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H2B Ubiquitylation Acts as a Barrier to Ctk1 Nucleosomal Recruitment Prior to Removal by Ubp8 within a SAGA-Related Complex

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SUMMARY

Histone modifications play an important role in transcription. We previously studied histone H2B ubiquitylation on lysine 123 and subsequent deubiquitylation by SAGA-associated Ubp8. Unlike other histone modifications, both the addition and removal of ubiquitin are required for optimal transcription. Here we report that deubiquitylation of H2B is important for recruitment of a complex containing the kinase Ctk1, resulting in phosphorylation of the RNA polymerase II (Pol II) C-terminal domain (CTD), and for subsequent recruitment of the Set2 methyltransferase. We find that Ctk1 interacts with histones H2A and H2B, and that persistent H2B ubiquitylation disrupts these interactions. We further show that Ubp8 enters the *GAL1* coding region through an interaction with Pol II. These findings reveal a mechanism by which H2B ubiquitylation acts as a barrier to Ctk1 association with active genes, while subsequent deubiquitylation by Ubp8 triggers Ctk1 recruitment at the appropriate point in activation.

INTRODUCTION

Posttranslational modification of histones, including acetylation, methylation, phosphorylation, and ubiquitylation, represents a major mechanism of eukaryotic transcriptional regulation (Berger, 2002). Modification of the N- and C-terminal tails of histones is thought to occur in patterns, which recruits specific effector proteins that alter chromatin structure and regulate gene expression.

The addition and removal of histone modifications are generally thought to have antagonistic effects; for example, lysine acetylation is activating, whereas deacetylation is usually repressing. One exception to this model, however, is lysine ubiquitylation of histone H2B. In yeast, H2B is monoubiquitylated at lysine 123 (H2BK123ub1) by the E2/E3 complex Rad6/Bre1 (Hwang et al., 2003; Robzyk et al., 2000; Wood et al., 2003). The analogous mammalian residue (K120) is ubiquitylated by enzymes homologous to those in yeast (Kim et al., 2005; Zhu et al., 2005). H2BK123ub1 increases at the promoter and open reading frames (ORFs) of numerous yeast genes during transcriptional activation (Henry et al., 2003; Kao et al., 2004; Xiao et al., 2005). Removal of transcription-associated H2BK123ub1 is mediated by the ubiquitin hydrolase Ubp8. Both the addition and removal of ubiquitin are important for activation; mutations that abolish the ubiquitylation site or Ubp8 activity lead to lowered transcription at numerous genes (Daniel et al., 2004; Henry et al., 2003).

H2BK123ub1 is linked to histone H3 methylation in a "*trans*-tail" modification pathway (Henry and Berger, 2002). H3 is methylated at K4, K36, and K79 by the enzymes Set1, Set2, and Dot1, respectively (Briggs et al., 2001; Krogan et al., 2002a; Miller et al., 2001; Nagy et al., 2002; Roguev et al., 2001; Strahl et al., 2002; van Leeuwen et al., 2002). In bulk histones, H2BK123ub1 is required for methylation at H3 K4 and K79, but not at K36 (Briggs et al., 2002; Dover et al., 2002; Sun and Allis, 2002). At specific genes, however, ubiquitylation and deubiquitylation affect relative levels of H3 K4 and K36 methylation in antagonistic ways (Henry et al., 2003). At the *GAL1* gene, mutation of the ubiquitylation site abolishes activation-associated K4 trimethylation (H3K4me3) but greatly increases K36 dimethylation (H3K36me2). Conversely, persistent H2BK123ub1 in a *ubp8Δ* strain causes H3K4me3 to increase, whereas H3K36me2 decreases. Thus, it appears that H2B ubiquitylation/deubiquitylation at *GAL1* functions to establish proper levels of the two methyl marks for optimal transcription.

Ubp8 is recruited to genes as part of the SAGA coactivator complex and is also part of the related SALSA/SLIK complex (Daniel et al., 2004; Henry et al., 2003). SAGA and SALSA are very similar in composition; however, SALSA contains truncated Spt7 and lacks Spt8 (Pray-Grant et al., 2002; Sterner et al., 2002; Wu and Winston, 2002). In addition to H2B deubiquitylation (H2B-dub), SAGA acetylates histones H3 and H2B via Gcn5 (Grant et al., 1998b). SAGA is modular in structure and function. In addition to the deubiquitylation (DUB; Ubp8 and Sgf11) and acetylation (HAT; Gcn5, Ada2, and Ada3) modules, SAGA contains specific subunits that function in TBP binding (Spt3, Spt7, and Spt8), activator interaction (Tra1), and complex assembly (Ada1 and Ada5; Belotserkovskaya et al., 2000; Dudley et al., 1999; Grant et al., 1998a; Sterner et al., 1999). These modules have distinct roles in transcription, as combined mutation of different modules leads to synergistic defects (Henry et al., 2003; Sterner et al., 1999).

The C-terminal domain (CTD) of the largest subunit of RNA polymerase II (Pol II) plays an important role in transcription and RNA processing (Buratowski, 2003). The CTD is comprised of a heptapeptide repeat (YSPTSPS) that is subject to phosphorylation at S2 and S5. S5 phosphorylation (CTDS5ph) is mediated by the TFIIH-associated kinase Kin28 (Buratowski, 2003; Komarnitsky et al., 2000). Rad6 and Set1 interact with CTDS5ph Pol II via the Paf1 complex, and subsequent H2BK123ub1 and H3K4me3 are dependent upon Kin28 activity (Krogan et al., 2003a; Ng et al., 2003; Xiao et al., 2005). S2 phosphorylation (CTDS2ph) by the cyclin-dependent kinase Ctk1 occurs during the later stages of transcriptional elongation (Cho et al., 2001; Komarnitsky et al., 2000). CTDS2ph recruits the RNA 3′ end processing machinery and is required for recruitment of Set2 and for H3K36me (Ahn et al., 2004; Komarnitsky et al., 2000; Krogan et al., 2003b; Licatalosi et al., 2002; Xiao et al., 2003).

In this study, we investigated mechanisms underlying Ubp8's role in transcription. We examined how the ubiquitylation state of H2B is linked to H3 methylation and whether there is a connection between H2B-dub and Pol II CTD phosphorylation. Our data offer insight into how H2B-dub functions to regulate transcription at SAGA-dependent genes. Further, our data suggest a function for a SAGA-related complex in transcriptional elongation, which is distinct from SAGA's role in transcription.

RESULTS

Set2 Recruitment to *GAL1*, but Not Pol II Recruitment, Is Reduced in *ubp8Δ*

Persistent H2BK123ub1 in *ubp8Δ* results in transcriptional defects and altered H3 methylation at *GAL1* (Henry et al., 2003). To determine whether these effects are due to defective recruitment of transcription factors during activation, we analyzed recruitment of factors to the *GAL1* promoter, 5′ ORF, and 3′ ORF in WT and *ubp8Δ* strains using chromatin immunoprecipitation (ChIP; Figure 1A).

To ensure that the signal from each primer set is distinct, we first analyzed localization of tagged TBP (Spt15) and Set2 in a WT strain during growth in glucose (*GAL1* repressing) and galactose (*GAL1* activating). Consistent with previous reports, we observed distinct localization patterns for TBP and Set2 along the *GAL1* gene (Kizer et al., 2005; Krogan et al., 2003b; Schaft et al., 2003). Upon induction, TBP recruitment was detected exclusively at the *GAL1* promoter (Figure 1B). In contrast, Set2 was not recruited to the promoter; however, we detected recruitment to the 5′ ORF and even higher levels at the 3′ ORF (Figure 1B). By recapitulating the known localization patterns of these proteins at *GAL1*, we establish that these regions of the gene are distinguishable by ChIP.

We were interested in determining why H3K36me2 at *GAL1* is altered in *ubp8Δ*. To determine whether the reduction is due to defective recruitment of Set2, we analyzed binding of tagged Set2 to *GAL1* by ChIP. Cells were collected in glucose, in raffinose (*GAL1* derepressing), and after galactose induction for 60 and 120 min (corresponding to RNA appearance and accumulation; see Figure S1A in the Supplemental Data available with this article online; Henry et al., 2003). In the WT strain, Set2 levels were low in glucose and steadily increased during growth in galactose (Figure 1C). In contrast, Set2 recruitment in *ubp8Δ* remained low throughout the time course at both ends of the gene. This lowered signal is not due to defective Set2 expression in *ubp8Δ* (Figure 1D). These data suggest that the reduced H3K36me2 we observed previously in *ubp8Δ* is due to decreased Set2 recruitment.

