subunit, resulting in histone deacetylation and repression of cryptic transcription initiation within the coding region (Carrozza *et al*, 2005; Joshi and Struhl, 2005; Keogh *et al*, 2005).

Recent studies have found that elongation factors such as Bur1–Bur2 and the Paf1 complex are required for specific histone modifications. *BUR1* and *BUR2* encode the catalytic and regulatory subunits of a cyclin-dependent protein kinase that is essential for normal growth and has a general role in transcription elongation (Yao *et al*, 2000; Keogh *et al*, 2003). The yeast Paf1 complex, which consists of Paf1, Ctr9, Cdc73, Rtf1, and Leo1 subunits (Mueller and Jaehning, 2002; Squazzo *et al*, 2002; Mueller *et al*, 2004), has been implicated

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in transcription elongation based on physical association with pol II and the elongation factors Spt4–Spt5 and yFACT, its localization to the ORFs of active genes *in vivo*, and mutant sensitivity to 6-azauracil and mycophenolic acid (Krogan *et al*, 2002; Mueller and Jaehning, 2002; Squazzo *et al*, 2002). The Rtf1, Paf1, and Ctr9 subunits of the Paf1 complex are required for histone H2B mono-ubiquitylation and histone H3 K4 di- and tri-methylation on actively transcribed genes (Wood *et al*, 2003; Krogan *et al*, 2003a; Ng *et al*, 2003b; Xiao *et al*, 2005). Proper recruitment of the Paf1 complex is dependent on Bur1–Bur2, and therefore mutations in *BUR1* and *BUR2* also cause defects in histone H2B mono-ubiquitylation and H3 K4 methylation (Larabee *et al*, 2005; Wood *et al*, 2005; Qiu *et al*, 2006). We recently showed that Bur1–Bur2 is also required for tri-methylation of histone H3 at K36 (Chu *et al*, 2006). The role of the Paf1 complex in histone H3 K36 methylation is unclear, however, as conflicting results have been reported (Ng *et al*, 2003a; Krogan *et al*, 2003b). Effects of Bur1–Bur2 and the Paf1 complex on histone acetylation have not been studied.

Although the precise mechanisms by which Bur1–Bur2 and the Paf1 complex influence elongation are not fully elaborated, it remains important to define and investigate their shared and unshared roles in regulating histone modifications and transcription. To gain more insight into these issues, we examined the methylation of H3 K36 in strains deleted for Paf1 complex subunits and the functional consequences of Bur1–Bur2 and Paf1 complex defects on histone acetylation and cryptic transcription. Our results indicate that the Bur1 and Paf1 complexes regulate histone acetylation and repress cryptic transcription by a mechanism that is distinct from the mechanism that regulates H3 K36 tri-methylation.

Results

Mutations in the Paf1 complex affect the histone H3 K36 methylation state

To identify factors that are required for the transition from the di-methylated to the tri-methylated state of histone H3 K36, we screened mutations in genes that have been functionally linked to histone modifications or transcription elongation. In particular, we focused on the multifunctional Paf1 complex, which is comprised of Paf1, Ctr9, Cdc73, Leo1, and Rtf1 (Krogan *et al*, 2002; Mueller and Jaehning, 2002; Squazzo *et al*, 2002; Mueller *et al*, 2004). Using antibodies specific for distinct methylated forms of H3 K36 in western blots of yeast cell lysates, we observed a strong reduction of H3 K36 tri-methylation in *paf1Δ* and *ctr9Δ* strains, a moderate reduction in the *cdc73Δ* strain, and no detectable K36 methylation defect in *rtf1Δ* and *leo1Δ* strains (Figure 1). The defect was restricted to tri-methylated H3 K36, as effects on mono- or di-methylated H3 K36 levels were either modest or absent. The dependence of K36 tri-methylation on only a subset of the Paf1 complex members was not due to allelic differences, since null alleles were used for each gene. Specific subunits of the Paf1 complex are therefore required for normal levels of histone H3 K36 tri-methylation.

Extensive genetic interactions between the Paf1 complex, Set2, the pol II CTD, and the Bur1–Bur2 kinase
Because mutations in a subset of the genes encoding the Paf1 complex share a similar H3 K36 tri-methylation defect as was

Figure 1 A subset of Paf1 complex mutations selectively affect histone H3 K36 tri-methylation. Whole-cell extracts were prepared from yeast strains with the genotypes shown at the top, and western blots were performed using antibodies specific for total H3, mono-, di-, or tri-methylated K36. The arrow indicates the position of mono-methylated H3 migrating slightly above a background band.

