ABSTRACT

Transcription by RNA polymerase II (RNAPII) is accompanied by a conserved pattern of histone modifications that plays important roles in regulating gene expression. The establishment of this pattern requires phosphorylation of both Rpb1 (the largest RNAPII subunit) and the elongation factor Spt5 on their respective C-terminal domains (CTDs). Here we interrogated the roles of individual Rpb1 and Spt5 CTD phospho-sites in directing co-transcriptional histone modifications in the fission yeast Schizosaccharomyces pombe. Steady-state levels of methylation at histone H3 lysines 4 (H3K4me) and 36 (H3K36me) were sensitive to multiple mutations of the Rpb1 CTD repeat motif (Y1S2P3T4S5P6S7). Ablation of the Spt5 CTD phospho-site Thr1 reduced H3K4me levels but had minimal effects on H3K36me. Nonetheless, Spt5 CTD mutations potentiated the effects of Rpb1 CTD mutations on H3K36me, suggesting overlapping functions. Phosphorylation of Rpb1 Ser2 by the Cdk12 orthologue Lsk1 positively regulated H3K36me but negatively regulated H3K4me. H3K36me and histone H2B monoubiquitylation required Rpb1 Ser5 but were maintained upon inactivation of Mcs6/Cdk7, the major kinase for Rpb1 Ser5 in vivo, implicating another Ser5 kinase in these regulatory pathways. Our results elaborate the CTD ‘code’ for co-transcriptional histone modifications.

INTRODUCTION

The RNA polymerase II (RNAPII) elongation complex coordinates transcription with key co-transcriptional events such as mRNA processing and histone post-translational modification. Association of processing factors and regulatory partners with the RNAPII elongation complex generally depends upon Rpb1, the RNAPII large subunit, and/or Spt5. Rpb1 and Spt5 have distinctive C-terminal domains (CTDs) comprising multiple repeats of a short motif; their CTDs interact with multiple regulators and are critical for assembly of a functional elongation complex (1,2). Whereas the canonical heptapeptide Rpb1 CTD motif (Y1S2P3T4S5P6S7) is conserved across diverse eukaryotic species, the number of repeats in the array increases with progression up the evolutionary scale. In contrast, the Spt5 CTD repeat array is variable, between taxa and within the Spt5 CTD array of a given species. The consensus repeated motif in human Spt5 is TP(M/L)YG(S/R)Q (3,4). The fission yeast Schizosaccharomyces pombe Spt5 contains a relatively homogeneous CTD consisting of 19 tandem copies of consensus TPAWNSGSK. The consensus repeat in budding yeast Spt5 (referred to as the CTR) is highly divergent (SAWGGQ) (5–7). Multiple components of the RNAPII elongation complex can bind to both CTD domains, and genetic analysis indicates that the two CTDs have overlapping functions in vivo (1,2,5,8–10).

The Rpb1 and Spt5 CTDs direct co-transcriptional histone modifications, which are present in a stereotyped pattern within the coding regions of transcribed genes. Signature features of this program are a 5′ peak of trimethyl H3K4 and a 3′ peak of trimethyl H3K36. Mono-ubiquitylated histone H2B (H2Bub1), methylated H3K79 and dimethyl forms of both H3K4 and H3K36 are all found broadly distributed across coding regions. These modifications have roles in nucleosome dynamics, mRNA processing and DNA repair (11–16). How the activities of the relevant histone-modifying enzymes are coordinated with the RNAPII elongation complex to generate this pattern is an important unresolved question (17).

Underlying the program of co-transcriptional histone modifications is an equally complex pattern of phosphorylation on the Rpb1 and Spt5 CTDs, which regulates their
interactions with elongation factors. All five of the potential phosphoacceptor sites within the Rpb1 CTD motif are phosphorylated at various stages of the transcription cycle (18). Most phospho-specific interactions with the Rpb1 CTD studied to date involve the phospho-Ser2 and phospho-Ser5 isoforms (Rpb1 S2-P and Rpb1 S5-P). Rpb1 S5-P and Rpb1 S2-P show characteristic distribution patterns within RNAPII transcription units: Rpb1 S5-P is preferentially enriched near promoters, whereas Rpb1 S2-P peaks toward gene 3′ ends. Numerous biochemical and structural studies of phospho-CTD interaction domains recognizing Rpb1 S2-P and Rpb1 S5-P have reinforced the notion that Rpb1 S5-P acts early in elongation, whereas Rpb1 S2-P acts later. For example, mRNA capping enzymes, which act on nascent mRNA 5′ ends, are directly engaged and allosterically activated by Rpb1 S5-P (8,19). On the other hand, factors involved in mRNA 3′ end cleavage and polyadenylation directly bind to Rpb1 S2-P (2,20).

The Spt5 CTD is typically phosphorylated on threonine of the repeat sequence (the variant Spt5 CTD in budding yeast is phosphorylated on serine) (4,6,7,21,22). Functional studies indicate that this modification (Spt5 T1-P) positively influences elongation (4,5,7). Fission yeast capping enzymes bind to the unphosphorylated Spt5 CTD; this interaction is antagonized by threonine phosphorylation (8,9).