H3K36me was recently shown to inhibit cryptic transcription initiation within gene ORFs (Carrozza et al., 2005; Joshi and Struhl, 2005; Keogh et al., 2005). Because we previously observed reduced H3K36me in *ubp8Δ*, we tested whether cryptic initiation is affected in this strain. We first performed RT-PCR on *GAL1* RNA collected from WT and *ubp8Δ* strains. At the 5′ ORF, the amount of *GAL1* transcript in *ubp8Δ* is significantly reduced relative to WT at the 120 and 180 min time points (Figure S1A). At the 3′ end, the difference between WT and *ubp8Δ* is considerably less. These data are consistent with previous observations, in which we saw only modest defects in transcription at the 3′ end of the gene (Henry et al., 2003). The differences observed at the 5′ and 3′ ends of the ORF suggest that deletion of *UBP8* results in a defect in the quality and/or stability of *GAL1* RNA.

To directly test whether these effects are due to increased cryptic initiation in *ubp8Δ*, we performed northern blots in WT and *ubp8Δ* strains, as well as an *spt6* temperature-sensitive (ts) strain that exhibits increased internal initiation at the restrictive temperature (Kaplan et al., 2003). First, we analyzed *FLO8* and *STE11*, two genes known to have cryptic start sites in their ORFs (Kaplan et al., 2003). We detected short, aberrant transcripts from *FLO8* and *STE11* in the *spt6* ts strain at the restrictive temperature; however, we detected no internal initiation in *ubp8Δ* (Figure S1B; data not shown). Because it is unclear

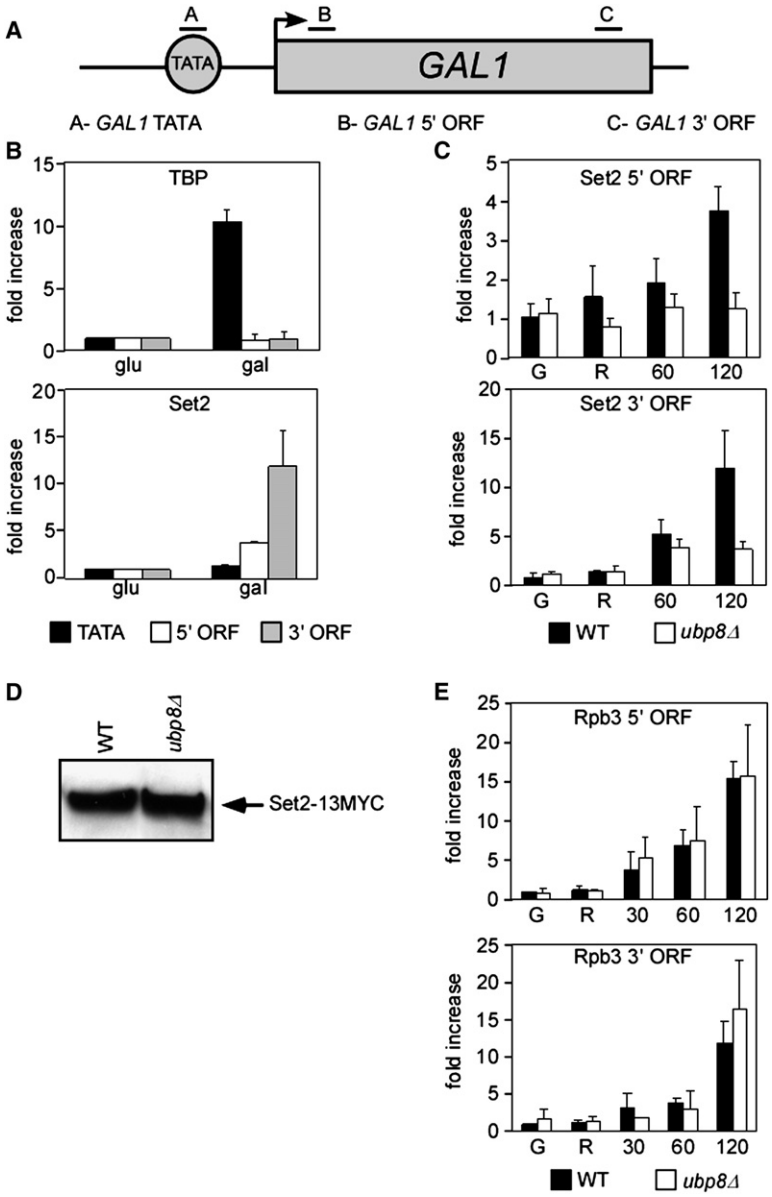

Figure 1. Set2 Recruitment to *GAL1* Is Impaired in *ubp8Δ*, but Pol II Recruitment Is Unaffected

ChIP data represent percent input normalized to an intergenic region on chromosome V (IntV). Following normalization to an isogenic untagged strain, signals are normalized to the WT glucose sample. Error bars represent standard deviation from the mean of the independent experimental values.

(A) Locations of primers used for ChIP analyses at *GAL1*.

(B) Recruitment of TBP and Set2 to *GAL1*. Recruitment was analyzed at the *GAL1* promoter, 5′ ORF, and 3′ ORF in glucose and 120 min after galactose addition. (Top) Anti-FLAG ChIP of TBP-3FLAG (n = 2). (Bottom) Anti-MYC ChIP of Set2-13MYC (n = 3).

(C) Set2 association at *GAL1*. Set2-13MYC binding in WT and *ubp8Δ* was analyzed at the *GAL1* 5′ and 3′ ORF (n = 3).

(D) Set2 stability in WT and *ubp8Δ* backgrounds. Anti-MYC IP/western of WT and *ubp8Δ* Set2-13MYC strains.

(E) Rpb3 recruitment to *GAL1*. Pol II binding in WT and *ubp8Δ* was analyzed by anti-Rpb3 ChIP. Data are represented as described above, minus normalization to an untagged strain (n = 3).

whether Ubp8 functions at these genes, we also assayed for cryptic initiation at the Ubp8-dependent genes *ADH1*, *GAL1*, and *PMA1* (data not shown; Bhaumik and Green, 2002; Henry et al., 2003). Analysis in the *spt6* ts strain, and in *ubp8Δ*, indicates that *ADH1*, *GAL1*, and *PMA1* do not contain cryptic start sites (Figure S1C; data not shown). Taken together, our RNA analysis indicates that H2B-dub and subsequent H3K36me likely function in regulating the ORF and RNA quality at *GAL1*, but that this role does not include the suppression of cryptic transcripts.

We next determined whether Set2 recruitment is directly affected in *ubp8Δ*, or whether it is caused by aberrant recruitment of an upstream factor. Because we observe defects in *GAL1* transcription in *ubp8Δ* (Figure S1A; Henry et al., 2003), we examined whether persistent H2BK123ub1 alters recruitment of Pol II via Rpb3 ChIP. The timing and level of Rpb3 recruitment are similar in WT and *ubp8Δ* (Figure 1E), suggesting that the defect in transcription in *ubp8Δ* is not due to altered Pol II recruitment.