seen previously in *bur1*, *bur2*, and *rpb1* CTD truncation strains (Xiao *et al*, 2003; Chu *et al*, 2006), we next tested whether genetic interactions could be detected by double mutant analysis. First, double mutants were constructed from a series of *rpb1* CTD truncation alleles and all Paf1 complex deletion mutations using a plasmid shuffle strategy. *paf1Δ* and *ctr9Δ* displayed strong synthetic lethality with CTD truncations, while *leo1Δ* and *rtf1Δ* exhibited no interactions with any CTD truncations, and *cdc73Δ* showed a synthetic growth defect only with the most severe CTD truncation, indicative of an intermediate functional interaction (Figure 2A). These double-mutant phenotypes strongly parallel the extent of the tri-methylation defect and the growth defect observed in individual Paf1 complex mutants (*paf1Δ* = *ctr9Δ* > *cdc73Δ* > *leo1Δ* = *rtf1Δ*). Second, a similar pattern of genetic interactions was observed between a subset of the Paf1 complex deletions and a deletion of *SET2*, with *set2Δ* causing strong synthetic growth defects with *paf1Δ* and *ctr9Δ*, synthetic lethality with *cdc73Δ* at 38.5°C and partial suppression at 30°C, and no interactions with *leo1Δ* and *rtf1Δ* (Figure 2B, and data not shown). These results differ from those obtained by a large-scale screen that described synthetic lethal interactions of all Paf1 complex deletion mutations, except *leo1Δ*, with *set2Δ* (Krogan *et al*, 2003b). The cause of this discrepancy is unknown but likely reflects limitations of the yeast deletion collection and the use of large-scale SGA screening methodology instead of creating fresh knockouts and tetrad analysis to follow double-mutant phenotypes. The consistent segregation of these interactions as detected by tetrad analysis, the overlap with the K36 tri-methylation defect, and concordance with the data of Biswas *et al* (2006) provide confidence in our results. Third, genetic interactions were also observed between Paf1 complex mutations and *bur2-1*, a mutation that causes a moderate growth defect by itself. Double-mutant combinations of *bur2-1* with *paf1Δ*, *ctr9Δ*, *cdc73Δ*, and *leo1Δ* all revealed synthetic growth defects, whereas *rtf1Δ* partially suppressed the *bur2-1* growth defect (Figure 2C and D). Although this pattern differed from that observed for the H3 K36 tri-methylation defect and for the genetic interactions with the CTD truncations and *set2Δ*, it is consistent with, and expands upon, prior results describing suppression of *bur1Δ* by *rtf1Δ* and synthetic growth defects between *bur1-23* and *leo1Δ*, *paf1Δ*, and *cdc73Δ* (Keogh *et al*, 2005). In summary, a subset of Paf1 complex mutations displays extensive genetic interactions with *rpb1*

Figure 2 Paf1 complex mutations interact genetically with CTD truncations, a *bur2* mutation, and *set2Δ*. (A) The growth phenotypes of strains containing deletions of Paf1 complex subunits and truncations of the pol II CTD. The numbers in the diagram on the right indicate the number of CTD repeats remaining. Strains were constructed by a plasmid shuffle approach and grown on 5FOA medium for 4 days at 30°C. (B) *set2Δ* causes synthetic sickness selectively with *paf1Δ* and *ctr9Δ*. Five-fold serial dilutions of the strains listed were spotted onto YPD plates and photographed after 3–4 days of growth at 30°C. (C) Summary of the growth phenotypes of double mutants between *bur2-1* and deletions of the Paf1 complex subunits. (D) Strains with the indicated genotypes were streaked onto a YPD plate and grown at 30°C for 3 days.

CTD truncations, *set2Δ*, and *bur2-1* that overlap significantly, but not completely, with the K36 tri-methylation defect. These systematic tests of double-mutant interactions further demonstrate functional distinctions between the subunits of the Paf1 complex and functional overlap with the pol II CTD, Set2, and the Bur1–Bur2 kinase complex.