Rpb1 S5-P, Rpb1 S2-P and Spt5 T1-P have all been implicated in co-transcriptional histone modification. Biochemical experiments have demonstrated direct interactions between the Set1C/COMPASS H3K4 methyltransferase complex and Rpb1 S5-P, whereas the H3K36 methyltransferase Set2 interacts with Rpb1 CTD peptides phosphorylated on both Ser2 and Ser5 (23–25). The PAF complex, a multi-functional elongation factor that promotes H2Bub1, H3K4me and H3K36me, interacts with diposphorylated Rpb1 S2-P/S5-P peptides as well as with Spt5 T1-P. These interactions involve the PAF subunits Ctr9 and Cdc73 (26,27). Rtf1, a protein that is functionally related to PAF and that is required for H2Bub1 and H3K4me, preferentially recognizes Spt5 T1-P (3,27–29). The human TAF complex, a multi-functional elongation factor that promotes H2Bub1, preferentially associates with Rpb1 S2-P in vitro (30).

The in vivo approach to validate these interactions has been largely limited to genetic or chemical genetic inactivation of CTD kinases that target multiple sites. Cdk7, a major kinase for Ser5 and Ser7 on the Rpb1 CTD, promotes H2Bub1 and H3K36me in human cells (31,32). A temperature-sensitive variant of the *Saccharomyces cerevisiae* Cdk7 orthologue Kin28 also affects H2Bub1, whereas chemical genetic inhibition of the *S. pombe* orthologue Mcs6 only affects H3K4me (22,33). Cdk9, which phosphorylates the Spt5 CTD and multiple sites on the Rpb1 CTD, is implicated in H2Bub1, H3K4me and H3K36me (22,34–39). The *S. cerevisiae* Cdk12 orthologue Ctk1, a major kinase for Rpb1 Ser2, positively regulates H3K36me and negatively regulates H3K4me (40–42). H3K36me depends on Cdk12 in the nematode *Caenorhabditis elegans* as well (43). While these studies have highlighted the importance of some combination of Rpb1 S5-P, Rpb1 S2-P and Spt5 T1-P for co-transcriptional histone modifications, they do not address the contributions of individual sites and how the sites functionally interact. The potential roles of other Rpb1 CTD phosphorylation sites in establishing the co-transcriptional histone modification pattern have not been assessed.

A more detailed understanding of the roles of individual residues in the Rpb1 and Spt5 CTD repeats is beginning to emerge from mutagenesis studies conducted in a variety of model systems (5,44–46). Here we systematically profile multiple co-transcriptional histone modifications across a series of Rpb1 and Spt5 CTD mutants, as well as in mutants affecting CTD kinases. Our findings provide novel insights into the mechanisms linking the histone and CTD modification programs.

**MATERIALS AND METHODS**

**pombe strains and media**

Strains harboring mutations at the *rpb1*+ and *spt5*+ loci, as well as analogue-sensitive alleles of *mcs6*+, *cdk9*+ and *lsk1*+, have been described previously (44,47,48). The *spt5-CTD-7 rpb1-S7A, spt5-T1A rpb1-S7A, cdk9*+ *lsk1*+ and *mcs6*+ *rpb1* strains were generated using genetic crosses and tetrad analysis (49). The relevant allele combinations were confirmed using diagnostic polymerase chain reaction (PCR) and western blotting. The ‘WT’ (wild-type) strain used in Figures 2–4, Supplementary Figures S1, S2 and S4 is strain JS78 and has been described previously (22). The *cdk9-T212A, set1*Δ and *hfb1-K119R* strains have been described previously (22,50). The *set1*Δ and *set9*Δ strains were constructed by replacement of the set2*+ or set9*+ coding regions with a hygromycin resistance cassette as described previously (51). The *lsk1*α allele was amplified by PCR and introduced at its native locus in the *rpb1-CTD-14* or the *rpb1-S2A* strain to create the combined *lsk1*α *rpb1* mutants. The *lsk1*Δ strain was generously provided by Jim Karagiannis (52).

Liquid cultures were grown at 30°C using standard YES media (yeast extract 5 g/l, D-glucose 30 g/l, supplemented with 250 mg/l each of histidine, leucine, adenine and uracil). 3-MB-PP1 (purchased from Toronto Research Chemicals) was dissolved in dimethyl sulfoxide (DMSO) at a final concentration of 50 mM. For inhibition of analogue sensitive alleles, cells were grown to early log phase (OD_{600} 0.2) and treated with the indicated concentration of 3-MB-PP1 for 3 h. Mycofenolic acid (MPA; purchased from Bioshop Inc.) was dissolved in DMSO and added to solid YES media at a concentration of 25 μg/ml.

**Immunoblotting**

Preparation of *S. pombe* whole-cell extracts, SDS-PAGE, and immunoblotting were performed as previously described (22). The following commercial antibodies were used: H2Bub1 (Active Motif #39623), H3K4me1 (Abcam #ab8895), H3K4me2 (Abcam #ab32356), H3K4me3 (Abcam #ab8580), H3K36me3 (Abcam #ab9050), histone H3 (Abcam #ab1791), Rpb1 (8WG16; Covance #MMS-126R-200), Rpb1 S2-P (clone 3E10; Millipore #04–1571), Rpb1 S5-P (clone 3E8; Millipore #04–1572). Antibodies against *S. pombe* Spt5 and Spt5 T1-P were described previously (22).
Chromatin immunoprecipitation (ChIP)

ChIP was carried out as previously described (28). The Spt5 antibody recognizing the CTD was used at a concentration of 5 μg/ml of extract. A strain lacking the entire CTD (spt5ΔCTD) was used as a negative control.