Kin28 Recruitment and CTDS5ph Are Not Affected in *ubp8Δ*

Because Pol II CTD phosphorylation is known to be important for recruitment of Set1 and Set2, and we have shown that overall Pol II recruitment to *GAL1* is unaffected in *ubp8Δ*, we examined whether persistent H2BK123ub1 affects the recruitment and/or activity of the CTD kinases. We performed ChIP for Kin28 and CTDS5ph at *GAL1*

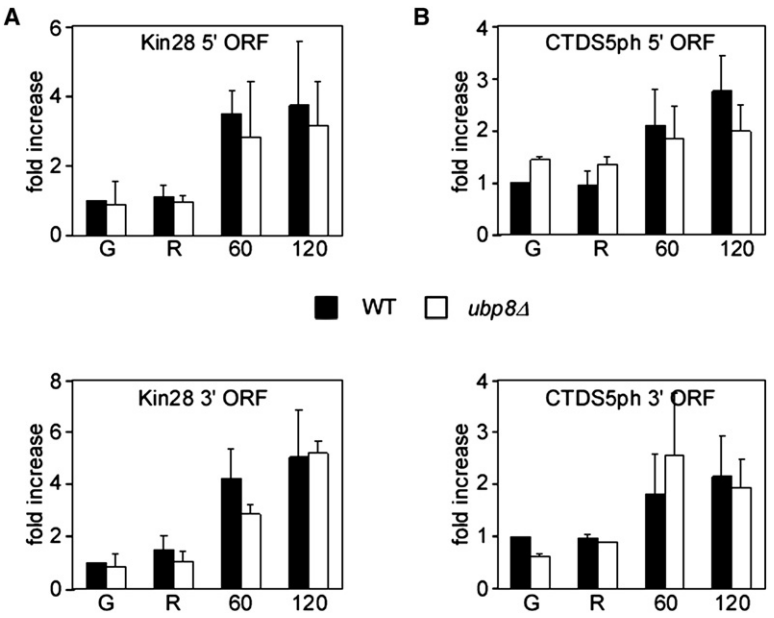

Figure 2. Kin28 Recruitment and Pol II CTDS5ph Are Not Affected in *ubp8Δ*
Recruitment of Kin28 and CTDS5ph in WT and *ubp8Δ* was analyzed as described in Figure 1E. (A) Anti-Kin28 ChIP. (B) Anti-CTDS5ph ChIP.

during a time course in galactose. Interestingly, we detected Kin28 (Figure 2A) and CTDS5ph (Figure 2B) at both ends of the *GAL1* ORF. While CTDS5ph is generally thought to occur at the 5′ end of genes, numerous reports suggest that it exists at the 3′ end as well (Ahn et al., 2004; Boehm et al., 2003; Kizer et al., 2005; Morris et al., 2005). Deletion of *UBP8* had no effect on Kin28 recruitment or kinase activity, as the timing and relative level of signal in WT and *ubp8Δ* are similar in both experiments (Figures 2A and 2B).

Ctk1 Recruitment and CTDS2ph Are Lowered in *ubp8Δ*

Because Set2 recruitment requires CTDS2ph by Ctk1, we speculated that *ubp8Δ* alters Ctk1 recruitment and/or activity. To test this, we performed ChIP using tagged Ctk1. Wild-type Ctk1 recruitment steadily increases at both the 5′ and 3′ ends of the *GAL1* ORF during activation (Figure 3A). In *ubp8Δ*, Ctk1 recruitment is reduced relative to WT throughout the time course. Similarly, Ctk1 levels are lower at the *ADH1* and *PMA1* genes in the absence of Ubp8 (Figure 3D), while overall Pol II levels are unaffected (data not shown). This lowered signal is not due to defective Ctk1 expression in *ubp8Δ* (Figure 3C). The level and timing of CTDS2ph, measured by ChIP, mimics that of Ctk1 recruitment in WT and *ubp8Δ* at *GAL1* (Figure 3B). Thus, deletion of *UBP8* diminishes CTDS2ph at a subset of SAGA-dependent genes, but not overall levels of Pol II. This disconnect between CTDS2ph and overall Pol II occupancy has been observed previously (Ahn et al., 2004; Laribee et al., 2005).

Our data suggest that persistent H2BK123ub1 in *ubp8Δ* blocks Ctk1 recruitment to *GAL1*, resulting in lowered CTDS2ph. Based on this model, one would predict that a loss of H2BK123ub1 in the *ubp8Δ* strain might restore normal Ctk1 binding. To test this theory, we analyzed Ctk1 recruitment in a *bre1Δ* strain, in which H2BK123ub1 is abolished, as well as a *bre1Δ/ubp8Δ* double mutant; we then compared the relative signals for these strains to WT and *ubp8Δ* (Figure 4A). Both *bre1Δ* and the *bre1Δ/ubp8Δ* double mutant displayed normal Ctk1 recruitment in galactose. Similarly, mutation of the ubiquitylation site on H2B (*htb1* K123R) in the *ubp8Δ* strain resulted in normal Ctk1 binding (Figure 4B). Analysis of Pol II recruitment in these strains showed a pattern similar to that of WT (data not shown), indicating that normal Ctk1 recruitment is restored. Thus, loss of H2BK123ub1 suppresses the defect in *ubp8Δ*, suggesting that persistent H2BK123ub1 is the cause of reduced Ctk1 binding.

We next tested whether the effects on CTDS2ph and H3K36me2 in *ubp8Δ* are global by performing western blots on lysates from WT and *ubp8Δ* strains. We detected only modest changes in CTDS2ph and no change in H3K36me2 in *ubp8Δ* (data not shown). We have previously observed that even modest levels of CTDS2ph result in normal H3K36me2 when assayed at the global level (T.X. and B.D.S., unpublished data). From these data, we conclude that Ubp8 controls these modifications at a specific subset of genes.

Elevated H2BK123ub1 in *ubp8Δ* Disrupts an Interaction between Ctk1 and Histones

Because our data suggest that H2BK123ub1 blocks Ctk1 recruitment to *GAL1*, we proposed that persistent H2BK123ub1 disrupts an interaction between Ctk1 and a specific chromatin-associated factor. To test this idea, we purified TAP-tagged Ctk1 and identified copurifying

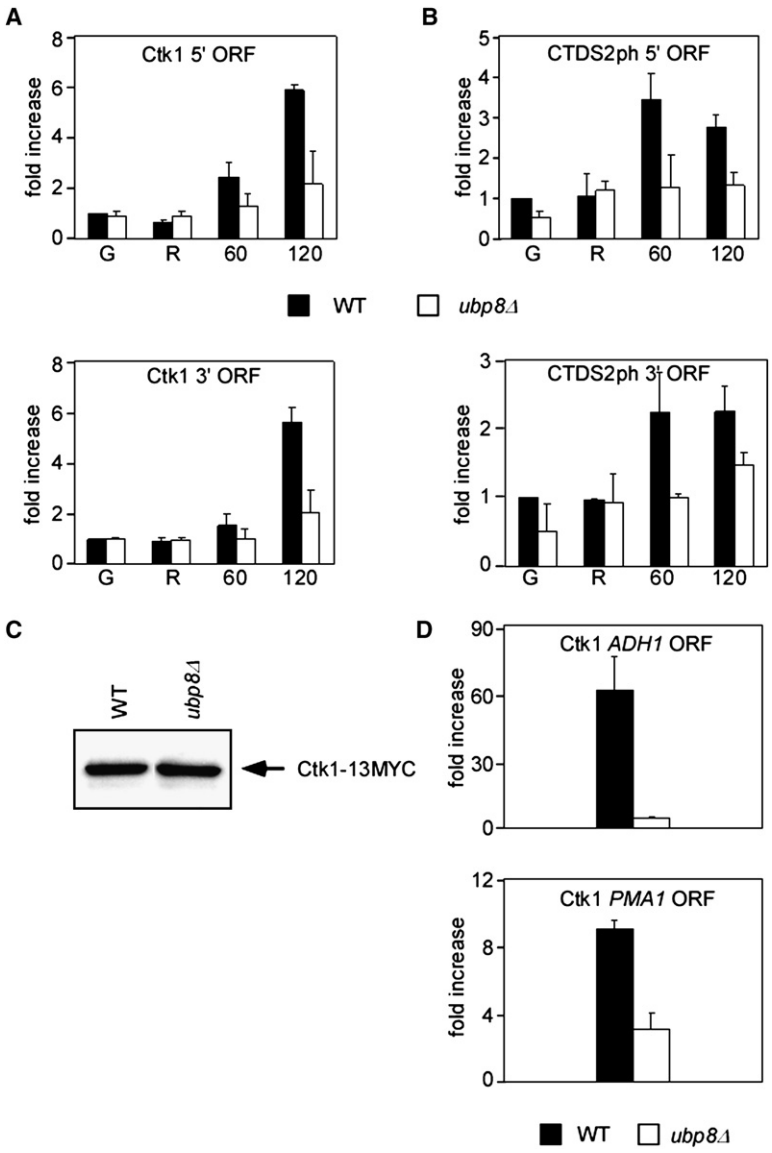

Figure 3. Ctk1 Recruitment and Pol II CTDS2ph Are Decreased in *ubp8Δ*

Ctk1 binding and CTDS2ph were analyzed by ChIP in WT and *ubp8Δ*.