Histone H3 K36 tri-methylation patterns at specific genes

Because H3 K36 tri-methylation defects were detected in *paf1Δ*, *ctr9Δ*, and *bur2Δ* crude extracts, it was expected that tri-methylation would also be affected at individual genes. ChIP assays were performed to independently assess K36 tri-methylation levels, to determine whether all genes being tested were equally affected, and to examine whether any detectable effects were equally distributed throughout the ORFs. Previous studies revealed that tri-methylation occurs throughout the ORF of transcribed genes, with a genome-wide average peaking toward the 3' end, although the 3' end peak does not occur at every gene (Pokholok *et al.*, 2005). Several genes were examined by ChIP, using primers specific for the 5' and 3' ends of their respective ORFs. In broad agreement with the results observed by western analysis, K36 tri-methylation at *PMA1*, *PYK1*, and *FLO8* was severely reduced in a *bur2Δ* strain (Figure 3A–C). This defect likely operates through the Paf1 complex, since *paf1Δ* and *ctr9Δ* strains displayed the same reduction of tri-methylated H3 at *PMA1*, *PYK1*, *FLO8*, *PDR5*, and *GLN4*, whereas little if any effect was observed in *cdc73Δ*, *leo1Δ*, and *rtf1Δ* strains (Figure 3D–F; Supplementary Figure 1). Interestingly, a greater reduction was consistently observed at the 5' end than at the 3' end in *bur2Δ*, *paf1Δ*, and *ctr9Δ* strains. The reduced tri-methylation signal is not due to a loss of histone H3 occupancy, since H3 levels are unaffected at *PMA1* and *FLO8* in *bur2Δ* and *paf1Δ* strains (Supplementary Figure 2). Three conclusions emerge from these ChIP results: strong Paf1c effects on H3 K36 tri-methylation were limited to *paf1Δ* and *ctr9Δ*, the greatest reduction occurred at the 5'

ends of the ORFs, and the reduction of 5' H3 K36 tri-methylation was shared with *bur2Δ*.

The reduction of H3 K36 tri-methylation in *bur2Δ* and *paf1Δ* mutants could conceivably result from decreased association of pol II or Set2 with transcribed genes. The occupancy of pol II as measured by ChIP assays with antibodies against unphosphorylated CTD (8WG16) or Ser5-phosphorylated CTD (H14), however, was only slightly reduced at the 5' and 3' ends of *PMA1* in *bur2Δ* and *paf1Δ* strains (Figure 4A and C; Supplementary Figure 3). At *FLO8*, pol II levels actually increased in the mutant strains (Figure 4B and D). These data indicate that the dramatic reduction of H3 K36 tri-methylation in *bur2Δ* and *paf1Δ* strains is not caused by a pol II recruitment defect. In contrast to the pol II results, deletion of *BUR2* or *PAF1* caused a significant decrease in Set2 levels at *PMA1* (Figure 4E and F), *PYK1*, and *GLN4* (Supplementary Figure 4C and D), without affecting total cellular levels of Set2 (Supplementary Figure 4A and B). This result agrees with previous observations of decreased Set2 recruitment in *rtf1Δ* and *cdc73Δ* strains (Krogan *et al.*, 2003b). Interestingly, in *paf1Δ* strains, association of Set2 was reduced more severely at the middle and 3' ends of *PMA1*, *PYK1*, and *GLN4* than at the 5' ends (Figure 4F; Supplementary Figure 4), even though the reduction in H3 K36 tri-methylation was more pronounced at the 5' ends (Figure 3). These results suggest that the H3 K36 tri-methylation defect does not correlate simply with a reduction in Set2 localization or levels. Consistent with this idea, deletion of *RTF1*, which did not affect H3 K36 methylation, and deletion of *PAF1*, which dramatically lowered H3 K36 tri-methylation, caused a similar decrease in Set2 recruitment (Figure 4F; Supplementary Figure 4C and D).

Histone acetylation increases at several active genes in *bur2Δ* and *paf1Δ* mutants

A functional role for histone H3 K36 methylation was described recently, in which methylated K36 recruits the Rpd3S

Figure 3 Histone H3 K36 tri-methylation levels at specific genes in *bur2Δ* and Paf1 complex mutants. (A–C) Total histone H3 or histone H3 tri-methylated at K36 was immunoprecipitated from extracts of formaldehyde-treated wild type, *bur2Δ*, and *set2Δ* strains. Levels of tri-methylated K36 or total H3 were assessed at the 5′ (black bars) and 3′ ends (gray bars) of the *PMA1* (A), *PYK1* (B), and *FLO8* (C) coding regions by ChIPs. (D–F) ChIPs of total histone H3 and H3 tri-methylated at K36 were performed in Paf1 complex deletion strains. Enrichment of tri-methyl K36 was measured at the 5′ (black bars) and 3′ (gray bars) ends of *PMA1* (D), *PYK1* (E), and *FLO8* (F). Tri-methyl K36 levels were normalized to total H3 levels. Standard error is provided for values representing three experiments.

