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New connections between ubiquitylation and methylation in the co-transcriptional histone modification network

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Abstract

Co-transcriptional histone modifications are a ubiquitous feature of RNA polymerase II (RNAPII) transcription, with profound but incompletely understood effects on gene expression. Unlike the covalent marks found at promoters, which are thought to be instructive for transcriptional activation, these modifications occur in gene bodies as a result of transcription, which has made elucidation of their functions challenging. Here we review recent insights into the regulation and roles of two such modifications: monoubiquitylation of histone H2B at lysine 120 (H2Bub1) and methylation of histone H3 at lysine 36 (H3K36me). Both H2Bub1 and H3K36me are enriched in the coding regions of transcribed genes, with highly overlapping distributions, but they were thought to work largely independently. We highlight our recent demonstration that, as was previously shown for H3K36me, H2Bub1 signals to the histone deacetylase (HDAC) complex Rpd3S/Clr6-CII, and that Rpd3S/Clr6-CII and H2Bub1 function in the same pathway to repress aberrant antisense transcription initiating within gene coding regions. Moreover, both of these histone modification pathways are influenced by protein phosphorylation catalyzed by the cyclin-dependent kinases (CDKs) that regulate RNAPII elongation, chiefly Cdk9. Therefore, H2Bub1 and H3K36me are more tightly linked than previously thought, sharing both upstream regulatory inputs and downstream effectors. Moreover, these newfound connections suggest extensive, bidirectional signaling between RNAPII elongation complexes and chromatin-modifying enzymes, which helps to determine transcriptional outputs and should be a focus for future investigation.

Keywords

Co-transcriptional histone modification; H2Bub1; H3K36me

Introduction

Post-translational modification of histones has long been associated with regulation of RNA polymerase II (RNAPII) transcription. Early, ground-breaking studies identified

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known transcriptional co-activators or co-repressors as histone acetyltransferases (HATs) or deacetylases (HDACs), respectively (Brownell et al. 1996; Taunton et al. 1996). These factors are recruited at or upstream of gene promoters by DNA-binding transcriptional activators or repressors, and so were quickly established as being instructive for gene expression, such that the acetylation state of promoter-proximal nucleosomes helped determine whether a particular gene was transcribed or not (Kuo et al. 1996, 2000; Kadosh and Struhl 1997; Rundlett et al. 1998). This conceptual framework is still in place today [although it is apparent that non-histone proteins are also important enzymatic targets of "HATs" and "HDACs" (Zhang and Dent 2005)]. This simple picture has been complicated, however, by the discovery of a diverse array of additional histone modifications and the genome-wide profiling of modification patterns by chromatin immunoprecipitation (ChIP). In addition to acting prior to transcriptional activation, many covalent marks are placed on gene-body nucleosomes during transcription, by chromatin-modifying enzymes recruited to elongating RNAPII complexes, and play important but still emerging roles in the transcription cycle. Here we review recent studies that illuminate functions and regulation of these co-transcriptional, covalent histone modifications.

One outcome of these studies is the discovery of a highly conserved, stereotypical pattern of histone modifications within the coding regions of RNAPII transcription units (Li et al. 2007a; Rando 2007; Tanny 2014). A metagene diagram depicting the best-characterized features of the histone modification landscape on typical protein-coding genes is shown in Fig. 1A. Acetylation of multiple histone lysine residues is most highly enriched on nucleosomes immediately surrounding the transcription start site (TSS), consistent with a generally permissive role in transcription initiation. In contrast, site-specific methylation of histone H3 and monoubiquitylation of histone H2B (H2Bub1) are highly enriched within the coding region. Rather than being simply permissive or non-permissive for transcription, these modifications are dependent on ongoing transcription. Moreover, recent evidence indicates that the majority of histone acetylation likewise occurs as a consequence of transcription (Martin et al. 2021). These findings have given rise to a new conceptual framework for understanding the genesis and function of histone modification. The importance of these questions is underscored by the numerous connections that have been uncovered between the relevant histone-modifying enzymes and human disease, most notably cancer (Marsh and Dickson 2019; McDaniel and Strahl 2017).

Regulatory crosstalk between the different modifications is critical for shaping this pattern. For example, H2Bub1 is required for histone H3 lysine 79 methylation (H3K79me) and, to some extent, histone H3 lysine 4 methylation (H3K4me) (Chandrasekharan et al. 2010). Furthermore, H3K4me and H3K36me both regulate histone acetylation in gene coding regions (Buratowski and Kim 2010). Thus, patterning of histone modifications by RNAPII transcription can be a multi-step or combinatorial process, which may serve to expand or fine-tune the repertoire of functional outcomes. Here we focus on new insights from our labs and others into regulatory crosstalk between H3K36me and H2Bub1, two modifications that had been thought to operate largely independently.