(A) Anti-MYC ChIP in strains expressing Ctk1-13MYC was analyzed as described in Figure 1C.

(B) Anti-CTDS2ph ChIP was analyzed as described in Figure 1E.

(C) Ctk1 stability in WT and *ubp8Δ* backgrounds. Ctk1-13MYC expression from WT and *ubp8Δ* strains was analyzed as described in Figure 1D.

(D) Ctk1 recruitment to *ADH1* and *PMA1*. Anti-MYC ChIP in WT and *ubp8Δ* strains expressing Ctk1-13MYC was analyzed at the *ADH1* and *PMA1* ORFs during growth in glucose. ChIP data represent percent input normalized to IntV, followed by normalization to an isogenic untagged strain (n = 2).

proteins by mass spectrometry. Not surprisingly, both Ctk2 and Ctk3, other components of the CTDK-I complex, were associated with Ctk1. Interestingly, substoichiometric amounts of Hta1, encoded by one of two histone H2A loci, copurified with Ctk1-TAP (data not shown), suggesting that Ctk1 may be recruited to genes in part through an interaction with histones.

To determine whether the CTDK-I complex directly binds histones in vitro, we first purified separate complexes via TAP-tagged Ctk1, Ctk2, and Ctk3, to confirm the identity of each subunit (Figure 4C, left). While we detected histone H2A by the more sensitive mass spectrometry approach, no proteins of that size were visible by silver staining. We then incubated immobilized Ctk1-TAP complex separately with each individual histone, as well as purified nucleosomes. The purified complex interacted directly and independently with histones H2A and H2B (Figure 4C, right). Histones H3 and H4, on the other hand, did not interact with the Ctk1-TAP complex. In the context of the nucleosome, we detected all four histones binding to the complex, suggesting that the CTDK-I complex may interact with whole nucleosomes in the cell.

To analyze interactions between the CTDK-I complex and histones in vivo, we generated strains coexpressing tagged Ctk1 with tagged histones H2A or H2B. We initially examined interaction between Ctk1-3FLAG and H2A-TAP and compared their coimmunoprecipitation (coIP) to the level detected in *ubp8Δ* and *bre1Δ* backgrounds, to determine whether aberrant levels of H2BK123ub1 alter the interaction. Ctk1-3FLAG reciprocally coimmunoprecipitated with H2A-TAP in all three strains (Figure 4D). However, the signal for the coprecipitating protein was significantly reduced in *ubp8Δ* compared to the other two strains. We conclude that persistent H2BK123ub1 in

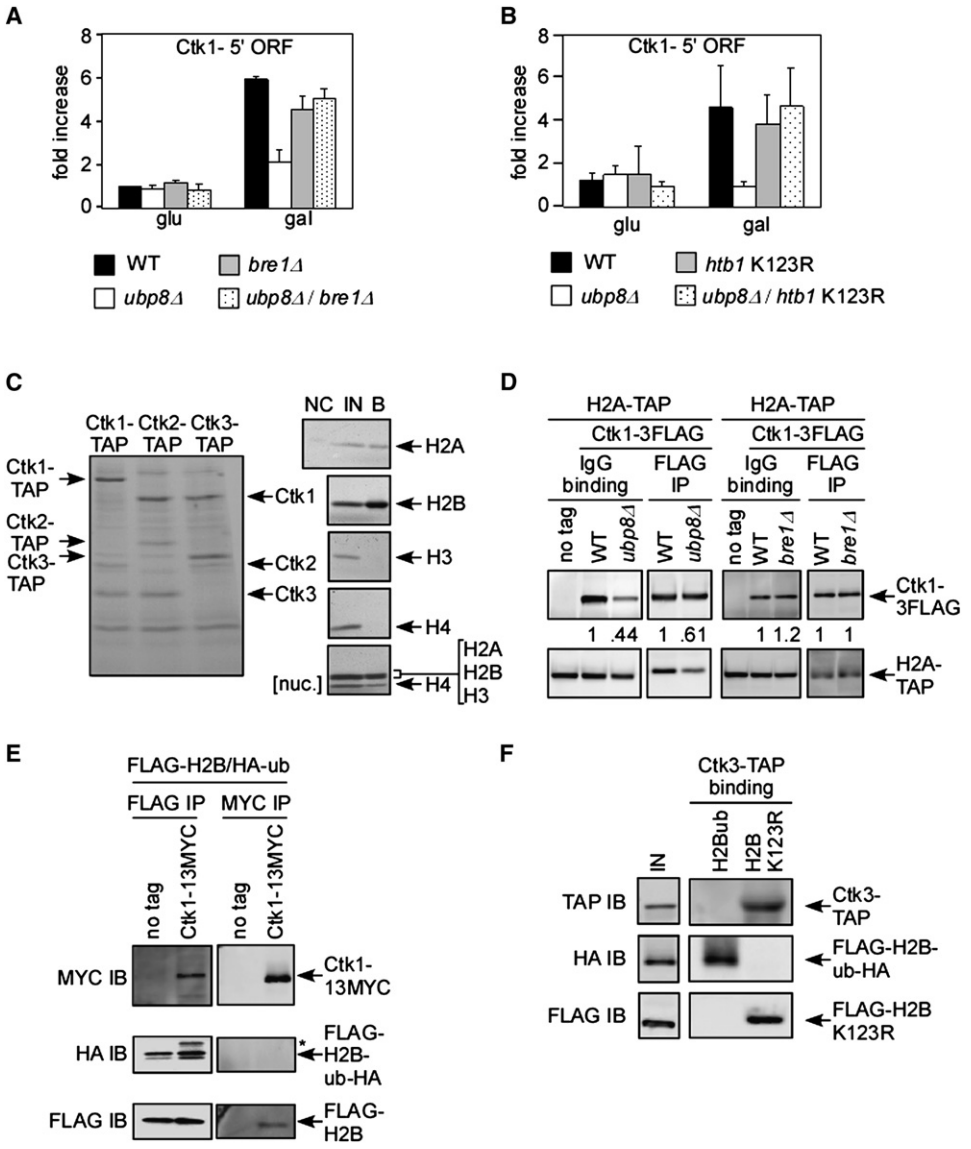

Figure 4. Persistent H2BK123ub1 Disrupts Ctk1 Recruitment

(A) Ctk1 recruitment in ubiquitylation mutants. Ctk1-13MYC recruitment in WT, *ubp8Δ*, *bre1Δ*, and *ubp8Δ/bre1Δ* backgrounds was analyzed by anti-MYC ChIP in glucose and galactose (120 min) as described in Figure 1C.

(B) Ctk1-13MYC recruitment in WT, *ubp8Δ*, *htb1* K123R, and *ubp8Δ/htb1* K123R backgrounds as described in (A).

(C) Direct binding of CTDK-I complex to histones in vitro. (Left) Complexes purified from Ctk1-TAP, Ctk2-TAP, or Ctk3-TAP strains were analyzed by SDS-PAGE and silver staining. Subunits are indicated. (Right) Silver stain of binding reactions. Immobilized Ctk1-TAP complex was incubated with the indicated histone or purified oligonucleosomes (nuc.). Bound proteins (B) were analyzed along with 10% inputs (IN). In addition, beads with no complex bound were incubated with H2A (NC).

(D) Ctk1 binding to histone H2A in vivo. WT, *ubp8Δ*, and *bre1Δ* strains expressing the indicated tagged proteins were incubated with IgG Sepharose or anti-FLAG agarose. Bound proteins were subjected to western blotting with anti-TAP and anti-FLAG HRP antibodies. Signal for the interacting protein was then quantified relative to the purified protein. Quantities for the *ubp8Δ* and *bre1Δ* strains were then normalized to WT, and the resulting values are indicated.

(E) Ctk1 binding to histone H2B in vivo. A *ubp8Δ* strain expressing FLAG-H2B, HA-ub, and either untagged or 13MYC-tagged Ctk1 was immunoprecipitated with anti-FLAG or anti-MYC antibodies. Bound proteins were subjected to western blotting with the indicated antibodies. The asterisk indicates an unidentified background band.