HDAC complex to the 3′ ends of ORFs, triggering histone deacetylation and the repression of cryptic intragenic transcripts (Carrozza *et al*, 2005; Joshi and Struhl, 2005; Keogh *et al*, 2005). The reduction of K36 tri-methylation in *bur2Δ* and *paf1Δ* strains allowed a test of whether a similar coupling of acetylation and K36 methylation occurs at the 5′ ends of ORFs. Antibodies that recognize di-acetylated H3 and tetra-acetylated H4 were used in ChIP assays to assess H3 and H4 acetylation at the 5′ and 3′ ends of several genes in *bur2Δ* and *paf1Δ* strains. Mirroring the reduction of K36 tri-methylation, acetylation of H3 and H4 was increased preferentially near the 5′ ends of *PMA1* and *FLO8* in a *bur2Δ* strain (Figure 5A and C; Supplementary Figure 5A and B). In *PYK1*, H3 and H4 acetylation was increased at the 5′ end, but also at the 3′ end in the *bur2Δ* strain (Figure 5B and D). Similar histone acetylation changes were detected in a *paf1Δ* strain, where histone H3 and H4 acetylation was increased at the 5′ ends of *PMA1* and *FLO8* (Figure 5E and G; Supplementary Figure 5G and H), and at both the 5′ and 3′ ends of *PYK1* (Figure 5F and H). The acetylation levels at two other genes were also tested in a *paf1Δ* strain; at *PDR5*, H3 and H4 acetylation increased at the 5′ end, while acetylation was unaffected at *GLN4* (Supplementary Figure 5). These data indicate that the loss of *BUR2* or *PAF1* results in significantly increased histone H3 and H4 acetylation at the 5′ ends of *PMA1*, *PYK1*, *FLO8*, and *PDR5*, and additionally affects the 3′ end of *PYK1*, while some genes, exemplified by *GLN4*, remain unaffected.

The increase in histone acetylation at the 5′ ends of genes is independent of H3 K36 methylation

Since we observed a parallel decrease in H3 K36 tri-methylation with an increase in histone H3 and H4 acetylation at the 5′ ends of several genes, we asked whether the changes in methylation and acetylation are functionally linked. The increased acetylation at the 5′ ends of *PMA1* and *PYK1*

observed in *bur2Δ* and *paf1Δ* strains did not occur in *set2Δ* or *set1Δ* strains (Figure 6A–D), indicating that complete loss of K36 or K4 methylation does not cause the increased histone acetylation at the 5′ end. Moreover, the increased acetylation observed in a *bur2Δ* strain is not reduced in *bur2Δ set2Δ* or *Δbur2Δ set1Δ* strains (Figure 6A–D), indicating that the increased acetylation is not dependent on either K36 or K4 methylation. Similarly, the increase in H3 and H4 acetylation at *PMA1* in a *paf1Δ* strain was *SET2* independent (Figure 6E and F). Based on these results, we conclude that the increased H3 and H4 acetylation observed near the 5′ ends of *PMA1* and *PYK1* in *bur2Δ* and *paf1Δ* strains does not require K36 methylation and is not caused by partial methylation of K36. It remains possible that the increased histone acetylation causes the reduced H3 K36 tri-methylation at the 5′ ends of genes, but a test of this hypothesis awaits identification of the responsible histone acetyltransferase (HAT) and HDAC.

Bur2 and Paf1 function redundantly with Set2 in regulating histone acetylation

The major difference observed at *PMA1* and *PYK1* in *bur2Δ* and *paf1Δ* strains was that acetylation increased only near the 5′ end of *PMA1* but at both the 5′ and 3′ ends of *PYK1* (Figure 5). This could be because *BUR2* and *PAF1* do not control acetylation near the 3′ end of *PMA1*, or because they function redundantly with another pathway. An excellent candidate is the Set2–Rpd3S pathway that controls histone acetylation and the appearance of cryptic transcripts at the 3′ ends of some ORFs (Carrozza *et al*, 2005; Joshi and Struhl, 2005; Keogh *et al*, 2005). To investigate whether *BUR2* and *PAF1* are redundant with the Set2–Rpd3S pathway, acetylation at the 3′ end of *PMA1* and *FLO8* in *bur2Δ* and *paf1Δ* strains was compared to that of *set2Δ*, *bur2Δ set2Δ*, and *paf1Δ set2Δ* strains (Figure 7). *set2Δ* by itself did not increase

Figure 4 Pol II and Set2 occupancy in *bur2Δ* and *paf1Δ* mutant strains. (A–D) 8WG16 antibody was incubated with chromatin samples to test the total pol II level at *PMA1* and *FLO8* from wild-type and *bur2Δ* strains (A, B) and from wild-type and *paf1Δ* strains (C, D). (E, F) ChIP assays measuring the recruitment of tagged Set2 to the promoter, 5' ORF, Mid ORF and 3' ORF of *PMA1* in *bur2Δ*, *paf1Δ* and *rtf1Δ* strains. Standard error is provided for values representing three experiments.

Figure 5 *bur2Δ* and *paf1Δ* affect histone acetylation at the 5' regions of ORFs. (A–D) ChIP analysis of wild type and *bur2Δ* strains using antibodies against acetylated histones H3 and H4. Levels of H3 acetylation and H4 acetylation were assessed at the 5' and 3' ends of *PMA1* (A, C) and *PYK1* (B, D). (E–H) ChIP analysis of wild type and *paf1Δ* strains using antibodies against acetylated histones H3 and H4 at the 5' and 3' ends of *PMA1* (E, G) and *PYK1* (F, H). Standard error is provided for values representing three experiments.

