A Positive Feedback Loop Links Opposing Functions of P-TEFb/Cdk9 and Histone H2B Ubiquitylation to Regulate Transcript Elongation in Fission Yeast

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Abstract

Transcript elongation by RNA polymerase II (RNAPII) is accompanied by conserved patterns of histone modification. Whereas histone modifications have established roles in transcription initiation, their functions during elongation are not understood. Mono-ubiquitylation of histone H2B (H2Bub1) plays a key role in coordinating co-transcriptional histone modification by promoting site-specific methylation of histone H3. H2Bub1 also regulates gene expression through an unidentified, methylation-independent mechanism. Here we reveal bidirectional communication between H2Bub1 and Cdk9, the ortholog of metazoan positive transcription elongation factor b (P-TEFb), in the fission yeast Schizosaccharomyces pombe. Chemical and classical genetic analyses indicate that lowering Cdk9 activity or preventing phosphorylation of its substrate, the transcription processivity factor Spt5, reduces H2Bub1 in vivo. Conversely, mutations in the H2Bub1 pathway impair Cdk9 recruitment to chromatin and decrease Spt5 phosphorylation. Moreover, an Spt5 phosphorylation-site mutation, combined with deletion of the histone H3 Lys4 methyltransferase Set1, phenocopies morphologic and growth defects due to H2Bub1 loss, suggesting independent, partially redundant roles for Cdk9 and Set1 downstream of H2Bub1. Surprisingly, mutation of the histone H2B ubiquitin-acceptor residue relaxes the Cdk9 activity requirement in vivo, and cdk9 mutations suppress cell-morphology defects in H2Bub1-deficient strains. Genome-wide analyses by chromatin immunoprecipitation also demonstrate opposing effects of Cdk9 and H2Bub1 on distribution of transcribing RNAPII. Therefore, whereas mutual dependence of H2Bub1 and Spt5 phosphorylation indicates positive feedback, mutual suppression by cdk9 and H2Bub1-pathway mutations suggests antagonistic functions that must be kept in balance to regulate elongation. Loss of H2Bub1 disrupts that balance and leads to deranged gene expression and aberrant cell morphologies, revealing a novel function of a conserved, co-transcriptional histone modification.


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Introduction

The elongation phase of transcription is a point of regulation for many genes transcribed by RNAPII in eukaryotes, and control of elongation is critical for coupling of transcription to downstream steps in gene expression [1,2]. Whereas the regulation of transcription at the initiation step has been studied extensively, many of the mechanisms governing elongation in vivo remain to be elucidated. Transcription is accompanied by post-translational modification of nucleosomal histones in a highly conserved pattern, stereotypical features of which include methylation of histone H3 Lys4 (H3K4me) and acetylation of histones H3 and H4 at 5′ ends, methylation of histone H3 Lys36 (H3K36me) towards 3′ ends, and mono-ubiquitylation of histone H2B at a conserved site in the carboxyl-terminus (H2Bub1) throughout coding regions of genes [3]. Conservation of this pattern suggests an important role in coordinating gene expression, but the precise functions of individual modifications in elongation control are poorly understood.

H2Bub1, which appears to play a central role in the interplay between chromatin and the RNAPII elongation complex, is catalyzed in budding yeast by the ubiquitin-conjugating enzyme Rad6 and the E3 ubiquitin ligase Bre1 [4]. Formation of H2Bub1 on transcribed chromatin also requires PAF, a conserved complex with multiple functions during elongation [5]. H2Bub1 is required...
Author Summary

Modification of histone proteins is an important transcriptional regulatory mechanism in eukaryotic cells. Although various histone modifications are found primarily within the coding regions of transcribed genes, how they influence transcription elongation remains unclear. Among these modifications is mono-ubiquitylation of histone H2B (H2Bub1), which is needed for co-transcriptional methylation of histone H3 at specific sites. Here we show that H2Bub1 and Cdk9, the kinase component of positive transcription elongation factor b (P-TEFb), are jointly regulated by a positive feedback loop: Cdk9 activity is needed for co-transcriptional H2Bub1, and H2Bub1 in turn stimulates Cdk9 activity toward one of its major substrates, the conserved elongation factor Spt5. We provide genetic evidence that the combined action of H2Bub1 on Spt5 phosphorylation and histone methylation accounts for the gene-regulatory effects of this modification. Surprisingly, our genetic and genome-wide studies indicate that P-TEFb and H2Bub1 act in opposition on elongating RNA polymerase. We suggest that the positive feedback linking P-TEFb and H2Bub1 helps to maintain a balance between their opposing actions. These results highlight a novel regulatory role for a conserved histone modification during transcription elongation.

for co-transcriptional generation of H3K4me by the methyltransferase Set1 in yeast, and contributes to global H3K4me levels in for co-transcriptional generation of H3K36me and consequent action of the

Bur1, the essential ortholog of Cdk9 in budding yeast, is required during transcription elongation. For example, in budding yeast, lethality of a BUR1 deletion is suppressed by deletion of the H3K36 methyltransferase Set2 [34,48]; and H2B de-ubiquitylation by Up1p—a component of the SAGA complex—is required for Cdk1 recruitment and Ser2 phosphorylation at some genes [49].

In fission yeast, the P-TEFb ortholog is the essential Cdk9/Pch1 complex. In vitro, Cdk9 phosphorylates the Rpb1 CTD at both Ser5 and Ser2 [50]; inhibition of Cdk9 in vivo reduced the apparent stoichiometry of Rpb1 phosphorylation but did not cause selective loss of either Ser5 or Ser2 signals [51]. Cdk9 also phosphorylates the CTD of S. pombe Spt5 [52]; spt5 mutations that prevent this phosphorylation cause slow growth, defects in transcript elongation and, when combined with partial truncations of the Rpb1 CTD, synthetic lethality [53]. Genetic epistasis suggests, moreover, that the Spt5 CTD contains an exclusive, albeit nonessential, target of Cdk9 in vivo [51]. The potential roles of CDKs and their substrates in regulating histone modifications in fission yeast have not been explored.

Here we uncover a mutual dependence of H2Bub1 and P-TEFb function in S. pombe that impair phosphorylation of Sp5 by Cdk9 diminish levels of H2Bub1 and, reciprocally, mutants unable to generate H2Bub1 have reductions in chromatin-associated Cdk9 and phosphorylated Sp5. Ablation of the preferred phosphoacceptor site in Spt5 combined with deletion of set1® phenocopies morphological defects of htb1-K119R mutants, suggesting that Cdk9 and Set1 govern separate, partially redundant pathways downstream of H2Bub1. Despite the dependencies between H2Bub1 and Sp5 phosphorylation, mutations that impair Cdk9 function suppress abnormal cell morphologies and reverse an RNAPII distribution defect, both due to loss of H2Bub1. Conversely, growth of htb1-K119R cells is resistant to selective deletion of H2Bub1. Taken together, biochemical and genetic results suggest that P-TEFb and H2Bub1 oppose one another to regulate RNAPII elongation, but that proper balance between the two is ensured via a positive feedback loop, in which phosphorylation by Cdk9 stimulates H2Bub1 and vice versa.