(F) CTDK-I complex binding to histone H2B in vitro. Purified FLAG-H2BK123ub1-HA or FLAG-H2B K123R was immobilized and incubated with purified Ctk3-TAP complex. Bound proteins were subjected to anti-TAP, anti-FLAG, or anti-HA immunoblotting. IN represents 10% input for Ctk3-TAP, and 50% input for FLAG-H2BK123ub1-HA and FLAG-H2B K123R.

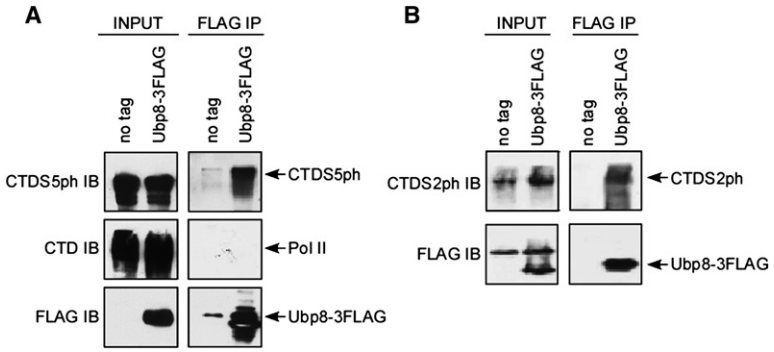

Figure 5. Ubp8 Associates with Phosphorylated Pol II

(A) Ubp8 interacts with CTDS5ph. Anti-FLAG IP of WT strains expressing untagged or 3FLAG-tagged Ubp8. Bound proteins, along with 0.67% inputs, were analyzed by western blotting with the indicated antibodies.

(B) Ubp8 interacts with CTDS2ph. IP of Ubp8-3FLAG was performed as described in (A). Bound proteins, along with 5% inputs, were analyzed by western blotting. Experiments were repeated multiple times with comparable results.

ubp8Δ disrupts the interaction between the CTDK-I complex and histone H2A, thereby reducing Ctk1 recruitment to *GAL1*.

To probe the interaction between Ctk1 and histone H2B in vivo, and to directly visualize binding to H2BK123ub1, we generated a strain expressing Ctk1-13MYC, FLAG-H2B, and HA-ubiquitin (HA-ub). In order to achieve a detectable level of H2BK123ub1, we tagged these proteins in a *ubp8Δ* strain, where bulk levels of H2BK123ub1 are significantly higher than WT (Henry et al., 2003). Tagging ubiquitin allowed us to distinguish H2BK123ub1 from H2B via anti-HA western blot; we have established via comparison with *htb1* K123R that the signal detected by HA western is specific for FLAG-H2BK123ub1-HA (Henry et al., 2003). As expected, FLAG IP purified H2B and H2BK123ub1, which we distinguish by anti-FLAG (H2B) and anti-HA (H2BK123ub1) western blotting (Figure 4E, left panel). Anti-MYC western blotting of this coIP indicates that Ctk1 coprecipitates with the H2B mixture. Reciprocally, Ctk1-13MYC IP and FLAG western blotting confirmed the interaction with nonubiquitylated H2B (Figure 4E, right panel). However, we failed to detect any coprecipitating H2BK123ub1 by anti-HA western, suggesting that Ctk1 interacts specifically with the nonubiquitylated form of H2B.

We further studied the specific interaction between the CTDK-I complex and nonubiquitylated H2B in vitro by separately purifying FLAG-H2BK123ub1-HA and FLAG-H2B K123R. FLAG IP in a FLAG-*htb1* K123R strain purifies nonubiquitylated H2B (Henry et al., 2003). Sequential FLAG and HA IPs in a *ubp8Δ* strain produce purified FLAG-H2BK123ub1-HA. Western blot of the purified histones detects no contamination between the ubiquitylated and nonubiquitylated forms (Figure 4F). To probe the binding of these purified histones to the CTDK-I complex, we incubated immobilized histones with purified Ctk3-TAP complex (Figure 4C, left). Anti-TAP western blotting of the binding reactions revealed an interaction between nonubiquitylated H2B (FLAG-H2B K123R) and Ctk3-TAP (Figure 4F). Strikingly, no interaction was detected between Ctk3-TAP and ubiquitylated H2B (FLAG-H2BK123ub1-HA), indicating that the CTDK-I complex interacts preferentially with nonubiquitylated H2B.

Ubp8 Functions in Gene ORFs

Our data indicate that H2B-dub affects recruitment of elongation factors to the coding regions of genes, suggesting that Ubp8 itself functions in the ORF. As mentioned previously, movement of Rad6/Bre1, Set1, and Set2 into gene ORFs requires an interaction with phosphorylated CTD. We thus tested whether Ubp8 also enters the ORF through an interaction with phosphorylated Pol II. We immunoprecipitated FLAG-tagged Ubp8, and probed for an interaction with unmodified and phosphorylated CTD by western blot. We detected CTDS5ph and CTDS2ph coprecipitating with Ubp8-3FLAG, indicating that Ubp8 interacts with elongating Pol II (Figures 5A and 5B). In contrast, we detected no interaction between Ubp8 and the unmodified CTD (Figure 5A), suggesting that Ubp8 interacts specifically with phosphorylated Pol II. Consistent with these findings, a phosphorylated CTD peptide used as bait in an affinity purification approach isolated several SAGA subunits (Phatnani et al., 2004).

The interaction of Ubp8 with elongating Pol II suggests that Ubp8 may function in gene ORFs during transcriptional elongation. H2BK123ub1 was previously shown to be present in the *GAL1* ORF during activation (Xiao et al., 2005). To determine whether Ubp8 deubiquitylates H2B in the *GAL1* ORF, we performed chromatin double IP (ChDIP) experiments in WT and *ubp8Δ* strains expressing FLAG-H2B and HA-ub (Figure 6A). As was previously observed, H2BK123ub1 is low throughout the *GAL1* gene in WT cells during growth in glucose (Henry et al., 2003; Kao et al., 2004; Xiao et al., 2005). Upon induction, H2BK123ub1 increases throughout the gene at 15 min, but returns to background levels by 30 min. In *ubp8Δ*, H2BK123ub1 levels are high throughout the gene under both repressing and inducing conditions (Figure 6A). This pattern is consistent with our previous observations at the *GAL1* promoter (Henry et al., 2003) and suggests that Ubp8 deubiquitylates H2B throughout the *GAL1* promoter and ORF. We then analyzed levels of H2BK123ub1 at *ADH1* and *PMA1* (Figure 6B). At each region tested, H2BK123ub1 was higher in *ubp8Δ* compared to WT. Similar alterations in H2BK123ub1 in *ubp8Δ* were observed at *ADH1* in a separate study (Shukla et al., 2006). We conclude that Ubp8 cleaves