Regulation and function of H3K36 methylation

Co-transcriptional deposition of H3K36me is triggered by phosphorylation of the carboxy-terminal domain (CTD) of the RNAPII large subunit Rpb1. The RNAPII CTD is composed of multiple repeats of a heptad motif with the consensus amino-acid sequence YSPTSPS (Jeronimo et al. 2016). It is an important binding target for many transcriptional regulators, and its interactions are regulated by phosphorylation of specific residues within the repeat. For example, phosphorylation of the serine 5 position (pSer5) peaks just downstream of the TSS, whereas phosphorylation of serine 2 (pSer2) is low at the TSS, increases in relative abundance with RNAPII passage through the coding region, and peaks downstream of the polyadenylation signal (PAS) (Fig. 1B). A family of transcription-associated cyclin-dependent kinases (CDKs) catalyze these phosphorylations during transcription. Cdk7 and Cdk9 are primarily implicated in placing pSer5, whereas Cdk9, Cdk12, and Cdk13 phosphorylate the Ser2 position (Sanso and Fisher 2013). The H3K36 methyltransferase Set2 associates directly with the RNAPII CTD phosphorylated at both serine residues (Kizer et al. 2005; Li et al. 2005). In vivo, the pSer2 form of the CTD is specifically required for H3K36 trimethylation (Youdell et al. 2008; Yoh et al. 2008). In yeast, all H3K36me is Set2-dependent; both the di-methyl and tri-methyl forms are enriched within gene coding regions (Fig. 1A) and have overlapping functions (Youdell et al. 2008; Li et al. 2009; DiFiore et al. 2020). Greater functional divergence between the di- and tri-methyl forms has been noted in mammalian cells, in which H3K36me2 is catalyzed by additional methyltransferases (such as NSD1 and NSD2) and can occur independently of RNAPII elongation (Kuo et al. 2011; Weinberg et al. 2019).

H3K36me acts by engaging factors that contain specific “reader” domains (McDaniel and Strahl 2017). H3K36me-specific readers harbor well-characterized methyl-lysine binding domains including PHD fingers, chromodomains, and PWWP domains (Arrowsmith and Schapira 2019). This last class of methyl-lysine binding domain is particularly enriched in H3K36me-binding factors and may be dedicated to H3K36me recognition. H3K36me function has been most extensively studied in budding yeast. Genetic and biochemical analyses have demonstrated that H3K36me is required for the function of Rpd3S, an HDAC complex that acts to maintain low levels of histone acetylation in the coding regions of active genes (Keogh et al. 2005; Carrozza et al. 2005). H3K36me is engaged by the chromodomain of the Eaf3 subunit of Rpd3S (Ruan et al. 2015). Mutations in Rpd3S components or in Set2 lead to hyperacetylation of histones in gene coding regions and also cause transcription to initiate inappropriately from these locations. These aberrant transcription events can result in ectopic sense transcripts that are truncated near their 5′ ends, or in antisense transcripts (Venkatesh et al. 2016; Li et al. 2007b). This phenotype, of aberrant intragenic transcription initiation, is characteristic of mutations thought to perturb chromatin structure during transcription, and was first described in connection with Spt6, an elongation factor and histone chaperone (Kaplan et al. 2003).

In addition to promoting Rpd3S function in gene coding regions, H3K36me blocks co-transcriptional histone exchange by regulating the histone chaperone Asf1 or the Isw chromatin remodeling complex; the latter function is mediated by interaction with a PWWP reader domain in Ioc4 (Smolle et al. 2012; Venkatesh et al. 2012). Histone hyperacetylation

correlates with increased histone exchange in the *set2*Δ mutant. Thus, the stabilization of transcribed chromatin by H3K36me may involve both enhanced deacetylase activity and reduced co-transcriptional histone exchange (Venkatesh et al. 2012).

Rpd3S is orthologous to the Sin3B HDAC complex in mammalian cells. A functional link to H3K36me is suggested by the fact that the MRG15 subunit of Sin3B is the ortholog of Eaf3 (Jelinic et al. 2011), although a role for the Sin3B complex in suppression of inappropriate initiation has not been established. H3K36me does participate in an analogous repressive mechanism, however, by recruiting the DNA methyltransferase DNMT3B to direct DNA CpG methylation in transcribed coding regions in embryonic stem cells (Baubec et al. 2015). Mutations that impair H3K36me binding by the DNMT3B PWWP domain lead to the accumulation of adventitious sense transcripts that initiate within gene coding regions (Neri et al. 2017).

Interestingly, Eaf3 is also a component of the HAT complex NuA4. Eaf3 forms a subassembly within this complex (with Eaf5 and the conserved Eaf7 subunit) that promotes its binding to H3K36me-containing nucleosomes (Sathianathan et al. 2016). There is also evidence that an Eaf3/5/7 complex associates with transcribed genes and regulates elongation as a module separate from the acetyltransferase (Rossetto et al. 2014). Other H3K36me-specific reader proteins also positively regulate elongation. For example, H3K36me helps to recruit the HAT complex NuA3, as well as the nucleosome disassembly factors NDF, LEDGF, and HDGF2 (all of which engage H3K36me through PWWP domains), to transcribed coding regions (Gilbert et al. 2014; Flury et al. 2017; Fei et al. 2018; LeRoy et al. 2019). Therefore, H3K36me seems to mediate a complex interplay among factors that make chromatin more or less permissive for transcription. How this balance is achieved at individual genes remains largely unknown.