Results

H2Bub1 influences global RNAPII distribution in S. pombe

Our previous results suggested that H2Bub1 promotes expression of a subset of genes [12]. To ask whether H2Bub1 might act more generally in transcription, we combined ChIP with hybridization of recovered DNA sequences to microarrays that cover the entire S. pombe genome at 200 base-pair intervals (ChIP-chip). We first mapped genome-wide distribution of H2Bub1 in wild-type cells with an antibody that specifically recognizes the ubiquitylated form of histone H2B [54], Figure S1A. A FLAG-epitope tag fused to the carboxyl-terminus of histone H2B allowed
us to normalize for total H2B occupancy by parallel ChIP-chip with anti-FLAG antibody. The FLAG-tagged H2B was ubiquitylated to a similar extent as the native protein [12], Figure S1B. To correlate the H2Bub1 pattern with transcription, we also performed ChIP-chip with an RNAPII-specific antibody. We grouped genes into quartiles according to their total levels of RNAPII enrichment and plotted average distributions of H2Bub1 within each group (Figure 1A). This analysis confirmed the presence of H2Bub1 throughout coding regions of transcribed genes in *S. pombe*, similar to its distribution in *S. cerevisiae* and metazoans [13,55–57]. Furthermore, H2Bub1 enrichment was correlated with that of RNAPII throughout the genome (r = 0.58).

Because of this genome-wide association, we sought to determine the impact of H2Bub1 loss on RNAPII distribution within genes, by ChIP-chip in wild-type and *htb1-K119R* mutant cells. This analysis revealed alterations in RNAPII occupancy within gene coding regions in the *htb1-K119R* mutant: a global, downstream shift in average RNAPII density, reflecting both a decrease in occupancy within the 5′ halves of genes and a more pronounced increase near the 3′ ends (Figure 1B and 1C). These changes occurred in all classes of genes, but varied quantitatively depending on their overall levels of RNAPII cross-linking (Figure 1D; Dataset S1). Genes highly enriched for RNAPII (Figure 1D; red line) had a significant (P<10exp-5) decrease in RNAPII 5′-end occupancy in the absence of H2Bub1, whereas the increase at 3′ ends failed to reach significance (P>0.01). In the other three classes of genes the increases in RNAPII occupancy at the 3′ end were the most significant changes (Figure 1D). Therefore, H2Bub1 globally affects RNAPII distribution in *S. pombe* genes.

Previous reports have shown that H2Bub1 affects nucleosome stability [9,14,58,59]. We performed ChIP-chip with an antibody that recognizes histone H3 to determine whether the changes in RNAPII distribution caused by H2Bub1 loss were correlated with changes in nucleosome occupancy. The average histone H3 occupancy in gene coding regions did not differ significantly between wild-type and *htb1-K119R* cells (Figure S2; Datasets S2 and S3). These data suggest that the observed changes in RNAPII occupancy are unlikely to be caused solely by altered nucleosome distribution.

To ascertain whether genes affected by the *htb1-K119R* mutation were associated with particular cellular functions, we searched for Gene Ontology (GO) terms that were significantly enriched among the 500 genes with the largest increases or decreases in RNAPII occupancy in the *htb1-K119R* mutant. The genes exhibiting loss of RNAPII occupancy were enriched for ribosomal protein genes (P = 3.5exp-26, 17.7%), consistent with a role for H2Bub1 in promoting RNAPII occupancy at heavily transcribed genes.

The ChIP-chip analysis revealed that RNAPII density was reduced throughout coding regions of 25 of 40 genes whose expression decreased by ≥2-fold in our previous microarray analysis of gene expression [12](Table S1). Furthermore, this group of genes is significantly enriched among the highest RNAPII-occupancy class that we defined by ChIP-chip (P = 7.96exp-5). Thus, the ChIP-chip results and our previous gene-expression data argue that loss of H2Bub1 has a particularly strong negative impact on steady-state levels of poly(A) mRNAs produced from highly transcribed genes. However, the global redistribution of RNAPII in *htb1-K119R* cells (Figure 1D) suggests that H2Bub1 might affect gene expression more broadly through additional, post-transcriptional mechanisms.

**P-TEFb activity promotes H2Bub1 in fission yeast**

Loss of H2Bub1 caused changes in cell morphology and RNAPII distribution that resemble those produced by inactivation of Rpb1 CTD kinases or loss of Rpb1-CTD Ser2 phosphorylation [12,54,60,61]. We therefore investigated possible interactions between H2Bub1 and three CDKs implicated in transcription: the TI1IH-associated Cdk7 ortholog Me36 [61], Cdk9, and the Cdk1 ortholog Lk1 [62]. First, we determined the effect on H2Bub1 of selective inhibition of Me36 or Cdk9, made possible by mutating the “gatekeeper” residue in each kinase to Gly, to render the enzyme sensitive to bulky adenine analogs that do not affect wild-type kinases [63]. We previously replaced *me36*, *cdk9* and *lkb1* with alleles encoding analog-sensitive (AS) mutant versions, each of which was sensitive to the inhibitory analog 3-MB-PP1 [51]. Selective inhibition of Cdk9, by addition of 20 μM 3-MB-PP1 to *cdk9*” cells, caused diminution of H2Bub1 signals in whole-cell extracts (Figure 2A). Cdk9 inhibition also decreased levels of H3K4 di- and trimethylation (H3K4me2 and H3K4me3, respectively) and H3K36 trimethylation (H3K36me3; Figure 2A and data not shown)—modifications likewise associated with actively transcribed genes [5]. Inhibition of the TI1IH-associated kinase in *me36*” cells had small or no effects on H2Bub1, H3K4me2, H3K4me3 or H3K36me3. These results suggest a predominant role for Cdk9 in co-transcriptional histone modification, and a possibly exclusive requirement in generating H2Bub1.

Burl in budding yeast and Cdk9 in mammalian cells are proposed to stimulate H2Bub1 by favoring recruitment of PAF, which can in turn associate directly with the H2B ubiquitylation machinery [10,37,38,64]. Inhibition of Cdk9 as decreased association of both the PAF component Rtf1 and the E2 ubiquitin-conjugating enzyme Rhp6 with transcribed chromatin (Figure S3A and S3B), suggesting faithful conservation of the P-TEFb-H2Bub1 pathway in *S. pombe*.