RESULTS

Comprehensive analysis of the roles of Rpb1 CTD phosphorylation sites in co-transcriptional histone modification

Our analysis employed a series of Rpb1 mutants in the fission yeast S. pombe in which a truncated but fully functional CTD (consisting of 14 consensus heptad repeats and the four non-consensus ‘rump’ repeats proximal to the body of Rpb1) was replaced with CTD variants harboring single amino acid substitutions in all or some of the repeats (44). To assess the role of the essential Ser5 position, we used either strains in which varying combinations of Ser5-Ala (SSA) repeats and wild-type Ser5 repeats were present, or strains in which lethality of complete S5A substitution of all consensus heptads was bypassed via Rpb1 fusion to the mammalian mRNA capping enzyme MCE1 (44,47). Steady-state levels of H2Bub1, H3K4me1, H3K4me2, H3K4me3 and H3K36me3 were reduced in the (S5)7(S5A)7 strain in which the CTD contains seven Ser5 heptads. Apparently, there is a threshold level of Ser5 content that can sustain H3K4me and H3K36me3 modifications. By contrast, H2Bub1 was only reduced in the rpb1-CTD-SSA-MCE1 strain, suggesting that this modification has a less stringent requirement for Rpb1 Ser5 content.

We observed a unique effect of Rpb1 Ser2 on H3K36 methylation, insofar as H3K36me3 levels, but not those of any other modification tested, were reduced in the S2A mutant strain (Figure 1A). Levels of H3K4me3 and H3K36me3 were also reduced, albeit less strongly, in Y1F, T4A and S7A mutant strains. The decrement in H3K4me3 levels observed in the S7A strain was smaller than that observed in the S2A-S7A double mutant, arguing that Ser2 and Ser7 have overlapping roles in positively regulating this modification. Thus, co-transcriptional methylation at H3K4 and H3K36 is influenced by multiple Rpb1 CTD phosphorylation sites. In contrast, global levels of H2Bub1 were not affected in the S2A, Y1F, T4A, S7A or S2A-S7A genetic backgrounds (Figure 1A).

We observed reduction of H2Bub1, H3K4me3 and H3K36me3 upon replacement of Ser7 with the phosphomimetic glutamate residue (Figure 1A). The fact that a constitutive negative charge at this position generally impedes co-transcriptional modifications points to the dynamics of Rpb1 CTD phosphorylation as an important factor in their regulation.

As a further test of the role of the Rpb1 CTD in co-transcriptional histone ubiquitylation and methylation, we assessed the levels of these modifications in strains expressing Rpb1 subunits with CTDs of varying repeat lengths (5). We found that the levels of H2Bub1 and H3K36me3 were similar in strains carrying CTDs with 16, 11, 10 or 9 consensus repeats. H3K4me3 levels were reduced in lockstep with reduction of CTD repeat number, underscoring the sensitivity of H3K4me3 to Rpb1 CTD structure (Figure 1B).
Global levels of Spt5 CTD phosphorylation are maintained in the absence of Rpb1 CTD Ser5 phosphorylation

Since H2Bub1 and H3K4me depend on Spt5 CTD Thr1 (presumably via its phosphorylation), we tested whether the impact of Rpb1 Ser5 mutations on these modifications could reflect an effect of Rpb1 Ser5 on Spt5 T1-P. Immunoblotting using antibodies that recognize total Spt5 or Spt5 T1-P showed no effect of the Rpb1 S5A mutations on levels of Spt5 T1-P in extracts. As a control for these experiments we included extracts derived from a strain harboring a deletion of the brl2Δ gene, encoding a ubiquitin ligase for histone H2B. We observed decreased levels of Spt5 T1-P in the brl2Δ strain, in accord with the previously described positive feedback loop linking Spt5 T1-P and H2Bub1 (22) (Figure 3A).

Chromatin immunoprecipitation (ChIP) assays demonstrated that association of Spt5 with the act1Δ, nup189Δ and SPBC354.10Δ genes was similar in the rpb1-CTD-S5A-MCE1 and the rpb1-CTD-S5A-MCE1 strains (Figure 3B). A control ChIP experiment using a strain lacking the Spt5 CTD confirmed the specificity of the ChIP signal. Therefore, neither phosphorylation of Spt5 nor its association with chromatin require Rpb1 Ser5. We conclude that Rpb1 Ser5 impacts co-transcriptional histone modifications independently of the Spt5 CTD.

Dual roles of Lsk1/Cdk12-dependent Rpb1-S2P in regulating co-transcriptional histone methylation

To determine whether the effects for Rpb1 CTD Ser2 and Ser5 mutations on histone modification were attributable to the lack of phosphorylation, we inhibited Rpb1 CTD kinases implicated in phosphorylation of these sites. We employed strains harboring engineered ‘analogue sensitive’
Figure 3. Loss of Rpb1 Ser5 does not impair Spt5 phosphorylation or its association with transcribed chromatin. (A) Whole-cell extracts derived from the indicated strains (top) were subjected to SDS-PAGE and immunoblotting with the indicated antibodies (right). ’WT’ in the left panel is as in Figure 1; ’WT’ in the right panel is as in Figure 2. (B) ChIP of Spt5 was carried out using a polyclonal antibody recognizing the Spt5 CTD in the indicated strains. A spt5/Delta1 strain lacking the entire CTD served as a negative control. ChIP signals were quantified by qPCR using primer pairs at the numbered locations across the act1+, nup189+ and SPBC354.10+ genes as shown and expressed as% of signal in the whole-cell extract (input). Error bars denote standard deviations from three independent experiments.