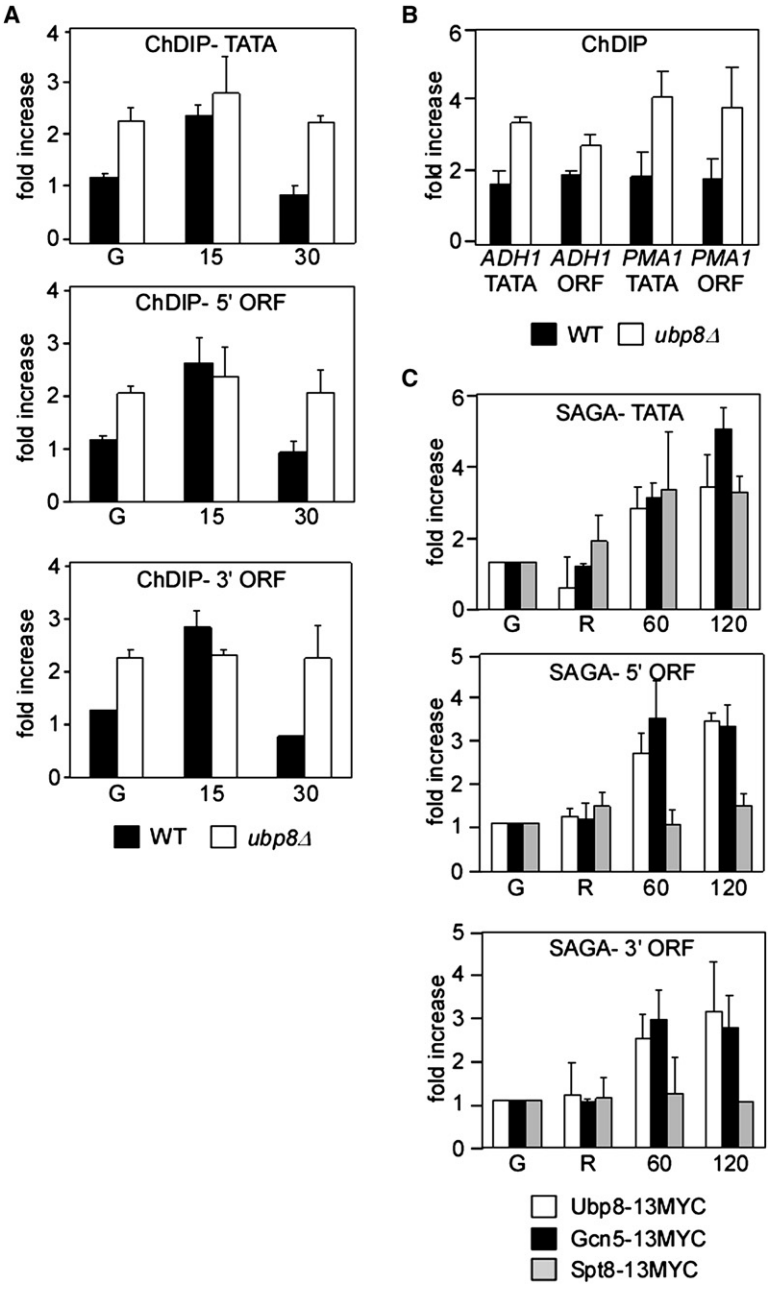

Figure 6. Ubp8 Activity and Recruitment during Transcriptional Elongation

(A) H2BK123ub1 along *GAL1* during activation. WT, *htb1* K123R, and *ubp8Δ* strains expressing FLAG-H2B and HA-ub were collected in glucose and after 15 or 30 min of growth in galactose. Samples were subjected to anti-FLAG and anti-HA ChDIP. Data represent percent input normalized to a telomeric region on chromosome VI. Signals were then normalized to the *htb1* K123R strain (n = 3).

(B) H2BK123ub1 at constitutive genes. WT, *htb1* K123R, and *ubp8Δ* strains were collected during growth in glucose and subjected to ChDIP as described in (A). H2BK123ub1 levels were analyzed at the promoters and ORFs of *ADH1* and *PMA1*.

(C) Localization of SAGA components along *GAL1*. Ubp8-13MYC, Gcn5-13MYC, and Spt8-13MYC recruitment along *GAL1* in a WT strain was analyzed by anti-MYC ChIP as described in Figure 1C.

H2BK123ub1 during transcriptional elongation at a subset of SAGA-dependent genes.

Ubp8 Functions in Elongation as Part of a SAGA-Related Complex

Ubp8 is recruited to the *GAL1* promoter as part of SAGA, and mutations that prevent Ubp8 incorporation into the complex result in a global increase in H2BK123ub1 (Henry et al., 2003; Ingvarsdottir et al., 2005; Lee et al., 2005). We examined whether Ubp8 enters the coding regions of genes as a component of SAGA, a portion of SAGA, or the DUB module alone. Ubp8, Gcn5, and Spt8 were tagged with 13MYC for ChIP analysis. As mentioned previously, Spt8 is absent from SALSA, so analysis of Spt8 allows us to distinguish SALSA from SAGA. During activation, we detected Ubp8, Gcn5, and Spt8 at the *GAL1* promoter (Figure 6C), as was seen previously (Bryant and Ptashne, 2003; Henry et al., 2003; Kao et al., 2004). While Ubp8 and Gcn5 were detected at both ends of the *GAL1* ORF, Spt8 localized exclusively at the promoter (Figure 6C). These results suggest that Ubp8 and Gcn5 have a role in *GAL1* transcript elongation. Conversely, Spt8 remains at the promoter, suggesting that SALSA or another SAGA-related complex enters the ORF.

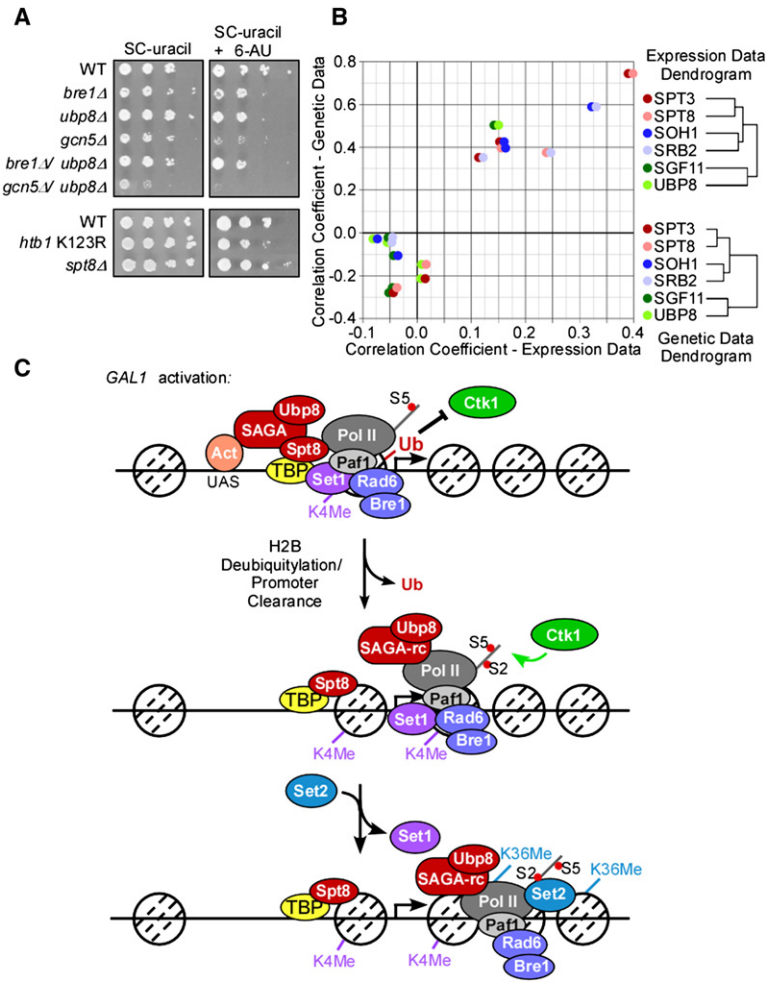

Figure 7. Analysis of SAGA Components in Transcriptional Elongation

(A) 6-AU assay of SAGA mutants. *URA3*$^+$ strains with the indicated gene deletions were plated onto SC-uracil containing no drug or 100 μg/ml of 6-AU as 10-fold serial dilutions. Similar results were obtained on plates containing 50 μg/ml of 6-AU (data not shown).

(B) Gene expression and genetic profiling of SAGA. A plot of pairwise correlation coefficients generated for Spt3, Spt8, Sgf11, Ubp8, Soh1, and Srb2 comparing genetic and gene expression profiling.

(C) A model for Ubp8 function in *GAL1* activation. Schematic representation of the *GAL1* gene showing the recruitment of transcription factors during activation. Illustration is not meant to indicate physical interactions or ordered recruitment. Only relevant components are depicted. See text for details.

To further probe the role of these subunits in transcriptional elongation, we performed a 6-azauracil (6-AU) plate assay. 6-AU reduces intracellular nucleotide pools, resulting in slow growth that is exacerbated by mutation of factors important for elongation. We tested *ubp8Δ*, *gcn5Δ*, and *spt8Δ* strains, as well as *bre1Δ/ubp8Δ* and *gcn5Δ/ubp8Δ* double mutants. For controls, we used *bre1Δ* and *htb1* K123R strains, which were previously shown to be 6-AU sensitive (Xiao et al., 2005). *ubp8Δ* and *gcn5Δ* are both sensitive to 6-AU, with *gcn5Δ* having a stronger sensitivity than *ubp8Δ* (Figure 7A). The *ubp8Δ* strain has a similar phenotype to *bre1Δ*, and combination of the two deletions does not increase sensitivity. In contrast, the *gcn5Δ/ubp8Δ* double mutant has a slow-growth phenotype without drug and appears unable to grow on 6-AU. Interestingly, *spt8Δ* is not 6-AU sensitive and may in fact be slightly resistant to the drug. This finding further suggests that Spt8 either has no role, or an inhibitory role, in transcriptional elongation.