**H2Bub1 depends on activated Cdk9 and phosphorylation of Spt5**

To further probe the dependence of H2Bub1 on P-TEFb, we tested effects of other mutations that perturb Cdk9 function (Figure 2B). Two mutant strains in which Cdk9 activity is reduced by lack of phosphorylation at Thr1212 of the activation segment (T loop)—*cdk9-T1212A* and *cdk1A*, which is missing the Cdk9-activating kinase (CAK) responsible for that phosphorylation [50]—had nearly undetectable levels of H2Bub1. In contrast, a *cdk9-T1212E* mutation, which substitutes a Gln residue for Thr1212 to mimic constitutive phosphorylation [65], had only a small negative effect on H2Bub1. Loss of H2Bub1 occurred uniquely in mutants with impaired Cdk9 function; neither *me36-S163A*, a T-loop mutation that renders Me36 refractory to CAK [66,67], nor deletions of *lbk1* or *lb1* [which encodes the cyclin partner of Lk1] [68], affected bulk H2Bub1 levels (Figure 2B). Therefore, among *S. pombe* CDKs implicated in transcript elongation, Cdk9 is uniquely required for H2Bub1.

Impaired Cdk9 function could lead to H2Bub1 loss by decreasing phosphorylation of the Spt5 CTD nonapeptide repeat TPAWNSG SK at Thr1, a site modified by Cdk9 in vitro [52]. To test this we measured H2Bub1 levels in a series of *spt5* truncation mutants: *spt5-5A*, which is missing the entire CTD; *spt5-5B*, one of many strains containing seven repeats, either of wild-type sequence (spt5-5WT), or with every Thr1 position mutated to Ala (spt5-5TiA), or to Gln (spt5-5TiE) [53]. H2Bub1 decreased in *spt5-5A* and *spt5-5TiA* mutants, but not in *spt5-5WT* or an spt5-5TiE mutant that mimics constitutive phosphorylation (Figure 2C). We conclude that Cdk9 activity and Sp5-CTD phosphorylation are required for H2Bub1 in vivo.

**Cdk9 activity is required for Spt5-Thr1 phosphorylation in vivo**

To investigate this connection, and to assess relative contributions of different CDKs to Spt5 phosphorylation in vivo, we
generated an antibody specific for the Sp5 CTD phosphorylated on the Thr1 residue (anti-Spt5-T1P), which recognized Sp5 only after it had been phosphorylated by Cdk9 in vitro (Figure S4A). In whole-cell extracts, the phosphorylated Sp5 signal was undetectable after treatment of cdk9as cells with 20 μM 3-MB-PP1, which had little or no effect on Sp5 phosphorylation in wild-type, mcs6as or lsk1as cells, and did not alter Sp5 expression levels in any of the strains (Figure 3A). Sp5-Thr1 phosphorylation was nearly abolished in cdk9as cells at 3-MB-PP1 doses as low as 300 nM (Figure S4B). In contrast, the IC50 for inhibition of cdk9as cell growth was ~10–15 μM, and Rpb1-CTD Ser2 phosphorylation was relatively resistant to inhibition of Cdk9 alone [51]. There was no additive sensitivity of Sp5-P to the analog in mcs6as cdk9as cells, nor any loss of Sp5-P signal in mcs6as cells treated with 3-MB-PP1.
A role for Spt5 phosphorylation downstream of H2Bub1

There is a precedent for H2Bub1 regulating the function of a CTD kinase [49]. We wondered whether Cdk9 and the H2Bub1 machinery could be reciprocally regulated, such that the H2Bub1-defective mutants hbl1-K119R and brl2A (which lacks the Br1 ortholog required for H2Bub1) [12] would have global alterations in Spt5-P. Indeed, Spt5-P was decreased relative to total Spt5 (detected with antibodies specific for wild-type Spt5-CTD irrespective of phosphorylation state; Figure S4D) in both hbl1-K119R and brl2A strains. Conversely, Spt5-P was slightly increased in cells lacking the ubiquitin-specific protease encoded by ubl6Δ (Figure 3B). Cells lacking a second putative H2B de-ubiquitylating enzyme, encoded by the UBP16 homolog ubl16Δ [69], had no change in levels of H2Bub1 or Spt5-P (data not shown). These data suggest a positive feedback loop connecting Cdk9 and the H2Bub1 machinery in vivo.

Previous reports have documented cooperative action of H2Bub1 and the FACT complex, a conserved elongation factor that controls nucleosome dynamics during RNP-II elongation [14,20,71]. This prompted us to ask whether FACT might also influence Spt5-P levels in vivo. There was no reduction in levels of Spt5-P, relative to total Spt5, in a strain deleted for pob3Δ, which encodes a subunit of FACT (Figure S5A). This argues that promotion of Spt5-P reflects a FACT-independent function of H2Bub1.

Another consequence of H2Bub1 loss is reduction in H3K4me—a chromatin modification strongly implicated in gene activation. We showed previously, however, that loss of H2Bub1 caused more severe phenotypes than did ablation of H3K4me due to deletion of set1Δ. Those phenotypes include increased frequency of cells with division septa in asynchronous populations, and the appearance of cells with abnormal septation patterns (multiple septa separating two nuclei and unseparated chains of cells in which septa had formed) [12]. To test whether impaired Spt5-Thr1 phosphorylation might account for the more severe phenotypes caused by H2Bub1 loss, relative to those due to absence of H3K4me, we constructed an spt5-T1A set1Δ double mutant; the combination, which abolished two protein modifications shown to depend on H2Bub1, phenocopied the increased and aberrant septation produced by H2Bub1 loss (Figure 3C, 3D). Unlike the hbl1-K119R and brl2A mutants, a set1Δ mutant showed no reduction in Spt5 phosphorylation (Figure 3E). Conversely, the spt5-T1A mutant retained detectable levels of H3K4me3 despite reduced H2Bub1 (Figure 3F). Therefore, H2Bub1 promotes Spt5-P and H3K4me by independent pathways. Importantly, the spt5-T1A mutation by itself did not cause morphological defects, even though it lowered H2Bub1 levels. We attribute this to the residual Set1 function present in the spt5-T1A strain (Figure 3F). A similar double mutant carrying spt5-T1E was morphologically normal, as was an spt5-T1A set2A strain, confirming the specificity of the genetic interaction (Figure 3C, 3D). (The pob3Δ mutation had no effect on cell morphology by itself, and did not modify phenotypes caused by hbl1-K119R, suggesting that these effects are also FACT-independent [Figure S3B]). Together, these results suggest that Cdk9 and Set1 operate downstream of H2Bub1 to modify components of the transcription machinery and chromatin, respectively, and thereby regulate transcript elongation.

Next, to investigate the basis for reduced Spt5-P in H2Bub1-deficient cells, we measured Spt5-P occupancy at specific genes in the hbl1-K119R mutant by ChIP. Control experiments confirmed that the Spt5-P antibody was suitable for immunoprecipitation and ChIP (Figure S6A, S6B). As predicted by immunoblot results, treatment of cdks9Δ spt5-myc cells with 3-MB-PP1 abolished the Spt5-P ChIP signal without affecting total Spt5 recruitment.