Figure 6 Increased acetylation at the 5' end is independent of histone methylation in *bur2Δ* and *paf1Δ* strains. (A–D) ChIP analysis with antibodies against acetylated histones H3 and H4 in wild type, *bur2Δ*, *bur2Δ set2Δ*, *bur2Δ set1Δ*, *set1Δ*, and *set2Δ* strains. Levels of H3 acetylation and H4 acetylation were assessed at the 5' ends of *PMA1* (A, B) and *PYK1* (C, D). (E, F) ChIP analysis of acetylated histone H3 and H4 in wild type, *paf1Δ*, *paf1Δ set2Δ*, and *set2Δ* strains at the 5' end of *PMA1* (E, F). Standard error is provided for these values representing three experiments.

histone H3 or H4 acetylation at the 3' end of *PMA1*, consistent with previous observations that H3 and H4 acetylation did not increase at the 3' end of *PMA1* in a strain lacking the Eaf3 subunit of the Rpd3S complex (Reid *et al*, 2004; Joshi and Struhl, 2005; Pokholok *et al*, 2005). In *bur2Δ set2Δ* and *paf1Δ set2Δ* double mutants, acetylation was increased at *PMA1* and *FLO8* compared with the single mutants (Figure 7). These results indicate that Bur2 and Paf1 have redundant functions with K36 methylation that are required to inhibit acetylation at the 3' ends of genes.

Bur2 and Paf1 function redundantly with Set2 in regulating cryptic transcription

To determine whether the increased acetylation at the 3' end has any functional consequences, we tested for the appearance of cryptic internal transcripts in single and double mutants. The effect of *bur2Δ*, *paf1Δ*, and *set2Δ* was examined at *FLO8*, which has been characterized as a reporter for cryptic transcription initiation (Kaplan *et al*, 2003; Carrozza *et al*, 2005). Paralleling the effects observed on 3' acetylation, the internal *FLO8* cryptic transcript was detected at a low level in *bur2Δ*, *paf1Δ*, *ctr9Δ*, and *set2Δ* single mutants, but a strong combinatorial effect was observed in *bur2Δ set2Δ*, *paf1Δ set2Δ*, and *ctr9Δ set2Δ* double mutants (Figure 8A–C). As expected for components of the same linear pathway, combinatorial effects on cryptic transcription were not ob-

served in *bur2Δ paf1Δ* double mutants (Figure 8D). These results were confirmed in two ways: by transforming *BUR2* and *SET2* plasmids into the *bur2Δ set2Δ* strain, and by using an *in vivo* assay in which the *FLO8* cryptic TATA box drives expression of a *HIS3* reporter (Prather *et al*, 2005). In the complementation assay, plasmids containing *BUR2* or *SET2* reduced internal *FLO8* transcript levels in the *bur2Δ set2Δ* strain to the same level as observed in a *set2Δ* strain or a *bur2Δ* strain (data not shown), indicating that the increased cryptic transcription was due to *bur2Δ* and *set2Δ*, and not some unsuspected mutations in that strain. In the *HIS3* reporter assay, *paf1Δ* and *set2Δ* individually caused an extremely weak His⁺ phenotype, whereas the *paf1Δ set2Δ* double mutant was stronger His⁺ (Figure 8E), confirming the northern results *in vivo*. Based on these combined results, the Bur1–Paf1 pathway and Set2 have redundant roles in regulating histone acetylation and cryptic transcription at the 3' ends of some genes.

Discussion

Expanded functions of the Bur1–Paf1c pathway

The genetic screens that originally identified Bur1–Bur2 and the Paf1 complex subunits relied on very different mutant phenotypes (Reed *et al*, 1988; Prelich and Winston, 1993; Di Como *et al*, 1995; Stolinski *et al*, 1997; Costa and Arndt,

Figure 7 Bur2 and Paf1 have redundant roles with Set2 in controlling histone acetylation at the 3' end of ORFs. (A–D) ChIP analysis with antibodies against acetylated histones H3 and H4 in wild type, *bur2Δ*, *bur2Δ set2Δ*, *bur2Δ set1Δ set1Δ*, and *set2Δ* strains. Levels of H3 acetylation and H4 acetylation were assessed at the 3' ends of *PMA1* (A, B) and *FLO8* (C, D). (E–H) ChIP analysis of acetylated histone H3 and H4 in wild type, *paf1Δ*, *paf1Δ set2Δ*, and *set2Δ* strains at the 3' ends of *PMA1* (E, F) and *FLO8* (G, H). Standard error is provided for values representing three experiments.