An additional role for H3K36me, in co-transcriptional mRNA processing, is suggested by interaction of Eaf3 with the Prp45 subunit of the spliceosome (Leung et al. 2019). MRG15 also participates in splicing regulation in mammalian cells, although it acts through the splicing regulator PTB and not through the spliceosome directly (Luco et al. 2010).

Regulation and function of H2B mono-ubiquitylation

H2Bub1 is a dynamic marker of transcribed chromatin that is catalyzed by a complex composed of the E2 ubiquitin conjugating enzyme Rad6 and the E3 ubiquitin ligase Bre1. Rad6 and Bre1 target histone H2B on a conserved C-terminal lysine residue (corresponding to K120 in humans, K123 in *S. cerevisiae*, or K119 in *S. pombe*). It is removed during transcription by the de-ubiquitylation (DUB) module of the SAGA (Spt-Ada-Gcn5 acetyltransferase) co-activator complex, the catalytic component of which is Ubp8 (Fuchs and Oren 2014). Co-transcriptional formation of H2Bub1 depends on the activity of a Cdk9/cyclin complex, known in metazoans as positive transcription elongation factor b (P-TEFb), an essential CDK needed for rapid elongation by RNAPII (Tanny 2014; Fuchs et al. 2014; Sanso et al. 2012; Pirngruber et al. 2009). The Cdk9 substrate most clearly linked to H2Bub1 is Spt5, one subunit of a conserved, heterodimeric transcription elongation and processivity factor known in metazoans as the DRB sensitivity-inducing

factor (DSIF) (Sanso et al. 2012; Mbogning et al. 2015). Cdk9 phosphorylates Spt5 on its carboxy-terminal repeats (CTRs, which may be functionally analogous to the CTD on the RNAPII large subunit); this form of Spt5 (pSpt5) peaks in abundance just downstream of the TSS, is thought to enhance RNAPII elongation rate, and is distributed throughout the coding region, as is the case for H2Bub1 (Fig. 1A,B) (Sanso et al. 2020; Parua et al. 2018; Cortazar et al. 2019). Another elongation factor, Rtf1, bridges pSpt5 and H2B ubiquitylation enzymes through physical interactions with both pSpt5 and Rad6 (Wier et al. 2013; Mayekar et al. 2013; Van Oss et al. 2016; Mbogning et al. 2013).

The action of H2Bub1 in promoting site-specific methylation of histone H3 on lysine 79, by Dot1, and on lysine 4, by COMPASS/MLL family methyltransferases, has been visualized at atomic resolution in recent cryo-EM structures. The details of these structures have been extensively reviewed elsewhere, but an important theme that emerged from this work is that H2Bub1 acts as an allosteric regulator of both methyltransferase classes, locking them in a conformation that is compatible with activity (Janna et al. 2020; Worden and Wolberger 2019). In vivo, H3K4 tri-methylation (H3K4me3) is enriched around the TSS (Fig. 1A), whereas the di-methyl (H3K4me2) form is distributed more broadly within the coding region. Mono-methylated H3K4 is largely absent from gene coding regions but is a prominent marker of enhancer regions in mammalian cells (Heintzman et al. 2007). H3K4me3 and H3K4me2 marks engage a variety of reader domains; the PHD finger is most often associated with H3K4me recognition, but chromodomains, Tudor domains, and WD40 domains can also have this property (Ruthenburg et al. 2007). H3K4me reader proteins are usually present in large transcriptional regulatory complexes implicated in activation or repression, including chromatin modifiers and general transcription factors (Vermeulen et al. 2010, 2007; Saksouk et al. 2009; Shi et al. 2006; Taverna et al. 2006). As is the case for H3K36me, H3K4me seems generally to pattern factor occupancy or function at transcribed genes, although deciphering how H3K4me-dependent interactions are coordinated at specific genes remains a work in progress. H3K4me can also function to block the association of heterochromatin proteins with chromatin, thus helping to demarcate boundaries between transcriptionally silent heterochromatin and transcriptionally active euchromatin (Ooi et al. 2007; Schuettengruber et al. 2007; Douillet et al. 2020).

In yeast, all H3K4me is catalyzed by a single COMPASS complex whose tri- and di-methyltransferase activity is strictly H2Bub1-dependent. In contrast, multiple methyltransferases contribute to H3K4me in mammalian cells; these enzymes have key roles in regulating gene expression during development (Shilatifard 2012). In these systems, the connection between H2Bub1 and H3K4me is complex, as the H3K4 methyltransferases have differing dependencies on H2Bub1 (Wu et al. 2013; Kwon et al. 2020). In ChIP-seq experiments conducted in mammalian cells, H3K4me is detected at highly expressed genes as a broad peak centered over the transcription start site (TSS), which extends into the coding region (Benayoun et al. 2014). Removal of H2Bub1 specifically decreases peak breadth, rather than peak height (Xie et al. 2017). This would be consistent with a role for H2Bub1 in stimulating H3K4me specifically during early elongation. The COMPASS complex containing the SET1A methyltransferase is required for broad H3K4me3 peaks at these genes and is strongly dependent on H2Bub1, further supporting this regulatory connection (Kwon et al. 2020; Sze et al. 2020).