We then compared cross-linking of Spt5-P to chromatin in hbl1Δ and hbl1-K119R cells, at the Cdk9-sensitive eng1Δ and Cdk9-insensitive amy1Δ genes [51] (Figure 4A and 4B); and at two loci (mp1809 and SPBC354.10Δ) that, according to our RNP-II ChIP-chip analysis, displayed a 3′ shift in RNP-II distribution in the absence of H2Bub1 (Figure 5A, 5B). A non-transcribed sequence served as a negative control (Figure S8). Although Spt5-P was broadly enriched within gene coding regions, as we observed for H2Bub1, the

Figure 2. H2Bub1 depends on Cdk9 activity and Spt5 phosphorylation. (A) Immunoblots of whole-cell extracts from wild-type (wt) (JS78) or A5 mutant strains (LV7, LV77, LV42), as indicated, grown in the absence (−) or presence (+) of 20 μM 3-MB-PP1, added 20 min prior to harvest. Antibodies are indicated at right. “T212A” and “T212E” denote strains cdk9-T212A (HD7-24) and cdk9-T212E (HG127). (B) Immunoblots of extracts from indicated strains probed for total histone H2B and H2Bub1, as indicated at right. “T212A” and “T212E” denote strains cdk9-T212A (HD7-24) and cdk9-T212E (HG127). (C) Immunoblots of extracts from wild-type (JS78) or indicated spt5 mutant strains. doi:10.1371/journal.pgen.1002822.g002

doses up to 40 μM (Figure S4B, S4C). We conclude that Cdk9 is the major, and possibly sole, kinase responsible for phosphorylation of Spt5-Thr1 in vivo, and that levels of Spt5-P are sensitive even to sublethal reductions in Cdk9 activity.
Figure 3. H2Bub1 independently stimulates Cdk9-mediated Spt5 phosphorylation and Set1-dependent H3K4 methylation. (A) Immunoblots of extracts from strains carrying spt5-myc with or without an as kinase allele as indicated (CS111, CS112, CS155, CS159, LV125 and LV167, respectively). Cultures were grown in the absence (−) or presence (+) of 20 μM 3-MB-PP1, added 20 min prior to harvest. Antibody reactivities are indicated at right. (B) Immunoblots of extracts from indicated strains (JS78, JTB67-1, JTB62-1, JTB331, HD7-24 and JTB297, respectively), probed for phosphorylated (Spt5-P) or total Spt5. (C) Fluorescent images of DAPI/calcofluor-stained cells from indicated strains (spt5-WT, JTB350, spt5-T1A, JTB352, spt5-T1E, JTB354, respectively). (D) Quantification of abnormal septation in strains of indicated genotypes (JTB67-1, spt5-WT, JTB350, spt5-T1A, JTB352, spt5-T1E, JTB354, JTB418 and JTB428, respectively). Error bars represent standard deviations from 2 independent experiments; at least 200
cells were counted in each. (E) Immunoblots of extracts from indicated strains [JTB204, JTB80-2, and JTB331, respectively], probed for phosphorylated (Spt5-P) or total Spt5. (F) Immunoblots of extracts from indicated strains [JTB204, JTB80-2, JTB67-1, spt5-W7, spt5-T1A, and spt5-T1E, respectively], probed for H3K4me3 or total H3.

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patterns of Spt5-P and H2Bub1 crosslinking across eng1" and amo1" differed, suggesting that the interdependence between these modifications is influenced by other, potentially locus-specific factors [Figure 4A, 4B, 4I, and 4J]. Nevertheless, levels of Spt5-P were reduced to varying degrees on all four genes in the htb1-K119R mutant [Figure 4A and 4B, Figure S7A and S7B]. Total Spt5 crosslinking was unchanged in htb1-K119R relative to wild-type cells, and the effect on Spt5-P was most evident near the 5' ends of genes we tested, suggesting that H2Bub1 promotes co-transcriptional phosphorylation (but not recruitment) of Spt5 during the transition from initiation to elongation (Figure 4C-4F; Figure S7C-S7F).

We hypothesized that the reduction in Sp5-P could be due to a defect in recruitment of Cdk9 to chromatin in the htb1-K119R mutant. To test this possibility we compared Cdk9 crosslinking to eng1", amo1", nap189" and SPT5 (or "nup189"") and SPBC2.54.10" by ChIP in htb1" cdk9-myc and htb1-K119R cdk9-myc strains [Figure 4G and 4H, Figure S7G and S7H]. This analysis revealed that Cdk9 recruitment was impaired at all four loci in htb1-K119R cells, consistent with the reduction in Sp5-P signals. Diminished Sp5-P and Cdk9 crosslinking to these loci was not an indirect consequence of reduced RNApol II recruitment, because RNApol II crosslinking and mRNA levels were not reduced at three of the four genes examined (Figure S9). We conclude that lack of H2Bub1 specifically impeded Cdk9 recruitment to transcribed chromatin, leading to impaired phosphorylation of Spt5 within gene coding regions.

**Mutual genetic suppression by P-TEFb and H2Bub1 pathway mutations**

Biochemical analyses revealed a reciprocal relationship between Sp5-P and H2Bub1, suggestive of a positive feedback loop. The synthetic phenotype of htb1-K119R mutant exacerbated 3-MB-PP1 sensitivity of H2Bub1 cells, consistent with the two pathways acting antagonistically. Absence of H2Bub1 did not fully bypass the requirement for Cdk9 activity, however, because higher doses of 3-MB-PP1 were still capable of arresting proliferation of cdk9" htb1-K119R cells.

In the absence of inhibitory analogs, cdk9" htb1-K119R cells displayed hyperseptated and branched morphologies characteristic of H2Bub1-defective mutants. After treatment with 10 μM 3-MB-PP1 for 7 hr, however, cell morphology reverted to normal (Figure 3B). This effect was reversible; after washout of 3-MB-PP1 and return to drug-free medium, the "reverted" cdk9" htb1-K119R cells re-acquired an aberrant, hyperseptated morphology.

To confirm that impairment of Cdk9 function could suppress phenotypes caused by the absence of H2Bub1, we combined a cdk9-T212E mutation, which prevents activation of Cdk9 by a CAK but allows cell viability [50], with htb1-K119R or htb2A. As was the case with chemical inhibition of Cdk9, cdk9-T212E rescued morphologic phenotypes of H2Bub1-defective mutants (Figure 3C, 3D). Importantly, there was no suppression of htb1-K119R or htb2A by lsk1 loss-of-function alleles (Figure 5D and data not shown), establishing the specificity of the interaction between Cdk9 and H2Bub1. Similarly, neither cdk9-T212E nor msc6-S165A could correct the aberrant morphologies of htb1-K119R cells (Figure 5C). Reversal of seption phenotypes by cdk9-T212A was accompanied by loss of the flocculation observed in htb1-K119R single mutants; ~15% of wild-type or cdk9-T212A htb1-K119R double mutant cells settled out of liquid cultures after 1 hr, compared to ~60% of cdk9" htb1-K119R cells (Figure 5E), consistent with suppression of htb1-K119R by reduction of Cdk9 activity.