2000), providing no obvious indication that these proteins were functionally related. Subsequent studies, however, revealed extensive functional overlap between Bur1–Bur2 and the Paf1 complex, including shared sensitivity to base analogs, similar *Spt[−]* phenotypes (Murray *et al*, 2001; Krogan *et al*, 2002; Squazzo *et al*, 2002; Keogh *et al*, 2003), association with the coding regions of active genes (Krogan *et al*, 2002; Pokholok *et al*, 2002; Keogh *et al*, 2003), and regulation of H2B ubiquitylation and K4 methylation (Laribee *et al*, 2005; Wood *et al*, 2005). The results presented here identify new functions for the Bur1 and Paf1 complexes, revealing shared roles in promoting K36 tri-methylation, reducing H3 and H4 acetylation, and cooperating with Set2 to repress cryptic transcription. The number of shared characteristics strongly implies that the Bur1–Bur2 and Paf1 complexes are

components of the same pathway for the regulation of histone methylation and acetylation. Indeed, Bur1–Bur2 is required for Paf1c occupancy at ORFs (Laribee *et al*, 2005; Wood *et al*, 2005; Qiu *et al*, 2006), although Paf1 is not required for Bur2 occupancy (Y Chu and G Prelich, data not shown). This ordering of the pathway suggests that the Paf1 complex might be a direct substrate for the Bur1 kinase. We have not been able to detect Bur1-dependent phosphorylation of the Paf1 complex *in vitro*, suggesting that Bur1 targets an additional component of the pathway. It is important to note that Bur1–Bur2 and the Paf1 complex also have roles that are independent of each other, exemplified by the near inviability of *bur1Δ* strains compared with the viable Paf1 complex deletion mutants, and by the observation that *set2Δ* partially suppresses *bur1Δ* and *bur2Δ*, yet causes synthetic growth

Figure 8 The Bur1 and Paf1 complexes are redundant with Set2 for repressing cryptic transcription. (A–D) Northern blot analysis of total RNA prepared from the indicated strains. Filters were probed with sequences complementary to the 3' region of *FLO8* (+1672/+2399), *PMA1* (+168/+2290), or *SCR1*. Ethidium bromide-stained gels showing the levels of ribosomal RNA serve as additional loading controls. The full-length and short cryptic transcript signals for *FLO8* are indicated. (E) Strains with the indicated genotypes were tested for effects on an integrated *pGAL1::FLO8-HIS3* reporter gene (Prather *et al*, 2005). Ten-fold serial dilutions were spotted onto SC and SC-His plates and plates were incubated at 30°C for 3 days. His⁺ growth is indicative of transcription from the internal *FLO8* promoter.

defects with *paf1Δ* and *ctr9Δ*. The molecular basis for these differences is not understood, but must be due to some unshared function.

Paf1 and Ctr9 are required for proper histone H3 K36 tri-methylation

An ongoing question in the chromatin field is whether the mono-, di-, and tri-methylation marks on histones confer different functions. Our study was motivated by the idea that identifying factors that are specifically required for K36 tri-methylation would be useful for investigating its potential roles. Indeed, we found that, like mutations in *BUR1*, *BUR2*, and some pol II CTD truncations, *PAF1* and *CTR9* are required for K36 tri-methylation, with little, if any effects on total mono- or di-methyl K36 level. Strikingly, the K36 tri-methylation function of the Paf1 complex is strongly dependent on Paf1 and Ctr9, with Cdc73 having a moderate role and Rtf1 and Leo1 having no detectable effect on K36 tri-methylation levels in crude extracts or at specific genes. This pattern contrasts with the essential role that Rtf1 plays in the ubiquitylation of H2B and methylation of H3 K4 and K79 (Wood *et al*, 2003; Ng *et al*, 2003a) and further supports the idea that the Paf1 complex carries out multiple functions during transcription elongation.

For any histone modification, functional significance is best inferred from its differential localization or by the identification of factors that recognize specific modified forms. The spatial distribution of tri-methyl K36, however, is similar to that observed for di-methyl K36 (Bannister *et al*, 2005; Pokholok *et al*, 2005; Rao *et al*, 2005), although the tri-methyl K36 pattern correlates more closely with transcriptional frequency. Moreover, the chromodomain of Eaf3, which is the only factor currently known to bind K36, binds equally well to mono-, di-, and tri-methylated K36 peptides (Carrozza *et al*, 2005; Joshi and Struhl, 2005; Keogh *et al*, 2005). Thus, by these criteria, the functional significance of K36 tri-methylation remains unknown. In genome-wide analyses, the level of K36 tri-methylation in wild-type strains correlated with transcriptional activity (Pokholok *et al*, 2005), but we have not observed a strict correlation between changes in K36 tri-methylation level and transcription in *bur2Δ* or *paf1Δ* mutant strains. For example, K36 tri-methylation levels are reduced at both the 5' and 3' ends of *FLO8* even though pol II occupancy increases. This suggests that the changes in K36 tri-methylation are not simply a consequence of altering transcription, but instead indicate that the Bur1–Paf1c pathway can affect the activity of Set2 or a K36 demethylase independent of transcription.