H3K79 methylation is catalyzed by the Dot1 family of methyltransferases, which are universally H2Bub1-dependent. H3K79 is located on the surface of the nucleosome and, in contrast to other histone methylation marks, H3K79me is not engaged by any known histone modification reader domain. H3K79me is distributed throughout RNAPII transcription units (Fig. 1A) and, like H3K4me, it seems to act by excluding the binding of transcriptional repressors and heterochromatin proteins (Vlaming and Leeuwen 2016).

H2Bub1 also has roles that are independent of downstream histone methylation. In vitro, H2Bub1 directly stimulates a nucleosome sliding activity of the chromatin remodeling enzyme Chd1, but inhibits remodeling activity by ISWI family enzymes (Levendosky et al. 2016; Dann et al. 2017). The SWI/SNF and Ino80 chromatin remodeling complexes have also been shown to respond to H2Bub1, although whether these effects are direct or indirect is not known (Shema-Yaacoby et al. 2013; Segala et al. 2016). H2Bub1 directly influences the co-transcriptional nucleosome assembly and disassembly activities of FACT (facilitates chromatin transcription), a nucleosome reorganizing complex and chaperone for H2A-H2B dimers (Pavri et al. 2006; Murawska et al. 2020). Together, these findings suggest that H2Bub1 is deeply involved in regulating structural transitions in chromatin that accompany elongation by RNAPII; indeed, yeast mutants lacking H2Bub1 have defects in genic chromatin structure, which are not due to loss of H3K4me or H3K79me (Murawska et al. 2020; Batta et al. 2011). The proximity of the consensus H2B ubiquitylation site to the “acidic patch” on the nucleosome surface, an important interaction site for several nucleosome-binding factors (including COMPASS and Dot1), suggests a common mechanism through which H2Bub1 may modulate these interactions (Fig. 2)(Worden and Wolberger 2019; Dann et al. 2017). There is evidence for H2Bub1 roles in other aspects of RNAPII elongation, notably mRNA splicing and export, although the relevant mechanisms have not been determined (Vitaliano-Prunier et al. 2012; Moehle et al. 2012). H2Bub1 may also promote elongation as a feedback regulator of Cdk9. In both *S. pombe* and mammalian systems, H2Bub1 and Cdk9 regulate one another: inactivation of Cdk9 leads to diminished H2Bub1, while mutations in the H2B ubiquitylation pathway impair phosphorylation of Cdk9 substrates such as Spt5 (Sanso et al. 2012; Wu et al. 2014). How this mutual dependence influences effects of H2Bub1 on co-transcriptional chromatin transitions and gene expression remains to be determined.

Evidence for crosstalk in H3K36me and H2Bub1 pattern formation

Crosstalk between H3K36me and H2Bub1 pathways would be consistent with the partly overlapping distributions of the two marks in transcribed genes (Fig. 1A). In general, the two have been considered to be functionally complementary but independent modifications acting during transcription elongation. Results of recent studies contain hints that the two pathways are in fact interconnected. The cryo-EM structures of Set2 bound to unmodified and H2Bub1-containing nucleosomes indicate that Set2 binding is positioned by H2Bub1 (Bilokapic and Halic 2019). Set2 engages the nucleosome where the DNA entering and exiting the nucleosome overlap (Fig. 2); in fact, Set2 binding displaces roughly one helical turn of DNA at one end from the histone octamer. Set2 stabilizes this partially unwrapped state through electrostatic interactions with histone H3 and with DNA. These interactions involve the catalytic SET domain of Set2 and position the H3 tail for K36 methylation.

This binding mode is distinct from that observed for established H2Bub1-dependent methyltransferases Dot1 and COMPASS/MLL, which relies on the “acidic patch” region and does not impinge on histone-DNA interactions (Fig. 2). Nonetheless, in Set2-nucleosome structures that included ubiquitin attached to H2B via disulfide linkage (to mimic authentic H2Bub1), a subset of density maps detected a defined position for ubiquitin in which its C-terminal ß strand was in close proximity to the AWS (associated with SET) domain of Set2. The AWS domain is immediately N-terminal to the SET domain in H3K36 methyltransferases related to Set2. Biochemical experiments suggested this interaction could be functional, as ubiquitin attachment modestly enhanced Set2 activity towards nucleosome substrates in vitro. To date, no structure—function analysis to ascertain the importance of AWS-domain residues for the stimulatory effect of H2Bub1, or for Set2 function in vivo, has been reported, leaving the physiological relevance of the H2Bub1 effect uncertain. However, these results offer intriguing hints that H2Bub1 can influence nucleosome interactions with distinct classes of chromatin-modifying enzymes.