Figure 4. Positive and negative effects of Lsk1/Cdk12 on co-transcriptional histone modifications. (A–C) Whole-cell extracts derived from the indicated strains (top) were subjected to SDS-PAGE and immunoblotting with the indicated antibodies (right). For strains carrying analogue sensitive (as) alleles, cultures were treated with DMSO (−) or 20 µM 3-MB-PP1 (+) for 3 h prior to extract preparation.
Lsk1/Cdk12 negatively regulates H3K4me via Rpb1 Ser2 phosphorylation. (A) Whole-cell extracts derived from the indicated strains (top) were subjected to SDS-PAGE and immunoblotting with the indicated antibodies (right). Cultures were treated with DMSO (−) or 20 μM 3-MB-PP1 (+) for 3 h prior to extract preparation. (B) Quantification of band intensities from three repeats of the experiments shown in (A). Intensities were determined using ImageJ software. In each experiment the H3K4me/H3 ratio in the DMSO-treated rpb1-CTD-14 sample was set to 1. Significant increases over this value (as determined by student’s t-test) are indicated (* denotes P < 0.1; ** denotes P < 0.05). Error bars denote standard deviations.

could indicate that this effect is highly sensitive to reduction in Lsk1 activity.

To determine whether regulation of H3K4me by Lsk1 occurred through phosphorylation of Rpb1 Ser2, we introduced the lsk1as allele into rpb1-CTD-14 or rpb1-S2A strains. In the rpb1-CTD-14 background, lsk1as caused a ∼50% increase in H3K4me3 and ∼30% increase in H3K4me2, as assessed by immunoblotting (Figure 5A and B). The lsk1as allele did not enhance H3K4me levels in the rpb1-S2A background, indicating that the effect of Lsk1 on H3K4me requires Rpb1 Ser2. Moreover, the S2A mutation by itself was sufficient to elicit increases in H3K4me2 and H3K4me3. Thus, Lsk1 negatively regulates H3K4me through phosphorylation of Rpb1 Ser2.

The roles of Mcs6/Cdk7 and Rpb1 S5-P

To examine the role of Rpb1 S5-P in co-transcriptional histone modifications we monitored modification levels upon inhibition of Mcs6as. Consistent with previous studies, treatment with 40 μM 3-MB-PP1 strongly reduced Rpb1 S5-P levels, but not those of Rpb1 S2-P, in the mcs6as

strain (Supplementary Figure S3) (48,58). Global levels of H2Bub1 and H3K36me3 were unaffected under these conditions, but H3K4me3 levels were reduced (Figure 6A). This indicates that the sensitivity of H3K4me3 levels to alteration of Rpb1 Ser5 is likely due to the loss of Rpb1 S5-P. However, phosphorylation of Rpb1 Ser7, which also depends on Mcs6 activity, may contribute to this effect (58).

Combined inhibition of Mcs6as and Cdk9as decreased the levels of H3K36me3 more strongly than inhibition of either kinase individually (Figure 6A). However, this effect may be attributed to decreased Rpb1 S2-P and/or loss of Spt5 T1-P (Supplementary Figure S3 and (22)). Thus, our kinase inhibition experiments failed to link the requirement of Rpb1 Ser5 for co-transcriptional H2Bub1 and H3K36me to activity of Mcs6, the major kinase for Rpb1 Ser5 in vivo.

We considered the possibility that residual Mcs6as activity toward the Rpb1 CTD, maintained in the presence of inhibitory analogue, was sufficient for these histone modifications to occur. We thus introduced the mcs6as allele into a strain harboring the rpb1-CTD-S5-MCE1 fusion. Rpb1 CTD function with respect to co-transcriptional histone modifications was partially compromised by its fusion to MCE1 (Figure 1A), but Cdk9-dependent phosphorylation of Spt5 was unaffected (Figure 3). We reasoned that rpb1-CTD-S5-MCE1 might sensitize cells to the effects of Mcs6as inhibition without affecting Spt5 T1-P. Levels of Rpb1 S5-P, Rpb1 S2-P and H3K4me3 were indeed more sensitive to inhibition of Mcs6as in this background as compared to those in a strain with an intact Rpb1 CTD, although Spt5 T1-P levels were unaffected (Figure 6B and Supplementary Figure S3). The enhanced reduction of Rpb1 S5-P was also associated with a partial loss of H2Bub1 (as compared to that caused by rpb1-CTD-S5A-MCE1; Figure 6C). However, levels of H3K36me3 remained insen-
Our data provide the first comprehensive examination of the roles of known Rpb1 and Spt5 phosphorylation sites in co-transcriptional histone modifications (summarized in Figure 7). We observed the most dramatic reductions in steady-state levels of one or more modifications upon loss of Rpb1 Ser2, Rpb1 Ser5 and Spt5 Thr1, pointing to these as key CTD phosphorylation sites regulating co-transcriptional histone modification pathways. Nevertheless, our data indicate that the complete CTD ‘code’ directing these modifications is more complex and includes Rpb1 Tyr1, Rpb1 Thr4 and Rpb1 Ser7. Although previous experiments have demonstrated that phosphorylation of Rpb1 Ser2 and Rpb1 Ser5 does not require any of these sites, the Y1F, T4A and S7A mutations may have subtle effects on levels of Rpb1-S2P and/or Rpb1-S5P that contribute to their effects on histone modifications (47).