Potential crosstalk between histone acetylation and H3K36 methylation

Several precedents establish that one histone modification can affect the appearance of a second histone modification (Fischle *et al*, 2003). Particularly relevant to this study is the established role of H3 K36 methylation in recruiting the Rpd3S complex and consequently causing histone deacetylation at the 3' ends of genes (Carrozza *et al*, 2005; Joshi and Struhl, 2005; Keogh *et al*, 2005). The more severe reduction of K36 tri-methylation in the 5' region of four different ORFs in *bur2Δ* and *paf1Δ* strains and the correlation of reduced tri-methylation with increased acetylation in these strains suggested that a similar process might operate at the 5' ends of genes. The links between acetylation and K36 methylation at the 5' and 3' ends of genes are mechanistically distinct, however, since acetylation at the 5' end did not increase in a *set2Δ* strain, and the increased acetylation observed in *bur2Δ* and *paf1Δ* strains was unaffected by loss of *SET2*. The increased 5' acetylation therefore is not caused by the loss of K36 methylation and does not require K36 methylation. Two other possibilities remain; the first is that the increased acetylation causes the decreased K36 tri-methylation, possibly by recruiting a K36 demethylase. Testing this model will require identification of the HAT, HDAC, and the K36 demethylase that act at the 5' end. The second possibility is that the increased acetylation and decreased K36 tri-methylation are coincidental. This seems unlikely, however, since four out of five genes that have increased 5' acetylation in *bur2Δ* or *paf1Δ* strains also have decreased K36 tri-methylation. The results presented here constitute another possible link between different histone modifications, indicate that the crosstalk between histone acetylation and H3 K36 methylation at the 5' region is different from the one existing at the 3' ends of ORFs, and raise the intriguing idea of a regulatory loop whereby acetylation reduces methylation at the 5' end, and methylation reduces acetylation at the 3' end via the Rpd3S pathway.

The Bur1–Paf1 pathway acts redundantly with Set2 for repressing cryptic transcription

Bur1–Bur2 and the Paf1 complex do not only function at the 5' ends of genes. Both complexes localize throughout ORFs (Keogh *et al*, 2003; Mueller *et al*, 2004) and individually contribute to repression of internal cryptic transcription (Kaplan *et al*, 2003; Nourani *et al*, 2006). Because *bur2Δ*, *paf1Δ*, and *set2Δ* each increase cryptic transcription and reduce K36 methylation, it was possible that they all increased cryptic transcription through the Set2–Rpd3S pathway. Our results, however, indicate that this is not the case, since a significant increase in cryptic transcription occurs in both *bur2Δ set2Δ* and *paf1Δ set2Δ* double mutants relative to the individual mutants. We infer that the Bur1–Paf1c pathway represses cryptic transcription by a mechanism that is redundant with the Set2–Rpd3S pathway. This result raises the questions of how many pathways control cryptic transcription and what their components are. Other factors known to affect cryptic transcription include histones, Spt6, Spt16, Spt10, Spt2, Hir1, and Elf1 (Kaplan *et al*, 2003; Prather *et al*, 2005; Nourani *et al*, 2006). Double-mutant analyses such as those reported here will help to elaborate the organization of the pathways and to identify additional components that repress spurious transcription of the gen-

ome. Because increased cryptic transcription at the 3' end of *FLO8* correlates with increased acetylation in *bur2Δ* and *paf1Δ* strains, we infer that the redundant pathway impacts directly or indirectly upon a HAT or HDAC that functions within transcribed ORFs. Efforts are underway to identify the responsible factor(s).

Two of the key results presented here are that mutations in the Bur1–Paf1 pathway reduce Set2-dependent tri-methylation of K36, yet act independent of the Set2 pathway to block cryptic transcription. At first glance, these results might appear to be contradictory. Our interpretation is that the Bur1–Paf1 pathway regulates K36 tri-methylation, perhaps by directly stimulating Set2 or inhibiting a K36-specific demethylase, or indirectly by stimulating histone acetylation. Bur1–Bur2 and the Paf1 complex affect cryptic transcription through another mechanism that is redundant with the residual Set2-dependent mono- and di-methylation that occurs in *bur2Δ* and *paf1Δ* strains. The Bur1 and Paf1 complexes are clearly multifunctional and can impact different histone modifications. Although the redundant function that cooperates with Set2 to repress cryptic transcription remains unknown, these results provide a strong foundation for future investigation of the phenomenon.