Signaling between H2Bub1 and H3K36me has been demonstrated to proceed in the reverse direction in the fission yeast *S. pombe*. That communication is mediated by the *S. pombe* NuA3 complex, which is a major histone H3 acetyltransferase previously implicated in transcription elongation. NuA3 is recruited to transcribed coding regions by a PWWP reader domain interaction with H3K36me (Gilbert et al. 2014; Flury et al. 2017). The key finding linking H3K36me to H2Bub1 is that NuA3 also acetylates the H2Bub1-specific E3 ligase Brl1, thereby enhancing its activity toward H2B (Flury et al. 2017). Acetylation of Brl1 was found to negatively regulate RNAi-mediated heterochromatin formation. Heterochromatin comprises constitutively repressed chromatin regions that harbor conserved molecular hallmarks: methylation of histone H3 at lysine 9, association of HP1 orthologs, and low levels of RNAPII. In *S. pombe*, pericentric heterochromatin formation also requires small non-coding RNAs generated by the RNAi pathway, akin to piRNA-directed silencing in the mammalian germline (Castel and Martienssen 2013). Small RNAs normally act to establish heterochromatin only within previously established heterochromatin domains; loss of Brl1 acetylation was found to relax this restriction (Flury et al. 2017). Brl1 acetylation occurred at a lysyl residue that is distant from the catalytic domain; mutation of this site decreased H2Bub1 levels in vivo by ~ twofold. It is unclear whether this modest reduction in H2Bub1, or a non-enzymatic function of Brl1 that is compromised upon loss of Brl1 acetylation, is responsible for the observed phenotype of ectopic heterochromatin formation. Further studies investigating the role of this residue in regulating Brl1 activity or protein—protein interactions will be needed to resolve this issue. Nevertheless, these data suggest that NuA3 supports a mutually reinforcing function of H3K36me and H2Bub1 during transcription elongation.

Evidence for crosstalk in H3K36me and H2Bub1 function

Our recent findings now provide evidence for a shared function of H3K36me and H2Bub1 in regulation of aberrant antisense transcription by the Rpd3S HDAC complex (Sanso et al. 2020). We identified a role for H2Bub1 in suppressing aberrant antisense transcription in *S. pombe* by strand-specific RNA-seq analysis. We then performed genetic epistasis analysis to relate this function to those of other known, negative regulators of antisense transcription.

We focused on Set2 and Rpd3S (Clr6-CII in *S. pombe*) because of their previously characterized roles in antisense suppression. We also included the CHD family chromatin remodeling factor Hrp3 because it has been implicated in antisense regulation (through a pathway distinct from Clr6-CII) and suggested as a potential target of H2Bub1 (Levendosky et al. 2016; Hennig et al. 2012; Pointner et al. 2012; Shim et al. 2012). The single mutants *htb1-K119R* (lacking the ubiquitylation site on H2B), *set2*Δ, *cph1*Δ (lacking Cph1, a Clr6-CII subunit) or *hrp3*Δ each displayed increased antisense transcription, as measured by strand-specific RT-qPCR at candidate genes. Combining *htb1-K119R* with each of the other mutations led to two different phenotypic outcomes with respect to antisense transcript levels. In the *htb1-K119R hrp3*Δ double mutant, there was an additive effect of the two mutations, arguing that the two individual mutations affect antisense transcription through different pathways. In contrast, antisense levels in the *set2*Δ *htb1K119R* and *cph1*Δ *htb1-K119R* double mutants were similar to those in the single *set2*Δ and *cph1*Δ mutants, indicating epistasis and suggesting that H2Bub1 regulates antisense transcription through the same pathway as Set2 or Clr6-CII. ChIP-seq analysis in the *htb1-K119R* mutant indicated that association of the Clr6-CII complex with transcribed coding regions was impaired genome-wide (by about twofold) in the absence of H2Bub1. This effect was unlikely to be an indirect result of changes in H3K36me, because no effects on Set2 occupancy or H3K36me levels were detected by ChIP-qPCR at select target genes. These results suggest that H2Bub1, like H3K36me, promotes the function of the Rpd3S/Clr6-CII complex.

The mechanistic linkage between H2Bub1 and Rpd3S/Clr6-CII has yet to be elucidated. Although loss of H2Bub1 reduced Clr6-CII occupancy on chromatin, we did not find evidence for increased histone H3 acetylation levels on the candidate genes we examined. This may reflect acetylation-site specificity or gene specificity of the H2Bub1 effect. When combined with inhibition of Cdk9, however, loss of H2Bub1 led to more dramatic, genome-wide decreases in Clr6-CII recruitment to gene bodies, relative to RNAPII occupancy, and synergistically increased levels of histone H3 acetylation on select genes we analyzed. These interactions roughly mirrored the combinatorial effects of H2Bub1 loss and Cdk9 inhibition on antisense suppression: (1) increases in the numbers of genes affected (i.e., those with increased antisense transcription) and (2) enhanced severity of the antisense de-repression detected at individual loci.

Alternatively, H2Bub1 may play a role in augmenting a non-enzymatic, chromatin-stabilizing role for the HDAC complex in antisense suppression (Chen et al. 2012). Further investigation will be required to test these possibilities. These results present an interesting contrast with H3K36me, which was found to promote Rpd3S HDAC activity while having little impact on its association with chromatin (Drouin et al. 2010; Govind et al. 2010). Thus, the two modifications may act on a shared target in different ways. Detailed biochemical and structural studies have shown that H3K36me affects Rpd3S activity directly; whether this is true for H2Bub1 remains to be determined. Intriguingly, the mammalian Sin3B subunit MRG15 is reported to bind directly to ubiquitylated histones (Wu et al. 2011). Our epistasis results are consistent with H2Bub1 and H3K36me both acting to suppress antisense transcription through Clr6-CII, but we cannot exclude the existence of an alternative pathway. Previous work in *S. pombe* suggests that functions of Set2 and Clr6-CII in antisense suppression are overlapping but distinct (Nicolas et al. 2007). The

nature of the putative Set2-dependent but Clr6-CII-independent pathway is not known, but links between Set2 and histone-exchange mechanisms or acetyltransferases point to other, potentially shared functions of H3K36me and H2Bub1 in antisense regulation.