We found that Lsk1-dependent Rpb1 S2-P positively regulates H3K36me3, in agreement with previous work on Cdk12 orthologues in budding yeast and in nematodes (42,43). Our data also provide insights into the regulation of H3K4me by Lsk1 and other Cdk12 orthologues. In budding yeast, Ctk1 functions to prevent the aberrant accumulation of H3K4me3 at the 3’ ends of transcribed genes, and thus loss of Ctk1 causes a reduction in H3K4me1 and an increase in H3K4me3 (40,41). This function was shown to depend on Ctk1 kinase activity, although the relevant target was not identified (41). Our data demonstrate that the negative regulation of co-transcriptional H3K4me1, H3K4me2 and H3K4me3 by Lsk1 occurs via Rpb1 Ser2 phosphorylation. The S2A mutation also led to reduced H3K4me3 levels when combined with S7A, suggesting that unphosphorylated Rpb1 Ser2 makes a positive contribution to H3K4me.

In mammalian cells, Rpb1 S2-P is proposed to directly interact with WAC, a WW-domain protein and subunit of the RNF20/40 ubiquitin ligase complex for H2Bub1 (30). In addition, a version of Rpb1 lacking Ser2 in its CTD repeats cannot support normal levels of H2Bub1 (39). The fact that WAC is not conserved outside of metazoans could explain why we found Rpb1 Ser2 to be dispensable for H2Bub1 in S. pombe. Whether WAC may also interact with other CTD phosphorylation sites, such as Spt5 T1-P, has not been tested.

Examination of strains expressing mutant forms of both CTDs allowed us to assess their additive effects on co-transcriptional histone modifications. Consistent with the independent effects of Spt5 Thr1 and Rpb1 Ser7 on H3K4me3, removal of both of these sites resulted in further reduction of this modification. Loss of H3K4me3 caused by spt5-T1A is most likely secondary to reduction in H2Bub1 due to the H2Bub1-H3K4me crossstalk pathway (16,57). H3K4me3 levels were reduced in the Y1F, S7A, T4A and S2A-S7A genetic backgrounds, upon truncation of the Rpb1 CTD, and upon inhibition of Mcs6 as. H2Bub1 levels were unaffected under these conditions. We think it likely that these effects reflect a direct interaction between the Set1C/COMPASS histone H3K4 methyltransferase complex and the phosphorylated Rpb1 CTD, an idea supported by previous reports (23,59,60). We thus envision Set1C/COMPASS as integrating independent signals from the Rpb1 and Spt5 CTDs during transcription.

Our data show that the Rpb1 and Spt5 CTDs have overlapping roles in the co-transcriptional formation of H3K36me. Although the ablation of Spt5 T1-P by itself has little effect on H3K36me3 levels, Spt5 T1-P becomes critical upon removal of Rpb1 Ser7. Whereas a primary role for the Rpb1 CTD in directing H3K36me is well established, a role for the Spt5 CTD has not been described previously. The apparent functional overlap could stem from impaired RNApolII elongation caused by defects in both CTD domains, although our MPA sensitivity experiments argued this was not the case in the mutant strains we employed.

H2Bub1 levels were insensitive to genetic alteration of most phospho-sites on the Rpb1 CTD, with the exception of Ser5. We were unable to assess functional overlap between these sites and Spt5 Thr1 as the levels of H2Bub1 in spt5-T1A mutants are already near the limits of detection in our immunoblots (22). However, Rpb1 Ser5 did not act through Spt5 Thr1, since levels of Spt5 T1-P were maintained and Spt5 crosslinking to several transcribed genes was not altered upon removal of Rpb1 Ser5. These data indicate that the deficit in H2Bub1 in SSA strains was not caused by a general impairment of Cdk9 activity toward Spt5 at sites of

Figure 7. A proposed CTD ‘code’ for co-transcriptional histone modifications. Grey ovals depicting the Rpb1 and Spt5 CTDs are positioned above the typical histone modification landscape of a transcribed gene (see (11)). The ovals are labeled with the specific phospho-sites implicated in each of the indicated histone modifications and are depicted as independent or overlapping. The sizes of the labels denote their relative functional importance for each histone modification. Arrows connect CTD kinases to functionally validated target phospho-sites. Broken arrow indicates negative regulation of H3K4me by Lsk1 and other Cdk12 orthologues. In mammalian cells, Rpb1 S2-P is proposed to directly interact with WAC, a WW-domain protein and subunit of the RNF20/40 ubiquitin ligase complex for H2Bub1 (30). In addition, a version of Rpb1 lacking Ser2 in its CTD repeats cannot support normal levels of H2Bub1 (39). The fact that WAC is not conserved outside of metazoans could explain why we found Rpb1 Ser2 to be dispensable for H2Bub1 in S. pombe. Whether WAC may also interact with other CTD phosphorylation sites, such as Spt5 T1-P, has not been tested.
transcription. Our previous work also argues against a role for Rpb1 Ser5 in recruitment of the PAF complex or of the Rtf1 orthologue Prf1 to transcribed genes (28). Thus, Rpb1 Ser5 seems to define an alternate mechanism involved in co-transcriptional formation of H2Bub1.