We next asked if aberrant morphologies due to htb1-K119R could be suppressed by mutations in the known Cdk9 substrates: Spt5 and Rbpl [51]. By itself, spo5-T1A did not modify htb1-K119R phenotypes (Figure 6A), implicating another Cdk9 target (or targets) in the observed suppression. We attempted to verify this by combining cdk9" with spo5-T1A in an htb1-K119R background, to allow selective inhibition of Cdk9 in the absence of Sp5-Thr1 phosphorylation. Unexpectedly, the triple-mutant cells grown in the absence of inhibitory analogs had nearly normal morphology—a seption index close to that of the wild-type strain, <5% multiseptated cells and no chained cells (Figure 6B). In vitro, the activity of Cdk9" was reduced ~3-fold relative to that of wild-type Cdk9, even in the absence of drugs (Figure S10). This is likely to be due to decreased affinity for ATP—a known consequence of the gatekeeper mutation in other AS kinases [72,73]. The data suggest that loss of Sp5-Thr1 phosphorylation, combined with the partial reduction of Cdk9 activity, suppressed morphological abnormalities caused by loss of H2Bub1.

These results reveal multiple, distinct roles for Sp5-Thr1 phosphorylation in mediating regulatory interactions between H2Bub1 and Cdk9. When Cdk9 activity levels are normal, Sp5-P serves, in combination with Set1, to promote H2Bub1 functions in maintaining normal cell morphology [Figure 3C, 3D]. When H2Bub1 is absent, however, Sp5-P, together with at least one other Cdk9-dependent pathway, contributes to aberrant and excessive septation (Figure 6B). A similar distinction between specific elimination of Sp5-P and a general reduction in Cdk9 activity is apparent in a set1A background, in which spo5-T1A resulted in cell-morphology defects but cdk9-T212A had no effect (Figure 3D). Thus, the positive feedback between H2Bub1 and Cdk9, involving a single Cdk9 target (Spo5), is genetically distinguishable from the antagonism, which involves multiple targets (Sp5 and one or more others).

Cdk9 also contributes to phosphorylation of Ser2 and Ser5 of RNApol II CTD repeats [50,51,74]. An rpb1-S2A mutation that replaced Ser2 in all repeats with Ala in the context of a truncated but still functional CTD [53] failed to suppress htb1-K119R in either a cdk9" or cdk9" background (Figure 6C). Combined loss of both Sp5-Thr1 and Rpb1-Ser2 in a cdk9" background also did not suppress htb1-K119R seption phenotypes. Similarly, in a cdk9" background, spo5-T1A but not rpb1-S2A suppressed flocculation due to an htb1-K119R mutation (Figure 6D). Therefore, diminished Cdk9 activity must rescue phenotypes caused by H2Bub1 loss through reduced phosphorylation of Sp5-Thr1 and another target (or targets) besides Rpb1-Ser2.

The suppression of hyperseptation and flocculation was not simply a consequence of reduced growth rate, because hyperseptated, flocculating cdk9" spo5-T1A htb1-K119R cells and suppressed cdk9" spo5-T1A htb1-K119R cells grew at similar rates in liquid culture. Moreover, the non-suppressed, cdk9" rpb1-S2A
Figure 4. H2Bub1 enhances Cdk9 recruitment and Spt5 phosphorylation at transcribed genes. (A,B) Spt5 phosphorylation was measured by ChIP using anti-Spt5-P in spt5-myc (MS265; black bars) and spt5-myc htb1-K119R (LV239; gray bars) strains and quantified at the indicated genes by qPCR. Enrichment is plotted as a percentage of input signal for each primer pair. Positions of PCR primer pairs within coding regions are indicated.
schematically at top. (C,D) Spt5-myc occupancy was measured by ChIP as in A and B. (E,F) Spt5-P enrichment normalized to total Spt5-myc occupancy. (G,H) Cdk9-myc occupancy was measured by ChIP in cdk9-myc (MS264; black bars) and cdk9-myc htb1-K119R (KL259; gray bars) strains. (I,J) H2Bub1 enrichment was measured by ChIP and normalized to H2B-FLAG occupancy. Error bars denote standard deviations from 3 independent experiments. Asterisks denote a significant difference between wild-type and mutant (**p < 0.04, ****p < 0.02, unpaired t-test). doi:10.1371/journal.pgen.1002822.g004

htb1-K119R strain grew more slowly than did the suppressed strain (Figure 6E). These data indicate that suppression of htb1-K119R is a specific consequence of reduced Cdk9 activity.

**RNAPII redistribution due to htb1-K119R depends on Cdk9 activity**

Our results thus far indicated that loss of H2Bub1 partially alleviated the cellular requirement for Cdk9 activity and that, conversely, selective impairment of Cdk9 function suppressed phenotypes of H2Bub1-defective mutants. This mutual suppression led us to hypothesize that P-TEFb and H2Bub1 might act antagonistically to control transcription elongation. We tested such an interaction by asking whether a reduction in Cdk9 activity could reverse the altered RNAPII distribution observed in an htb1-K119R mutant. We analyzed genome-wide RNAPII crosslinking by ChIP-chip in cdk9-T212A and cdk9-T212A htb1-K119R strains (Figure 7; Datasets S4 and S5). The cdk9-T212A mutation caused a global redistribution of RNAPII occupancy that was essentially opposite to that observed in the htb1-K119R strain: an increase in RNAPII density in the 5′ halves of genes, and a decrease toward the 3′ ends of genes that was particularly significant at highly transcribed loci (compare Figure 7C and Figure 1D). Impaired Cdk9 activity also led to reversal of the RNAPII 3′ shift caused by htb1-K119R (Figure 7F). Global RNAPII occupancy profiles in cdk9-T212A htb1-Thr7 and cdk9-T212A htb1-K119R mutants were nearly identical, suggesting that, when Cdk9 activity was reduced, H2Bub1 had little or no effect on RNAPII dynamics at the majority of genes (Figure 7A–7E). Loss of H2Bub1 reduced RNAPII occupancy within the 5′ halves of genes even in the context of reduced Cdk9 activity, however, suggesting that this effect is partially Cdk9-independent (Figure 7D, 7E). Nonetheless, full Cdk9 activity is required for the increase in RNAPII density at the 3′ ends of genes in the absence of H2Bub1 (Figure 7E). These data argue that P-TEFb and H2Bub1 antagonize one another to govern the distribution of elongating RNAPII within genes (Figure 8).