Another recent study also documented a role for H2Bub1 in antisense suppression (Murawska et al. 2020). This work focused on a potential role for H2Bub1 in regulating the FACT nucleosome reorganizing complex, which had emerged from previous work in budding yeast and in vitro. FACT and H2Bub1 were required for antisense suppression at different genomic loci, but a dual loss-of-function mutant had an effect similar to that of FACT ablation alone. It was therefore suggested that H2Bub1 loss leads to increased antisense transcription through an aberrant activity of FACT. However, correction of aberrant FACT activity could also reflect the effect of H2Bub1 on Rpd3S/Clr6-CII, as genetic interactions in budding yeast suggest that H3K36me and the Rpd3S complex act in opposition to FACT (Biswas et al. 2006; Stevens et al. 2011). Deciphering how Rpd3S/Clr6-CII and FACT mediate the interplay between H2Bub1 and H3K36me requires further investigation.

H2Bub1 and H3K36me both affect regional gene silencing in *S. pombe*

H2Bub1 and H3K36me are abundant in the actively transcribed, euchromatic regions of the genome and are excluded from or depleted in pericentric and telomeric heterochromatin. However, both H2Bub1 and H3K36me regulate heterochromatin, likely through indirect but conserved mechanisms that are beginning to emerge. The reduced NuA3 localization to gene coding regions caused by loss of Set2 activity in *S. pombe* allows the complex to bind to chromatin promiscuously, leading to some association with heterochromatin. This leads to inappropriate acetylation (of unknown targets) and destabilizes the transcriptionally repressed state (Flury et al. 2017; Georgescu et al. 2020). A similar sequestration mechanism involving H3K36me has been shown to regulate heterochromatin formation during development in the nematode *C. elegans* (Cabianca et al. 2019).

In *S. pombe*, Set2 is also important for repression of highly condensed, subtelomeric chromatin domains (termed “knobs”) that are ~ 50 kilobases away from the telomere and distinct from telomeric heterochromatin (Matsuda et al. 2015). These domains are not associated with H3K9 methylation or HP1 and instead require the shugoshin ortholog Sgo2 for their establishment or maintenance (Matsuda et al. 2015; Tashiro et al. 2016). Shugoshin normally functions to protect sister chromatid cohesion at centromeres; its function at subtelomeres is not yet clear. Given that H3K36me is not abundant at subtelomeric chromatin it is likely to exert its function indirectly. Interestingly, sequestering of NuA3 only partially accounts for Set2-dependent repression in these regions (Georgescu et al. 2020). This suggests that H3K36me may sequester additional factors to prevent their association with subtelomeric chromatin domains.

Loss of H2Bub1 *enhances* transcriptional repression in and around *S. pombe* heterochromatin. This is accompanied by an increase in H3K9 trimethylation within heterochromatin domains, as well as invasion of H3K9me from pericentric regions into the central core of the centromere (Zofall and Grewal 2007; Sadeghi et al. 2014). We and

Murawska et al. both found that subtelomeric “knob” regions were also hyper-repressed in H2Bub1 mutants, despite the fact that these regions harbor relatively low levels of H2Bub1 in wild-type cells (Fig. 3A)(Murawska et al. 2020). This hyper-repression was not due to inappropriate spread of telomeric heterochromatin, as it was maintained in a double mutant with *sir2*Δ (Fig. 3B). By analogy to the sequestration function of H3K36me, we hypothesize a related function for H2Bub1 that, when disrupted, leads to aberrant action of a repressive factor in heterochromatin or subtelomeric regions. Evidence in support of this type of mechanism includes the finding that reduced FACT function partially alleviates hyper-repression at subtelomeres. We found that the *cph1*Δ and *alp13*Δ mutations that impair Clr6-CII function (Alp13 is the ortholog of Eaf3 and MRG15) have a similar effect—relief of hyper-repression—at the candidate subtelomeric genes that we tested (Fig. 3C). However, there is as yet no clear-cut evidence from ChIP experiments that loss of H2Bub1 leads to redistribution of Clr6-CII or FACT, as is clearly the case for NuA3 when H3K36me is removed. Therefore, another factor regulated by H2Bub1 may be involved in hyper-repression at heterochromatin and subtelomeric regions.