The finding that H2Bub1 is independent of Mcs6 activity in *S. pombe* is in contrast to previous work on Cdk7 in mammalian cells and Kin28 in budding yeast. The requirement of Cdk7 activity for H2Bub1 was also uncovered by inhibition of an AS kinase expressed at physiologic levels, and can be explained by the CDK-activating function of this enzyme in mammalian cells, which is required for the activity of Cdk9 (31). In both fission and budding yeast, the CDK-activating function resides in an unrelated kinase (50,61). The role of Kin28 in H2Bub1 formation in yeast, the CDK-activating function resides in an unrelated kinase (50,61). The role of Kin28 in H2Bub1 formation in budding yeast was ascertained using a temperature-sensitive kinase (50,61). The role of Kin28 in H2Bub1 formation in *S. pombe* and *S. cerevisiae* is supported by the finding that the *mcs6*/*kin28* null mutant is synthetically lethal with a null allele of *rpb1-CTD* (23). In budding yeast, the role of Kin28 in H2Bub1 formation is supported by the finding that the *mcs6*/*kin28* null mutant is synthetically lethal with a null allele of *rpb1-CTD* (23). In budding yeast, the role of Kin28 in H2Bub1 formation is supported by the finding that the *mcs6*/*kin28* null mutant is synthetically lethal with a null allele of *rpb1-CTD* (23).

How do we reconcile the requirement of Rpb1 Ser5 for H3K36me with the relative insensitivity of this mark to loss of Mcs6/Cdk7 activity? Phosphorylation of both Ser5 and H3K36me with the relative insensitivity of this mark to loss of Mcs6 activity is in contrast to previous work on Cdk7 in mammalian cells and Kin28 in budding yeast. The requirement of Cdk7 activity for H2Bub1 was also uncovered by inhibition of an AS kinase expressed at physiologic levels, and can be explained by the CDK-activating function of this enzyme in mammalian cells, which is required for the activity of Cdk9 (31). In both fission and budding yeast, the CDK-activating function resides in an unrelated kinase (50,61). The role of Kin28 in H2Bub1 formation in budding yeast was ascertained using a temperature-sensitive *kin28* allele (*kin28ts-16-HA*), inactivation of which has been shown to disrupt the entire TFIIH complex at gene promoters (33,62). Thus, the effect of this allele on H2Bub1 may be unrelated to Kin28 activity per se.

We think it more likely that Rpb1 Ser5-P catalyzed by a kinase other than Mcs6/Cdk7 is necessary for co-transcriptional formation of H3K36me (and perhaps, to a lesser extent, H2Bub1). Cdk9 is the strongest candidate to fulfill this role for several reasons. First, genome-wide ChIP experiments in budding yeast demonstrate that the activities of Kin28 and Bur1 are both required for Rpb1 S5-P at promoter-distal locations within gene bodies (63). Second, although Cdk9 is thought to primarily target serine 2 on the Rpb1 CTD in vivo, experiments in vitro show that its preferred site on Rpb1 CTD-derived peptide substrates is in fact serine 5 (64). Finally, Cdk9 colocalizes with Rpb1 S5-P in ‘transcription factories’ visualized in live mammalian cells (65). Our data suggest a potential physiological role for Cdk9-dependent Rpb1 S5-P. An important goal of future work will be to uncover the mechanistic basis for the functional difference between Rpb1 Ser5-P catalyzed by Mcs6/Cdk7 and that catalyzed by Cdk9 or other kinases.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

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**REFERENCES**

1. Hartogz, G.A. and Fu, J. (2013) The Spt4-Spt5 complex: a multi-faceted regulator of transcription elongation. *Biochim. Biophys. Acta*, 1829, 145–115.

2. Hsin, J.P. and Manley, J.L. (2012) The RNA polymerase II CTD coordinates transcription and RNA processing. *Genes Dev.*, 26, 2119–2137.

3. Wier, A.D., Mayekar, M.K., Heroux, A., Arndt, K.M. and Vandemark, A.P. (2013) Structural basis for Spt5-mediated recruitment of the Paf1 complex to chromatin. *Proc. Natl. Acad. Sci. U.S.A.*, 110, 17290–17295.

4. Yamada, T., Yamaguchi, Y., Inukai, N., Okamoto, S., Mura, T. and Handa, H. (2006) P-TEFb-mediated phosphorylation of hSpt5 C-terminal repeats is critical for processive transcription elongation. *Mol. Cell.*, 21, 227–237.

5. Schneider, S., Pei, Y., Shuman, S. and Schwer, B. (2010) Separable functions of the fission yeast Spt5 carboxyl-terminal domain (CTD) in capping enzyme binding and transcription elongation overlap with those of the RNA polymerase II CTD. *Mol. Cell. Biol.*, 30, 2353–2364.

6. Liu, Y., Warfield, L., Zhang, C., Luo, J., Allen, J., Lang, W.H., Ranish, J., Shokat, K.M. and Hahn, S. (2009) Phosphorylation of the transcription elongation factor Spt5 by yeast Bur1 kinase stimulates recruitment of the PAF complex. *Mol. Cell. Biol.*, 29, 4852–4863.

7. Zhou, K., Kuo, W.H., Fillingham, J. and Greenblatt, J.F. (2009) Control of transcriptional elongation and cotranscriptional histone modification by the yeast BUR kinase substrate Spt5. *Proc. Natl. Acad. Sci. U.S.A.*, 106, 6956–6961.

8. Doamekpor, S.K., Sanchez, A.M., Schwer, B., Shuman, S. and Lima,C.D. (2014) How an mRNA capping enzyme reads distinct RNA polymerase II and Spt5 CTD phosphorylation codes. *Genes Dev.*, 28, 1323–1336.

9. Doamekpor, S.K., Schwer, B., Sanchez, A.M., Schwer, B., Shuman, S. and Lima, C.D. (2015) Fission yeast RNA triphosphatase reads an Spt5 CTD code. *RNA*, 21, 113–123.