**Discussion**

Here we combined chemical genetics and genomics to reveal reciprocal relationships between a CDK and a co-transcriptional histone modification in fission yeast. First, we showed interdependence of Cdk9-mediated Spt5 phosphorylation and H2Bub1—a specific and sensitive indicator of Cdk9 activity allowed us to uncover another, unexpected relationship between P-TEFb and H2Bub1 in vivo: Cdk9-mediated phosphorylation of Spt5 depends on H2Bub1. ChIP analysis suggested a possible explanation—decreased recruitment of Cdk9 to H2Bub1-deficient chromatin. In mammalian cells, basal association of P-TEFb with the HIV-1 promoter decreased upon depletion of the Br1 ortholog RNPF20, consistent with a conserved role for H2Bub1 in P-TEFb recruitment [80]. Based on our data and results in budding yeast, we propose that a positive feedback loop connects Cdk9 activity and H2Bub1 through the sequence: 1) phosphorylation of Spt5 by Cdk9; 2) PAF recruitment by Spt5-P; 3) recruitment and/or activation of the Rhp6 (S. pombe ortholog of Rad6)-Brl1/2 complex by PAF, to generate H2Bub1; and 4) Cdk9 recruitment, leading to reiteration of the cycle (Figure 8). Although the mechanism by which H2Bub1 facilitates Cdk9 recruitment remains to be determined, there is precedence in metazoan P-TEFb association with modified histones [44,45].

**Two independent protein modification pathways downstream of H2Bub1**

Our results indicate that H3K4me and Spt5-P are independently stimulated by H2Bub1 in vivo. Importantly, elimination of either modification alone did not cause septation phenotypes, but their combined ablation phenocopied the aberrant cell morphologies produced by H2Bub1 loss. Therefore, we have identified a molecular intermediate—Spt5-P—in the H3K4me-independent pathway downstream of H2Bub1 (Figure 6). A challenge for the future will be to ascertain how Set1 cooperates with Cdk9, acting through Spt5-Thr1, to regulate gene expression.

Cdk9-H2Bub1 antagonism during RNAPII elongation

The mutually reinforcing relationship between Cdk9 activity and H2Bub1 seemed to predict the two would act in concert during transcription. Instead, genetic analysis indicated they work in opposition: loss of H2Bub1 partially relieved the requirement for Cdk9 activity in vivo, and reduction in Cdk9 activity suppressed cell-morphology phenotypes of H2Bub1-deficient
mutants. Simultaneous mutation of two known sites of Cdk9-dependent phosphorylation (Sp5-Thr1 and Rpb1-Ser2) did not suppress htb1-K119R, implicating at least one other Cdk9 target in opposing H2Bub1 function. Consistent with the genetic interaction, Cdk9 and H2Bub1 exerted inverse effects on global RNA Pol II distribution: whereas RNA Pol II density shifted toward the 3′ ends of genes in an htb1-K119R mutant, lowering Cdk9 activity produced the opposite effect in an htb1− background, and prevented the 3′ shift in the double-mutant strain.

The antagonism we observe between Cdk9 activity and H2Bub1 is incomplete—loss of H2Bub1 did not entirely bypass the essential function of Cdk9, and led to altered RNA Pol II occupancy in the 5′ portions of genes even when Cdk9 activity was reduced. Conversely, although the presence of cdk9-T212A eliminated aberrant cell morphologies associated with htb1-K119R, the growth rate of double mutant cells was slower than that of either single mutant (data not shown). These results imply that other pathways can modulate the functions of Cdk9 and H2Bub1 in transcript elongation.

Our results strengthen the notion that P-TEFb activity is required to overcome repressive effects of histone modifications, first suggested by work in budding yeast. In S. cerevisiae, the instructive genetic interactions occurred between BUR1 and the H3K36 methyltransferase Set2; lethality of a bur1 null mutation was suppressed by deletion of SET2 [34,48]. In fission yeast, however, set1 deletion did not suppress growth arrest due to inhibition of Cdk9 in vivo (L.V. and R.P.F., unpublished observations). Interestingly, bur1Δ was also weakly suppressed by deletion of RAD6 [40], implying that a regulatory interaction between P-TEFb and H2Bub1 might be conserved in budding yeast.

In mammalian cells, the Brf1/Bril ortholog RNF20 selectively represses transcription of genes implicated in tumorigenesis; its knockdown reduces H2Bub1, enhances the transcriptional response to growth factors and promotes cellular transformation [13]. These effects were recently shown to depend on the transcription elongation factor TFIIH [81]. This extends the parallel between mammals and fission yeast, and suggests that H2Bub1 selectively modulates gene expression in both settings by restraining transcript elongation, albeit through different factors—TFIIH or P-TEFb, respectively. Given the established dependence of H2Bub1 on Cdk9 activity in mammalian cells [30,31], it will now be important to ask if metazoan P-TEFb function is also reciprocally influenced by H2Bub1.

Evidence for a chromatin-based checkpoint in RNA Pol II elongation

How can we reconcile biochemical evidence for mutual dependence of Cdk9 activity and H2Bub1 with genetic evidence for their antagonism? We propose a homeostatic mechanism whereby Cdk9-H2Bub1 interdependence ensures a balance between their opposing functions. In this scenario, Cdk9 promotes H2Bub1 to regulate its own effects on elongation (Figure 8). In an htb1-K119R mutant, the balance is disrupted, resulting in Cdk9-dependent accumulation of RNA Pol II in 3′ regions of genes. We suggest this pattern reflects unchecked elongation that is poorly responsive to mRNA processing signals, and may imply enhancement of the RNA Pol II pausing normally associated with mRNA 3′ end processing [82,83]. H2Bub1 has been linked to various aspects of RNA processing in budding yeast and in mammalian cells, including splicing, 3′ end formation, and nuclear export [30,57,84–86]. In fission yeast, Cdk9 has been implicated in mRNA 5′-end formation, through its role in recruiting a capping enzyme to transcribed chromatin [87]. Still to be determined are the full range of H2Bub1-dependent functions that depend on P-TEFb activity and the mechanism(s) by which H2Bub1 and Cdk9 influence each other.

In cell division, faithful genome duplication and segregation are ensured by checkpoints—extrinsic signaling pathways that enforce dependency between intrinsically independent events [88]. Transcription and mRNA-processing could be intrinsically coupled through chromatin structure, because removal of nucleosomal barriers is likely to be a limiting step for elongation [89]. Here we have uncovered a role for a covalent chromatin modification, H2Bub1, in regulating elongation through an extrinsic pathway involving Cdk9 and Sp5. We propose a checkpoint-like function of H2Bub1, to set thresholds of Cdk9 and Sp5. In an htb1-K119R mutant, the balance is disrupted, resulting in Cdk9-dependent accumulation of RNA Pol II in 3′ regions of genes. We suggest this pattern reflects unchecked elongation that is poorly responsive to mRNA processing signals, and may imply enhancement of the RNA Pol II pausing normally associated with mRNA 3′ end processing [82,83]. H2Bub1 has been linked to various aspects of RNA processing in budding yeast and in mammalian cells, including splicing, 3′ end formation, and nuclear export [30,57,84–86]. In fission yeast, Cdk9 has been implicated in mRNA 5′-end formation, through its role in recruiting a capping enzyme to transcribed chromatin [87]. Still to be determined are the full range of H2Bub1-dependent functions that depend on P-TEFb activity and the mechanism(s) by which H2Bub1 and Cdk9 influence each other.