Perspectives

We are still far from a complete understanding of the interplay between chromatin structure and the transcription machinery. A defining feature of both H2Bub1 and H3K36me is that they are placed predominantly or exclusively during the act of transcription and are thus stringently regulated by the factors that govern RNAPII elongation. Indeed, the enzymes most intimately linked to the relevant histone-modifying activities are the CDKs that phosphorylate Rpb1, Spt5 and other components of the transcriptional machinery to coordinate the RNAPII cycle. Cdk9, the rate-limiting kinase for RNAPII elongation in metazoans and fission yeast (Jonkers et al. 2014; Booth et al. 2018), appears to play a central role in regulating H2Bub1 (and H3K4me) and H3K36me in *S. pombe*, but other transcriptional CDKs clearly contribute (Sanso et al. 2012; Mbogning et al. 2015). These functions of Cdk9 in regulating chromatin modification have been conserved in metazoans, but prominent roles in these pathways have also been ascribed to Cdk7, the kinase component of the initiation factor TFIIH, which in mammalian cells is also the activating kinase for Cdk9, Cdk12 and Cdk13 (Pirngruber et al. 2009; Larochelle et al. 2012; Ebmeier et al. 2017; Rimel et al. 2020). Just as H2Bub1 and H3K36me appear to influence each other, signaling between the CDKs and histone modifications such as H2Bub1 and H3K36me is bidirectional, with examples of crosstalk and feedback that have not been fully explained. For example, despite the mutual dependence of H2Bub1 and pSpt5 in vivo, loss-of-function mutations in the H2Bub1 and Cdk9 pathways can have opposing, additive or synergistic effects on downstream events including antisense transcription (Sanso et al. 2012, 2020). Moreover, the two pathways collaborate to govern recruitment and function of Clr6-CII genome-wide, but also appear to work independently to suppress unscheduled antisense transcription at specific gene sets, possibly through Clr6-CII-independent mechanisms.

Here we have summarized evidence for regulatory and functional crosstalk between H2Bub1 and H3K36me that highlights the extent to which events during transcription are interconnected. Signaling between H2Bub1 and H3K36me is apparently unique in that it is

bidirectional, suggesting a mutually reinforcing relationship. Determining the mechanistic basis for that relationship will require addressing several key questions: (1) What is the significance of non-histone acetylation by NuA3 and perhaps other acetyltransferase complexes? (2) Do H2Bub1 and H3K36me act in combination to engage shared targets such as Rpd3S/Clr6-CII? (3) How are the sequestering functions of these modifications coordinated to balance their regulatory effects at transcribed genes and at heterochromatin? Answers to these questions will have broad implications for understanding the regulation and function of co-transcriptional histone modification. Further investigation is also needed to unravel the communication between the RNAPII elongation complex and histone modification pathways, which appears to be conserved in evolution and relevant to human disease processes; the CDKs implicated in this signaling have recently emerged as potential anti-cancer drug targets (Parua and Fisher 2020).

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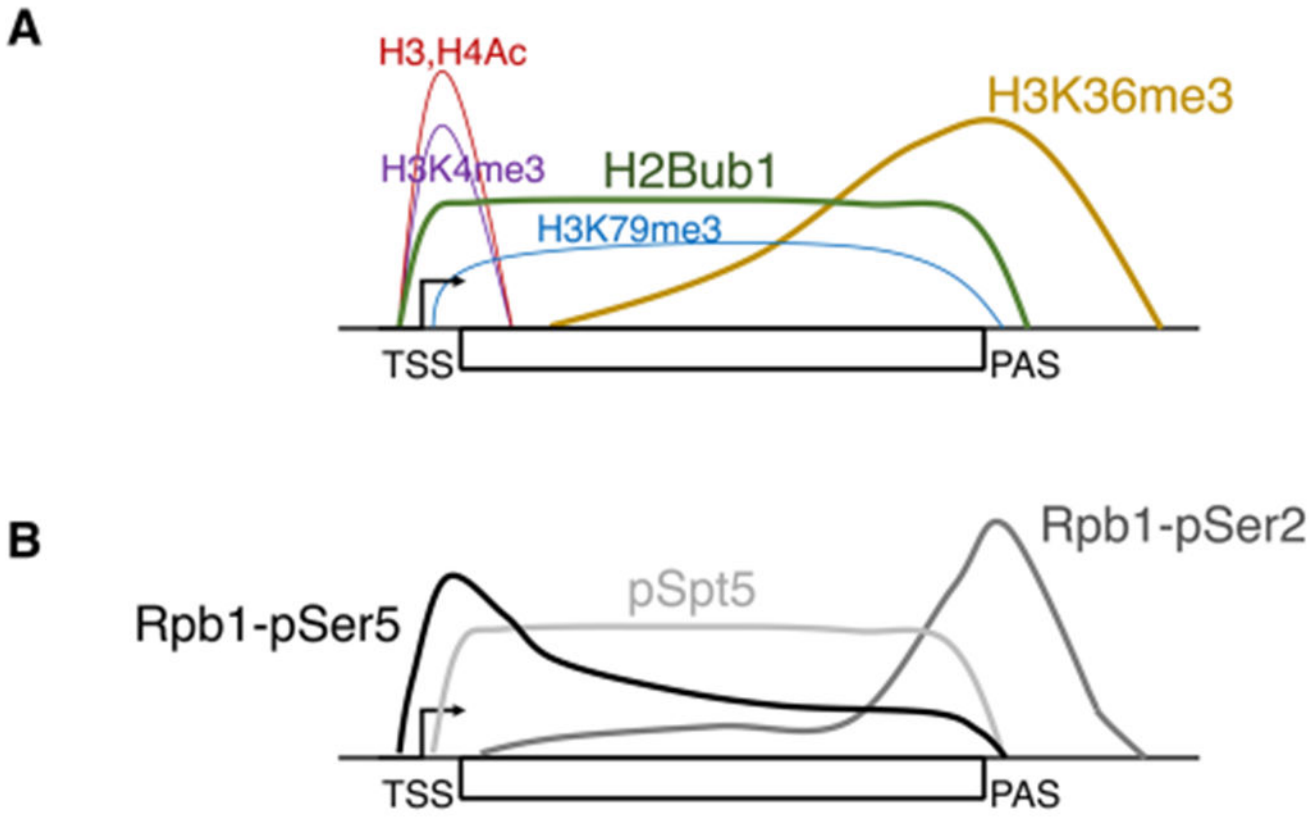

Fig. 1.