10. Mayer, A., Schrieck, A., Lidschreiber, M., Leike, K., Martin, D.E. and Cramer, P. (2012) The Spt5 C-terminal region recruits yeast 3′ RNA cleavage factor I. *Mol. Cell. Biol.*, 32, 1321–1331.

11. Smolle, M. and Workman, J.L. (2013) Transcription-associated histone modifications and cryptic transcription. *Biochim. Biophys. Acta*, 1829, 145–115.

12. Bannister, A.J. and Kouzarides, T. (2011) Regulation of chromatin by histone modifications. *Cell Res.*, 21, 381–395.

13. Campos, E.I. and Reinberg, D. (2009) Histones: annotating chromatin. *Annu. Rev. Genet.*, 43, 559–599.

14. Wagner, E.J. and Carpenter, P.B. (2012) Understanding the language of Lys36 methylation at histone H3. *Nat. Rev. Mol. Cell Biol.*, 13, 115–126.

15. Fuchs, G. and Oren, M. (2014) Writing and reading H2B monoubiquitylation. *Biochim. Biophys. Acta*, 1839, 694–701.

16. Shilatifard, A. (2012) The COMPASS family of histone H3K4 methylases: mechanisms of regulation in development and disease pathogenesis. *Annu. Rev. Biochem.*, 81, 65–95.

17. Tanny, J.C. (2014) Chromatin modification by the RNA Polymerase II elongation complex. *Transcription*, 5, e988093.

18. Eick, D. and Geyer, M. (2013) The RNA polymerase II carboxy-terminal domain (CTD) code. *Chem. Rev.*, 113, 8456–8490.
19. Ho,C.K. and Shuman,S. (1999) Distinct roles for CTD Ser-2 and Ser-5 phosphorylation in the recruitment and allosteric activation of mammalian mRNA capping enzyme. Mol. Cell, 3, 405–411.
20. Jasnovidova,O. and Stefl,R. (2013) The CTD code of RNA polymerase II: a structural view. Wiley Interdiscip. Rev. RNA, 4, 1–16.
21. Pei,Y. and Shuman,S. (2003) Characterization of the Schizosaccharomyces pombe Cdk9/Pcf1 protein kinase: Sp5 phosphorylation, autophosphorylation, and mutational analysis. J. Biol. Chem., 278, 43346–43356.
22. Sanso,M., Lee,K.M., Viladevall,L., Jacques,P.E., Page,V., Nagy,S., Phatnani,H.P., Jones,J.C. and Greenleaf,A.L. (2004) Expanding the CTD code of RNA polymerase II. Proc. Natl. Acad. Sci. U.S.A., 101, 17636–17641.
23. Xiao,T., Shiibata,Y., Rao,B., Laribee,R.N., O’Rourke,R., Buck,M.J., Osborne,M.A. and Strahl,B.D. (2005) Histone H2B ubiquitylation is associated with transcription elongation in fission yeast. PLoS Genet., 1, e1000222.
24. Pei,Y., Du,H., Singer,J., St Amour,C., Granitto,S., Shuman,S. and Strahl,B.D. (2005) Phosphorylation of RNA polymerase II CTD regulates H3 methylation in yeast. Mol. Cell, 20, 699–718.
25. Li,M., Phatnani,H.P., Guan,Z., Sage,H., Greenleaf,A.L. and Zhou,P. (2004) Solution structure of the Set2-Rpb1 interacting domain of human Set2 and its interaction with the hyperphosphorylated C-terminal domain of Rpb1. Proc. Natl. Acad. Sci. U.S.A., 102, 17636–17641.
26. Phatnani,H.P., Jones,J.C. and Greenleaf,A.L. (2004) Expanding the functional repertoire of CTD kinase I and RNA polymerase II: novel phospho-CTD-associating proteins in the yeast proteome. Biochemistry, 43, 15702–15719.
27. Li,M., Huang,Y. and Hinnebusch,A.G. (2012) Pol II CTD kinases Bur1 and Kin28 promote Sp5 CTR-independent recruitment of Paf1 complex. EMBO J., 31, 3494–3505.
28. Xiao,T., Shiibata,Y., Yuan,J.C., Jacques,P.E., Page,V., Nagy,S., Racine,A., St Amour,C.V., Zhang,C., Shuman,S. and Strahl,B.D. (2004) A novel domain in Set2 mediates RNA polymerase II interaction and couples histone H3 K36 methylation with transcription elongation. Mol. Cell, 25, 3305–3316.
29. Li,M., Pei,Y., Shuman,S. and Shuman,S. (2003) Characterization of the Schizosaccharomyces pombe Cdk9/Pcf1 protein kinase: Sp5 phosphorylation, autophosphorylation, and mutational analysis. J. Biol. Chem., 278, 43346–43356.
30. Sanso,M., Lee,K.M., Viladevall,L., Jacques,P.E., Page,V., Nagy,S., Racine,A., St Amour,C.V., Zhang,C., Shokat,K.M. et al. (2012) A positive feedback loop links opposing functions of P-TEFb/Cdk9 and histone H2B ubiquitylation to regulate transcription elongation in fission yeast. PLoS Genet., 8, e1002822.
31. Li,M., Pei,Y., Shuman,S. and Shuman,S. (2003) Characterization of the Schizosaccharomyces pombe Cdk9/Pcf1 protein kinase: Sp5 phosphorylation, autophosphorylation, and mutational analysis. J. Biol. Chem., 278, 43346–43356.
32. Sanso,M., Lee,K.M., Viladevall,L., Jacques,P.E., Page,V., Nagy,S., Racine,A., St Amour,C.V., Zhang,C., Shokat,K.M. et al. (2012) A positive feedback loop links opposing functions of P-TEFb/Cdk9 and histone H2B ubiquitylation to regulate transcription elongation in fission yeast. PLoS Genet., 8, e1002822.
33. Pei,Y., Du,H., Singer,J., St Amour,C., Granitto,S., Shuman,S. and Strahl,B.D. (2005) Phosphorylation of RNA polymerase II CTD regulates H3 methylation in yeast. Mol. Cell, 20, 699–718.
34. Li,M., Phatnani,H.P., Guan,Z., Sage,H., Greenleaf,A.L. and Zhou,P. (2004) Solution structure of the Set2-Rpb1 interacting domain of human Set2 and its interaction with the hyperphosphorylated C-terminal domain of Rpb1. Proc. Natl. Acad. Sci. U.S.A., 102, 17636–17641.
35. Li,M., Pei,Y., Shuman,S. and Shuman,S. (2003) Characterization of the Schizosaccharomyces pombe Cdk9/Pcf1 protein kinase: Sp5 phosphorylation, autophosphorylation, and mutational analysis. J. Biol. Chem., 278, 43346–43356.
36. Sanso,M., Lee,K.M., Viladevall,L., Jacques,P.E., Page,V., Nagy,S., Racine,A., St Amour,C.V., Zhang,C., Shokat,K.M. et al. (2012) A positive feedback loop links opposing functions of P-TEFb/Cdk9 and histone H2B ubiquitylation to regulate transcription elongation in fission yeast. PLoS Genet., 8, e1002822.
37. Li,M., Pei,Y., Shuman,S. and Shuman,S. (2003) Characterization of the Schizosaccharomyces pombe Cdk9/Pcf1 protein kinase: Sp5 phosphorylation, autophosphorylation, and mutational analysis. J. Biol. Chem., 278, 43346–43356.
38. Sanso,M., Lee,K.M., Viladevall,L., Jacques,P.E., Page,V., Nagy,S., Racine,A., St Amour,C.V., Zhang,C., Shokat,K.M. et al. (2012) A positive feedback loop links opposing functions of P-TEFb/Cdk9 and histone H2B ubiquitylation to regulate transcription elongation in fission yeast. PLoS Genet., 8, e1002822.
39. Li,M., Pei,Y., Shuman,S. and Shuman,S. (2003) Characterization of the Schizosaccharomyces pombe Cdk9/Pcf1 protein kinase: Sp5 phosphorylation, autophosphorylation, and mutational analysis. J. Biol. Chem., 278, 43346–43356.
57. Racine, A., Page, V., Nagy, S., Grabowski, D. and Tanny, J.C. (2012) Histone H2B ubiquitylation promotes activity of the intact Set1 histone methyltransferase complex in fission yeast. J. Biol. Chem., 287, 19040–19047.