Materials and Methods

S. pombe strains and media

Strains used in this study are listed in Table S2. Cells were grown in YE medium containing 250 mg/L each of adenine, leucine, histidine, and uracil (YES). Strains were constructed by standard genetic techniques [90].

Immunological methods

To detect Sp5 phosphorylation by immunoblotting, S. pombe whole-cell extracts were prepared as previously described [37,91]. To detect chromatin modifications, cells were lysed in trichloroacetic acid, as described [92]. The Sp5-P antibody was raised against the peptide acetyl-NSGNK[pT]PAWNVGNK[pT]-PAWNSC-amide injected into rabbits, and purified from whole
Figure 6. Cdk9 activity towards multiple substrates is required for abnormal morphologies of H2Bub1-deficient cells. (A) Quantification of abnormal septation patterns in the indicated htb1-FLAG strains (JTB378, JTB351, JTB379, JTB353, JTB380, JTB355). Error bars represent standard deviations from 2 independent experiments; at least 200 cells were counted in each. (B) As in (A) for the indicated cdk9as strains (KL289, KL291, KL293). Cells were grown in the presence of either DMSO or 3-MB-PP1 as described in Figure 5B. (C) As in (A) for the indicated htb1-FLAG strains (MS260, MS256, MS261, MS257, MS272, MS259). (D) Flocculation of indicated strains (JS78, JTB67-1, JTB351, JTB353, KL291, KL293, MS259) was quantified as described in Materials and Methods. (E) Growth rates in liquid rich medium (YES) were measured for strains analyzed in (D).

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serum after depletion with the same peptide in unphosphorylated form immobilized on resin, by adsorption to and elution from resin-bound acetyl-AWNSGSK[pT]PAWNSGSC-amide by 21st Century Biochemicals (Marlboro, MA). Other antibodies are listed in Text S1.

Chromatin immunoprecipitation and microarray analysis

ChIP was carried out as described previously [12,93]. Amplification, labeling, and hybridization of control and immunoprecipitated DNA samples for ChIP-chip were carried out as described previously and normalized data are included as Datasets.
S1, S2, S3, S4, S5 [94]. Further details are included in Text S1. Sequences of primers used for qPCR analysis are available upon request.

**Microscopy**

_S. pombe_ cells were fixed and stained with diamino-phenylindole (DAPI) and calcofluor as described previously [51]. Cells were viewed using a Leica DM5000b microscope and photographed with a CCD camera. Images were processed using Volocity software.

**Chemical genetic methods**

Cultures were treated with 3-MB-PP1 at indicated doses, or with vehicle (DMSO), for indicated times at 30°C prior to extract preparation. Dose-response to 3-MB-PP1 was determined as previously described [51]. To analyze effects of 3-MB-PP1 on cell morphologies, cells were grown at 30°C to OD600~0.1 in YES and treated with 10 μM 3-MB-PP1 or DMSO for 7 hr, then fixed and stained for microscopy immediately or after return to growth in drug-free YES for 7 hr.

**Flocculation assays**

Assays were carried out as described previously [95].

**Supporting Information**

**Dataset S1** Microarray data for ChIP-chip performed with a histone H3 antibody (wild-type).

**Dataset S2** Microarray data for ChIP-chip performed with a histone H3 antibody (htb1-K119R).

**Dataset S3** Microarray data for ChIP-chip performed with a RNAPII antibody (cdk9-T212A).

**Dataset S4** Microarray data for ChIP-chip performed with a RNAPII antibody (cdk9-T212A htb1-K119R).

**Dataset S5** BED file containing microarray data for ChIP-chip performed with a RNAPII antibody (cdk9-T212A htb1-K119R).

**Figure S1** Specificity of H2Bub1 antibody for ChIP in _S. pombe_. (A) H2Bub1 occupancy was measured by ChIP in wild-type (JTB62-1) and _htb1-K119R_ (JTB67-1) strains and quantified at the inactive _hsp16* gene_ and constitutive _act1* gene_. Values were normalized to H2B-FLAG occupancy measured in parallel to control for differences in nucleosome density. Positions of primer pairs used for qPCR are indicated in schematic at top. (B) Immunoblots of whole-cell extracts from untagged (JTB204), _htb1-FLAG_ (JTB62-1), and _htb1-K119R-FLAG_ (JTB67-1) strains using the indicated antibodies.

**Figure S2** Loss of H2Bub1 does not significantly alter nucleosome density in _S. pombe_. (A) Average distribution of histone H3 at 540 _S. pombe_ genes, as determined by ChIP-chip in wild-type strain (JTB62-1). Genes were grouped according to total levels of RNAPII enrichment (see key at top). The grey box in the “average gene” representation at bottom denotes the gene coding region; 5’ and 3’...
untranslated regions are denoted by thin black lines. The arrow denotes the transcription start site. (B) As in (A), determined in an *htb1-K119R* mutant strain (JTB67-1). Gene groupings were created using wild-type RNAPII enrichment values. (C) Average distribution of differences between mutant and wild-type histone H3 enrichment grouped according to RNAPII enrichment in wild-type cells. The key below the graph illustrates the statistical significance of the differences for each group at 50 positions along the average gene. The rows of the key are color-coded according to the graph. Open squares denote p>0.01; light shading denotes 0.01>p>10exp-5 (one-sample t-tests; \( \mu_0 = 0 \)).

**Figure S3** Chromatin association of Rhp6 and the PAF protein Rtf1 requires Cdk9 activity. (A) Rhp6-TAP occupancy was measured by ChIP in the indicated strains and quantified at the *eng1* gene by qPCR. Enrichment is plotted as percentage of the input signal for each primer pair. Positions of PCR primer pairs are indicated in the schematic at top. (B) Rtf1-TAP occupancy was measured by ChIP in the indicated strains and quantified at the *act1* gene by qPCR. Enrichment is plotted as percentage of the input signal for each primer pair. Positions of PCR primer pairs are indicated in the schematic at top. In all cases, treatment of the *cdk9* strains was with either DMSO (−) or 20 μM 3-MBP-PP1 for 3 hr prior to harvesting. Error bars denote standard deviations from three independent experiments.