Materials and methods

Strains and media

The *S. cerevisiae* strains used in this study are listed in Supplementary Table 1. All media, including yeast-peptone-dextrose (YPD), synthetic complete drop-out (e.g., SC-Ura), 5-fluoroorotic acid (5FOA), synthetic dextrose minimal (SD), and sporulation media were prepared as described previously (Rose *et al*, 1990). Standard genetic methods for mating, sporulation, transformation, and tetrad analysis were used throughout this study (Rose *et al*, 1990). Gene disruptions were constructed using a PCR-based method (Ausubel *et al*, 1997) and confirmed by PCR analysis. The C-terminus of Set2 was tagged with six copies of the HA epitope by PCR amplification from pYM3-6HA-klTRP1 (Knop *et al*, 1999), or with 13 copies of the c-Myc epitope by PCR amplification of pFA6a-13Myc-kanMX6 (Longtine *et al*, 1998), and transformation of the PCR product into a diploid strain. To test for genetic interactions between truncations of the pol II CTD and mutations in genes for members of the Paf1 complex, plasmid shuffle experiments were performed with *LEU2*-marked plasmids encoding wild-type Rpb1 (pRP114) or Rpb1 truncations containing 17 4/7 CTD repeats (pV7), 13 3/7 repeats (pV5), 11 2/7 repeats (pC1), 10 5/7 repeats (pC3), 9 3/7 repeats (pN15), or 6 3/7 repeats (pN56) (Nonet *et al*, 1987).

Western analysis

Preparation of yeast whole-cell lysates and western blotting were as described previously (Chu *et al*, 2006). Antibodies that recognize total histone H3 (ab1791), mono-methylated histone H3 K36 (ab9048), and tri-methylated histone H3 K36 (ab9050) were obtained from Abcam, and the antibody that recognizes dimethylated histone H3 K36 (07-369) was obtained from Upstate. Preparation of yeast extracts and western analysis of Set2-HA and Set2-Myc expression were performed as described previously (Shirra *et al*, 2005), using anti-HA antibody or anti-c-Myc antibody (Santa Cruz).

Chromatin immunoprecipitation

Yeast strains were grown at 30°C in YPD to a concentration of approximately $1\text{--}3 \times 10^7$ cells per ml. Chromatin was isolated and sonicated as described previously (Shirra *et al*, 2005). Anti-HA beads (Santa Cruz) or antibodies specific for polymerase II (8WG16 and H14) (Covance), c-Myc (Covance), histone H3 tri-methylated at K36, total H3, acetylated H3 (K9, K14) (Upstate, 06-599), or acetylated H4 (K5, K8, K12, K16) (Upstate, 06-866) were added to sonicated chromatin. Immunoprecipitation was performed for 4 h

or overnight at 4°C. Immune complexes were captured by incubating with Protein A-coupled sepharose (Amersham Biosciences) for histone antibodies or Protein G-coupled sepharose (Amersham Biosciences) for c-Myc antibody and 8WG16 at 4°C for 2 h. Immune complexes with the H14 antibody were captured by incubating with Protein G-coupled sepharose beads (Sigma) at room temperature for 90 min. Precipitated DNA was purified using Qiagen PCR purification columns or phenol extraction and analyzed by PCR essentially as described (Komarnitsky *et al*, 2000; Keogh and Buratowski, 2004). Primers used for ChIP assays are listed in Supplementary Table 2. PCR signals were quantitated using a phosphorimager. For Set2-Myc ChIP assays, the average immunoprecipitated signal (IP) was divided by the average input signal for three independent chromatin preparations assayed in duplicate. For the recruitment of Set2-HA, pol II, and H3 occupancy, ChIP was quantified by normalizing band intensities for each sample using the following calculation: (specific gene IP/TEL IP)/(specific gene input/TEL input) as described (Komarnitsky *et al*, 2000; Carrozza *et al*, 2005). The background amplification of the chromosome V telomere region was performed as described (Komarnitsky *et al*, 2000). The normalized IP value calculated for

H3 K36 tri-methyl, acetylated H3, or acetylated H4 was divided by the normalized IP value calculated for total H3.

RNA isolation and northern analysis

Cells were grown in YPD medium to a concentration of $1-2 \times 10^7$ cells per ml. Total RNA was isolated and analyzed by northern analysis as described previously (Swanson *et al*, 1991). DNA probes were prepared by random prime labeling of PCR products for *PMA1* (+168/+2290), *FLO8* (+1672/+2399), and *SCR1*.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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