To further assess the roles of the different modules within SAGA and SALSA in elongation, we examined their relationships to one another by E-MAP (epistatic *m*iniarray *p*rofile) analysis. For every pair of genes in an E-MAP, correlation coefficients can be calculated and strains can be organized by two-dimensional hierarchical clustering according to the similarities of their genetic interaction profiles. Clustering of all the genes in the E-MAP reveals that subunits within several SAGA modules are most similar to each other, as was previously described (Ingvarsdottir et al., 2005). Specifically, Spt3 and Spt8 are most similar to each other and are very different from Ubp8 and Sgf11, which in turn are highly correlated with one another (Figure 7B). Interestingly, two genes (*SOH1* and *SRB2*) encoding components of the transcriptional initiation complex Mediator highly correlated with each other and with Spt3 and Spt8, but not with Ubp8 and Sgf11. Gene expression profiles generated on a large subset of the gene deletions analyzed in the E-MAP were subjected to a similar clustering analysis. An almost identical pattern was observed in that the Spt3/Spt8 module behaved more similarly to the Mediator subunits (Soh1/Srb2) than to Ubp8/Sgf11 (Figure 7B). These patterns are highly suggestive of the Spt3/Spt8 module, and not the Ubp8/Sgf11 module, functioning with Mediator during transcriptional initiation and are consistent with

Ubp8/Sgf11 playing a different role in transcription, potentially during elongation.

DISCUSSION

In this study, we investigated the mechanism underlying sequential H2B ubiquitylation/deubiquitylation during transcription. We show that deletion of *UBP8* leads to reduced Ctk1 recruitment and CTDS2ph at a subset of SAGA-dependent genes. Our data suggest that the reduction in Ctk1 recruitment is due to persistent H2BK123ub1, which disrupts an interaction between Ctk1 and histones H2A/H2B. We also provide evidence that Ubp8 carries out these functions as part of a SAGA-related complex that lacks Spt8. We propose that SAGA is recruited to the *GAL1* promoter during initiation, while SALSA or another form of the complex is recruited into the ORF with Pol II.

A New Function for H2B Ubiquitylation/Deubiquitylation in Transcription

Previous models for H2BK123ub1 in transcription suggest that it functions as either a "wedge" or a "bridge" (Henry and Berger, 2002). The wedge model proposes that the large ubiquitin moiety disrupts chromatin structure in a somewhat nonspecific manner. The bridge model, on the other hand, proposes that H2BK123ub1 provides an altered binding surface for the recruitment of downstream factors.

Our findings suggest an additional function for H2BK123ub1 in transcription at SAGA-dependent genes, where H2BK123ub1 acts as a "barrier" to block interaction of Ctk1 with histones, thereby reducing CTDS2ph and subsequent H3K36me. We hypothesize that this mechanism functions as a checkpoint, ensuring the proper timing of phosphorylation and methylation during activation. In this sense, H2BK123ub1 both promotes and inhibits the progression of transcription—it is required for H3K4me3 by Set1 (bridge), but at the same time its physical presence partially inhibits recruitment of Set2 and H3K36me2 (barrier). H2B-dub by Ubp8 removes the barrier at the appropriate time, thereby allowing CTDS2ph and the subsequent shift from H3K4me3 to H3K36me2.

Although H2B is the only known target of Ubp8, it is possible that Ubp8 deubiquitylates other proteins during transcription as well. Importantly, the Ctk1 recruitment defect we observed in *ubp8Δ* was suppressed by deletion of *BRE1* or mutation of H2BK123 (Figures 4A and 4B). Thus, while we do not rule out the possibility that Ubp8 deubiquitylates other proteins, our data strongly suggest that the partial defect in Ctk1 recruitment at *GAL1* is due to persistent H2BK123ub1 in *ubp8Δ*.

Ctk1 recruitment and CTDS2ph are reduced, but not completely abrogated, in *ubp8Δ* at every gene tested (Figure 3). The presence of low-level CTDS2ph would likely allow some recruitment of downstream elongation factors, explaining why milder phenotypes are observed in *ubp8Δ* compared to a *ctk1Δ* strain.

The fact that Ctk1 recruitment is not completely lost in *ubp8Δ* suggests that the kinase interacts with multiple factors at *GAL1*. We speculate that the CTDK-I complex may possess one surface for interaction with histones (Figure 4), and a different domain that interacts with Pol II. Blocking Ctk1 interaction with histones may destabilize its association, without completely abolishing it, because it might still contact other factors at the gene.

Simultaneous interaction with histones H2A/H2B and Pol II might also explain the specific recruitment of Ctk1 to coding regions, as it would only stably bind where Pol II is present. Further, the presence or absence of modifications on H2A/H2B may also dictate where Ctk1 is recruited, because different modifications occur in transcriptionally active versus silent regions, and in gene promoters versus coding regions. Thus, while our data strongly suggest that Ctk1 recruitment to SAGA-dependent genes is affected by its ability to interact with histones, further study is required to fully understand this process.

We propose that Ctk1 recruitment to SAGA-dependent genes relies, in part, on the activity of Ubp8. However, this begs the question of how Ctk1 is recruited to SAGA-independent genes, where Ubp8 is not present. One possibility is that H2BK123ub1 remains low at SAGA-independent genes; however, current data suggest that this is unlikely (Briggs et al., 2002; Dover et al., 2002; Pokholok et al., 2005; Sun and Allis, 2002). Another possibility is that a second H2B deubiquitylase, such as Ubp10, targets H2BK123ub1 at SAGA-independent genes. Ubp10 was shown to regulate genes throughout the genome, including some that are not regulated by Ubp8 (Gardner et al., 2005; Orlandi et al., 2004). Further, there may be additional, presently unknown proteases in yeast that remove ubiquitin from H2B (Amerik et al., 2000).

Consistent with numerous studies, our ChIP data suggest that CTDS5ph and CTDS2ph coexist along the length of the *GAL1* gene (Boehm et al., 2003; Kim et al., 2004; Kizer et al., 2005; Morris et al., 2005; Phatnani and Greenleaf, 2006). There is an apparent disconnect between the levels of CTDS2ph and Set2 at *GAL1*, because Set2 levels are concentrated at the 3′ end of the gene (Kizer et al., 2005; Krogan et al., 2003b; Schaft et al., 2003). However, Set2 recruitment is regulated by factors beside CTDS2ph (Krogan et al., 2003b); how these factors collaborate with the CTD to establish specific Set2 patterns is an important area for future study.

Deletion of *UBP8* results in increased H2BK123ub1 along the *GAL1* gene even in repressed conditions, when SAGA is thought to be absent (Figure 6B; Henry et al., 2003). We speculate that Ubp8, or the entire DUB module of SAGA, may have an additional nontargeted role to maintain constitutively low H2BK123ub1. However, there is currently no direct evidence for a second targeting mechanism for Ubp8 to genes, so it remains to be seen how Ubp8 regulates H2BK123ub1 at *GAL1* in its repressed state.

A Function for a SAGA-Related Complex in Transcriptional Elongation

In this study, we identify a role for a SAGA-related complex in transcriptional elongation. Combined deletion of *UBP8* and *GCN5* causes synergistic elongation defects (Figure 7A), suggesting that the roles of the HAT and DUB modules in elongation are functionally distinct. Consistent with these findings, a *ubp8Δ/gcn5Δ* double mutant exhibits greater transcription defects than either single mutation (Henry et al., 2003). Our data strongly suggest that Spt8 is absent from the complex that enters the *GAL1* ORF. However, further study is required to determine whether this complex is SALSA or another SAGA-related complex lacking Spt8.

Recently, Gcn5 and other SAGA subunits were shown to enter gene ORFs during activation (Govind et al., 2007). Movement of Gcn5 into the ORF requires CTDS5ph, which supports our observation that Ubp8 interacts specifically with phosphorylated Pol II (Figure 5). Interestingly, C-terminally tagged Spt7 was reported to enter the *GAL1* ORF, suggesting that at least some of the complex that enters the coding region contains full-length Spt7 (Govind et al., 2007). Thus, it is possible that the elongation-specific form of the complex is not SALSA but another SAGA-related complex that possesses full-length Spt7 and lacks Spt8.