**Figure S4** Validation of Spt5 as an exclusive Cdk9 target using a phospho-specific Spt5 antibody. (A) Kinase reactions containing recombinant GST-Spt5 substrate and indicated combinations of purified, recombinant Cdk9/Pch1 and Csk1 were analyzed by immunoblotting with: affinity-purified antibody specific for phosphorylated Spt5 (Spt5-P); crude serum specific for wild-type Spt5 CTD repeats, regardless of phosphorylation state (Spt5-CTD; see Text S1); or anti-GST antibody (GST). (B,C) Immunoblots of whole-cell extracts from indicated strains and Cdk9 was measured by ChIP using anti-myc or anti-Spt5-P in wild-type (JTB62-1; black bars) and *htb1-K119R* (LV239; gray bars) strains and quantified at the indicated genes by qPCR. In all graphs of this figure the enrichment is plotted as percentage of the input signal for each primer pair, positions of PCR primer pairs within coding regions are indicated in the schematic at top, error bars denote standard deviations from 3 independent experiments and asterisks denote a significant difference between wild-type and mutant (\(^*^\) \( p<0.04 \), \(^{**}\) \( p<0.02 \); unpaired t-test). (D) Spt5-myc occupancy was measured by ChIP as in C and D. (E,F) Spt5-P enrichment normalized to total Spt5-myc occupancy. (G,H) Cdk9-myc occupancy was measured by ChIP in *cdk9*-myc (MS264; black bars) and *cdk9*-myc *htb1-K119R* (KL259; gray bars) strains.

**Figure S5** The FACT subunit Pob3 has functions distinct from those of H2Bub1. (A) Immunoblots of whole-cell extracts from wild-type (JTB204), *brl2* (JTB331), *cdk9*-T212A (HD7-24), and *pob3A* (JTB281-1) strains using the indicated antibodies. (B) Quantification of abnormal septation in strains of indicated genotypes (JTB317 and JTB318, respectively). Error bars represent standard deviations from 2 independent experiments; at least 200 cells were counted in each.

**Figure S6** Anti-Spt5-P specifically immunoprecipitates Spt5-P from wild-type and *spt5-myc* strains. (A) Spt5-P immunoprecipitation from whole-cell extracts. Extracts from indicated strains (bottom) were incubated with or without anti-Spt5-P (top), and immunoprecipitates were analyzed by immunoblotting with antibodies indicated at right. The *cdk9* strain was treated prior to extract preparation with either DMSO or 5 μM 3-MBP-PP1, as indicated at bottom, for 20 min. For each treatment we analyzed 10% of input extract ("input"), 10% of supernatant after precipitation ("sup") and 50% of immunoprecipitated material ("IP"). Antibodies are indicated at right. (B) ChIP of Spt5-P and Spt5-myc at *eng1*. Patterns of Spt5-P occupancy on *eng1* are similar in wild-type ("WT"; top; JST70) and *spt5-myc* (middle; CS111) strains. Distribution of total Spt5 on *eng1* was measured in the *spt5-myc* strain (bottom). (C) Spt5-myc (top) and Spt5-P (middle) occupancies were measured by ChIP in a *cdk9*/*spt5-myc* strain and quantified at indicated positions within *eng1* by qPCR. Cells were treated prior to lysis with either DMSO or 5 μM 3-MBP-PP1 for 20 min. Enrichment is plotted as percentage of input signal for each primer pair. The ratios of *Spt5-P/Spt-myc* at each position are plotted at bottom.

**Figure S7** Impact of H2Bub1 on Spt5 phosphorylation and Cdk9 recruitment at *sup189* and *SPB3*4*10*. (A,B) Spt5 phosphorylation was measured by ChIP using anti-Spt5-P in *spt5-myc* (MS265; black bars) and *spt5-myc *htb1-K119R* (LV239; gray bars) strains and quantified at the indicated genes by qPCR. In all graphs of this figure the enrichment is plotted as percentage of the input signal for each primer pair, positions of PCR primer pairs within coding regions are indicated in the schematic at top, error bars denote standard deviations from 3 independent experiments and asterisks denote a significant difference between wild-type and mutant (\(^*^\) \( p<0.04 \), \(^{**}\) \( p<0.02 \); unpaired t-test). (C,D) Spt5-myc occupancy was measured by ChIP as in C and D. (E,F) Spt5-P enrichment normalized to total Spt5-myc occupancy. (G,H) Cdk9-myc occupancy was measured by ChIP in *cdk9*-myc (MS264; black bars) and *cdk9*-myc *htb1-K119R* (KL259; gray bars) strains.

**Figure S8** ChIP of Spt5-P, Spt5-myc, and Cdk9-myc at an intergenic sequence. (A) Occupancy of total Spt5, phospho-Spt5 and Cdk9 was measured by ChIP using anti-myc or anti-Spt5-P in wild-type strains (MS265 for Spt5-myc and Spt5-P IP; MS264 for Cdk9-myc IP), followed by qPCR using primers covering the 5’ end of *eng1* (*eng1*-1) and an intergenic region as control (control). In all graphs of this figure the enrichment is plotted as percentage of the input signal for each primer pair and error bars denote standard deviations from 3 independent experiments. (B) Same experiment as in (A) in *htb1-K119R* strains (LV239 for Spt5-myc and Spt5-P IP; KL259 for Cdk9-myc IP).

**Figure S9** Impact of H2Bub1 on RNAPII occupancy and mRNA expression at individual genes. (A-D) RNAPII occupancy was measured by ChIP in wild-type (JTB62-1; black bars) and *htb1-K119R* (JTB67-1; gray bars) strains and quantified at the indicated genes by qPCR. Enrichment is plotted as percentage of the input signal for each primer pair, and positions of PCR primer pairs within coding regions are indicated in the schematic at top of each graph. (E) Levels of mRNA from the indicated genes were quantified by qRT-PCR and normalized to *act1*. Throughout this figure error bars denote standard deviations from 3 independent experiments and asterisks denote a significant difference between wild-type and mutant (\(^*^\) \( p<0.04 \), \(^{**}\) \( p<0.02 \); unpaired t-test).

**Figure S10** Kinase activity of Cdk9 and Cdk9*mut* complexes purified from *S. pombe*. Lysates from *pcm1-myc* (CS145) or *cdk9*- *pcm1-myc* (CS165) strains were incubated either with anti-myc-bound Protein G beads (α-myc-beads) to co-immunoprecipitate Cdk9 bound to Pcm1-myc or with protein G alone (mock). Input lysate amounts in each immunoprecipitation were varied as...
indicated. Immunoprecipitates were analyzed by immunoblotting (top panels) and for Rpb1-CTD kinase activity (middle panels). Phosphorylation signals were visualized by autoradiography and quantified with a phosphorimager (bottom).

(PDF)

Table S1  RNAPII distributions at H2Bub1-stimulated genes in the htb1-K119R strain.


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Table S2  S. panbe strains used in this study.


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Text S1  Supplemental materials and methods.


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References


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Author Contributions

Conceived and designed the experiments: P-EJ FR. Performed the experiments: MS LV KML VP SN AR JCT. Analyzed the data: P-EJ FR MS JCT. Contributed reagents/materials/analysis tools: CVSA CZ KMS BS. Wrote the paper: MS LV KML RPF JCT.