58. St Amour, C.V., Sanso, M., Bosken, C.A., Lee, K.M., Larochelle, S., Zhang, C., Shokat, K.M., Geyer, M. and Fisher, R.P. (2012) Separate domains of fission yeast Cdk9 (P-TEFb) are required for capping enzyme recruitment and primed (Ser7-phosphorylated) Rpb1 carboxyl-terminal domain substrate recognition. Mol. Cell. Biol., 32, 2372–2383.

59. Krogan, N.J., Dover, J., Wood, A., Schneider, J., Heidt, J., Boateng, M.A., Dean, K., Ryan, O.W., Golshani, A., Johnston, M. et al. (2003) The Paf1 complex is required for histone H3 methylation by COMPASS and Dot1p: linking transcriptional elongation to histone methylation. Mol. Cell, 11, 721–729.

60. Ng, H.H., Robert, F., Young, R.A. and Struhl, K. (2003) Targeted recruitment of Set1 histone methylase by elongating Pol II provides a localized mark and memory of recent transcriptional activity. Mol. Cell, 11, 709–719.

61. Yao, S. and Prelich, G. (2002) Activation of the Bur1-Bur2 cyclin-dependent kinase complex by Cak1. Mol. Cell. Biol., 22, 6750–6758.

62. Kanin, E.I., Kipp, R.T., Kung, C., Slattery, M., Viale, A., Hahn, S., Shokat, K.M. and Ansari, A.Z. (2007) Chemical inhibition of the TFIIH-associated kinase Cdk7/Kin28 does not impair global mRNA synthesis. Proc. Natl. Acad. Sci. U.S.A., 104, 5812–5817.

63. Bataille, A.R., Jeronimo, C., Jacques, P.E., Laramée, L., Fortin, M.E., Forest, A., Bergeron, M., Hanes, S.D. and Robert, F. (2012) A universal RNA polymerase II CTD cycle is orchestrated by complex interplays between kinase, phosphatase, and isomerase enzymes along genes. Mol. Cell, 45, 158–170.

64. Czudnochowski, N., Bosken, C.A. and Geyer, M. (2012) Serine-7 but not serine-5 phosphorylation primes RNA polymerase II CTD for P-TEFb recognition. Nat. Commun., 3, 842.

65. Ghamari, A., van de Corput, M.P., Thongjuea, S., van Cappellen, W.A., van Ijcken, W., van Haren, J., Soler, E., Eick, D., Lenhard, B. and Grosveld, F.G. (2013) In vivo live imaging of RNA polymerase II transcription factories in primary cells. Genes Dev., 27, 767–777.