Deletion of *UBP8* causes only mild effects on overall *GAL1* transcript levels (Figure S1; Henry et al., 2003). It is often the case that histone modification regulatory mechanisms have slight effects, due to partial redundancy between modifications (Henry et al., 2003; Kao et al., 2004). Nonetheless, we observed that the defect in *ubp8Δ* was more pronounced at the 5′ end of the gene than at the 3′ end, suggesting that deletion of *UBP8* affects the quality and/or stability of *GAL1* RNA.

H3K36me2 was recently shown to inhibit cryptic initiation at certain genes (Carrozza et al., 2005; Joshi and Struhl, 2005; Keogh et al., 2005). Because deletion of *UBP8* results in reduced H3K36me2, we hypothesized that Ubp8 may also function in this pathway. We show here that *ubp8Δ* fails to trigger internal initiation at genes with known cryptic start sites, such as *FLO8* and *STE11* (Figure S1B; data not shown). However, our data suggest that Ubp8 regulates H3K36me at a subset of SAGA-dependent genes, and there is currently no evidence to suggest that *FLO8* or *STE11* belong to this subset. Further, we were unable to detect cryptic start sites in any of the Ubp8-dependent genes we tested (Figure S1C; data not shown). Thus, while it is reported that all actively transcribing genes possess H3K36me (Pokholok et al., 2005), not all genes possess cryptic start sites within their ORFs (Figure S1C; data not shown; V. Cheung and F. Winston, personal communication). It is therefore highly likely that H3K36me has a role in transcription besides suppressing internal initiation, because this function would not be required at genes with no internal start sites. Because we detect differences in RNA levels at the 5′ versus 3′ end of *GAL1* in the *ubp8Δ* strain (Figure S1A), we propose that H2B-dub regulates the ORF and RNA quality at *GAL1*. However, the mechanistic basis of this regulation remains unclear.

Our data suggest that deletion of *UBP8* leads to reduced Ctk1 recruitment and CTDS2ph at a subset of SAGA-dependent genes (Figure 3). CTDS2ph by Ctk1 is required for recruitment of the 3′ mRNA processing machinery (Ahn et al., 2004; Komarnitsky et al., 2000; Licatalosi et al., 2002) and for proper polyadenylation of mRNA transcripts. Thus, it is possible that H2B-dub promotes RNA stability by permitting CTDS2ph by Ctk1, leading to the recruitment of these RNA processing factors. Further study is required to determine the extent of the downstream effects of lowered Ctk1 recruitment to SAGA-dependent genes in *ubp8Δ*.

A Model for the Role of Ubp8 in *GAL1* Activation

Based on our data and previous reports, we propose a model for *GAL1* activation shown in Figure 7C. Upon induction in galactose, SAGA and Rad6/Bre1 are recruited to the promoter (Bryant and Ptashne, 2003; Henry et al., 2003; Kao et al., 2004). Kin28 is recruited as well and phosphorylates the Pol II CTD at S5. CTDS5ph triggers loading of Rad6/Bre1 and Set1 onto Paf1 for subsequent H2BK123ub1 and H3K4me3 (Krogan et al., 2003a; Ng et al., 2003; Xiao et al., 2005). Meanwhile, the presence of H2BK123ub1 physically blocks the interaction between Ctk1 and histones H2A and H2B, destabilizing its recruitment to the gene and preventing CTDS2ph.

The next critical step is H2B-dub by Ubp8. Once ubiquitin has been removed from H2B, the CTDK-I complex is recruited, in part, through its interaction with histones H2A and H2B, and phosphorylates the CTD at S2. CTDS2ph leads to recruitment of Set2 (Krogan et al., 2003b; Xiao et al., 2003), and as Set1 dissociates from the transcription machinery there is a shift from H3K4me3 to H3K36me2 as Pol II moves toward the 3′ end of the gene.

SAGA is recruited to the *GAL1* promoter at an early point in activation, where Spt3 and Spt8 recruit TBP to the TATA box (Dudley et al., 1999; Sterner et al., 1999). We propose that the complex is later loaded onto Pol II for movement down the gene; however, the complex appears to lack Spt8, as this subunit remains exclusively at the promoter. Loading of the complex onto Pol II appears to occur sometime after CTDS5ph (Figure 5; Govind et al., 2007). Once loaded onto Pol II, we propose that the complex transits the *GAL1* gene and that both its HAT and DUB activity play important roles in elongation (Figures 7A and 7C).

In summary, we provide evidence for a function of H2BK123ub1 as a barrier to downstream elongation factor recruitment during transcription of SAGA-dependent genes. In addition, we uncover a role for H2B-dub in transcriptional elongation and provide evidence that the elongation-specific roles of Ubp8 and Gcn5 are carried out within a SAGA-related complex lacking Spt8. Future studies will reveal the mechanisms by which Ubp8's activity

and loading onto Pol II are regulated and will uncover the importance of the activity of the HAT and DUB modules in elongation.

EXPERIMENTAL PROCEDURES

S. cerevisiae Strains

Yeast strains are listed in Table S1. Gene deletions and epitope tagging were performed as described previously (Longtine et al., 1998).

GAL1 Induction

Yeast strains were grown in YP-2% glucose to mid-log phase. Cells were harvested, washed, and resuspended in YP-2% raffinose. Following 2 hr of growth, galactose was added to a final concentration of 2%.

ChIP

ChIP and ChDIP were performed as described previously (Burke et al., 2000; Henry et al., 2003). Extracts were precleared prior to incubation with antibody. DNA was purified using a Qiaquick PCR purification kit (QIAGEN) following the manufacturer's protocol. Purified DNA and 10% inputs were analyzed by qPCR. For ChDIP, inputs consisted of 10% of eluate from FLAG ChIP. qPCR primers are listed in Table S2. Antibodies are listed in Table S3.

Purification of Ctk1-TAP Complex for Mass Spectrometry and In Vitro Binding Experiments

Proteins were tagged, purified, and prepared for mass spectrometry and binding as previously described (Krogan et al., 2002b).

In Vitro Binding Experiments

Ctk1-TAP complex was dialyzed against calmodulin binding buffer (CBB; Krogan et al., 2002b) and bound to calmodulin sepharose (Amersham). Equal amounts of immobilized complex or blank beads were incubated with individual calf thymus histones (Roche) or purified oligonucleosomes. Bound proteins were washed in CBB and eluted in calmodulin elution buffer (Krogan et al., 2002b).

FLAG-H2B and FLAG-H2B K123R were purified as previously described (Henry et al., 2003). To isolate FLAG-H2B-ub-HA, total purified FLAG-H2B was immunoprecipitated with anti-HA antibody (Roche). Equal concentrations of immobilized histones were incubated with purified Ctk3-TAP in extraction buffer (EB; Ingvarsdottir et al., 2005) containing 100 mM NaCl (100 mM EB), washed in 100 mM EB, and eluted by boiling in SDS-PAGE sample buffer.

Protein Purification

Details of protein extraction, immunoprecipitation, and western blotting are presented in the Supplemental Data. Briefly, equal concentrations of protein extracts were incubated with the appropriate immobilized antibody. Bound proteins were washed in the binding buffer and eluted by boiling in SDS-PAGE sample buffer. Antibodies are listed in Table S3.

6-AU Plate Assay

Strains expressing *URA3* were grown in SC-uracil and plated as 10-fold serial dilutions onto SC-uracil plates containing no drug, 50 μg/ml of 6-AU, or 100 μg/ml of 6-AU. Images were taken after 2 days of growth at 30°C.

Genetic Interaction and Gene Expression Profiling

Genetic interaction profiles using E-MAP technology (Schuldiner et al., 2005) and effects of deletions on gene expression were identified as previously described (Keogh et al., 2005).

RNA Analyses

Details for RNA purification, RT-PCR, and northern blotting are described in the Supplemental Data.

Supplemental Data

Supplemental Data include one figure, three tables, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at http://www.molecule.org/cgi/content/full/27/2/275/DC1/.

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