Landscape of histone modifications (**A**) and CDK-dependent phosphorylations (**B**) in and around RNAPII transcription units. TSS, transcription start site; PAS, polyadenylation site. See text for details

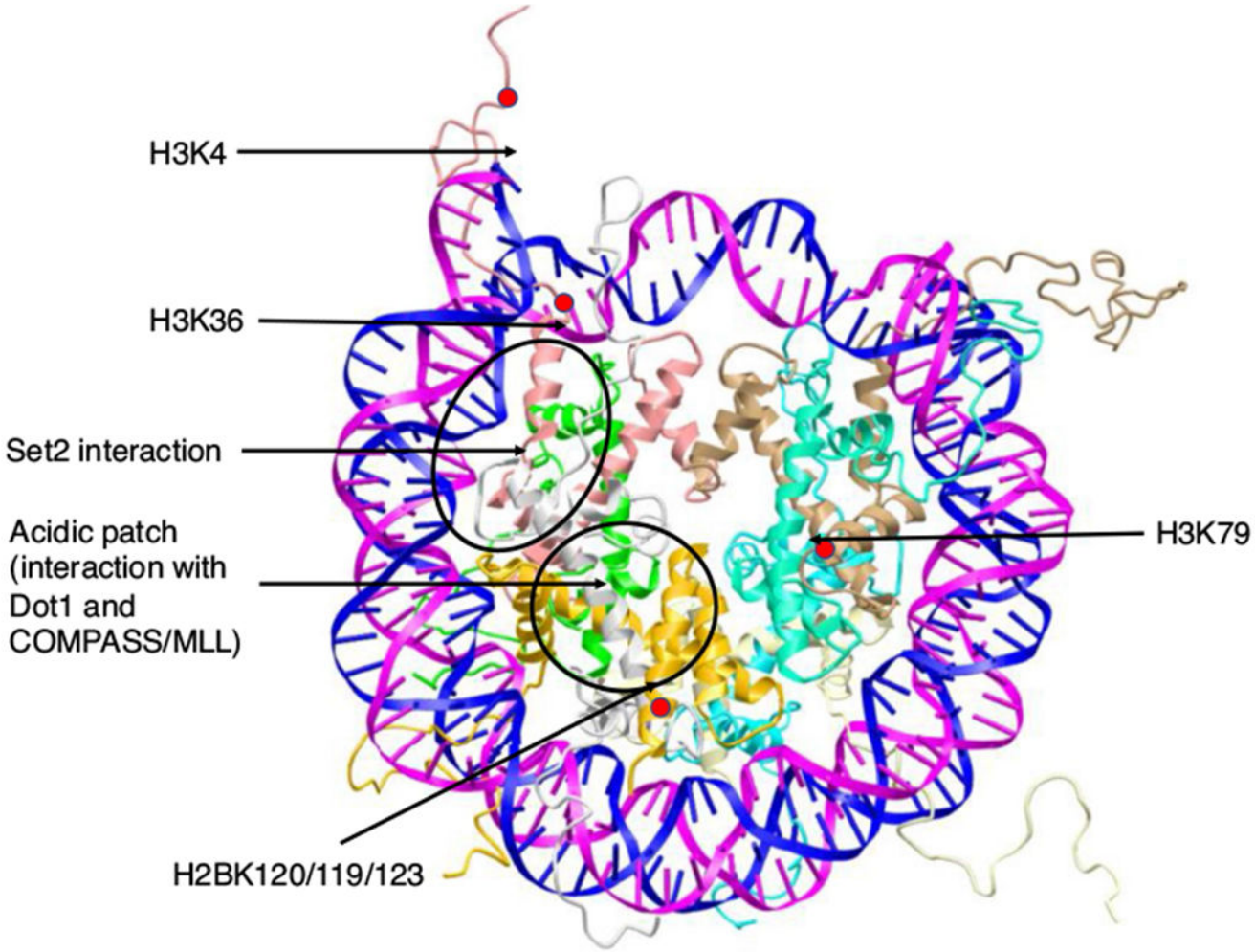

Fig. 2.

Interaction surfaces for Set2, Dot1, and COMPASS/MLL on the nucleosome. Sites of interaction (circles) and relevant histone modification sites (red dots) are highlighted on a model of the nucleosome crystal structure (PDB 1KX5). In this view, COMPASS/MLL and Set2 methylate the histone H3 monomer on the left side of the dyad axis (coloured red), whereas Dot1 methylates the histone H3 monomer on the right (coloured brown)

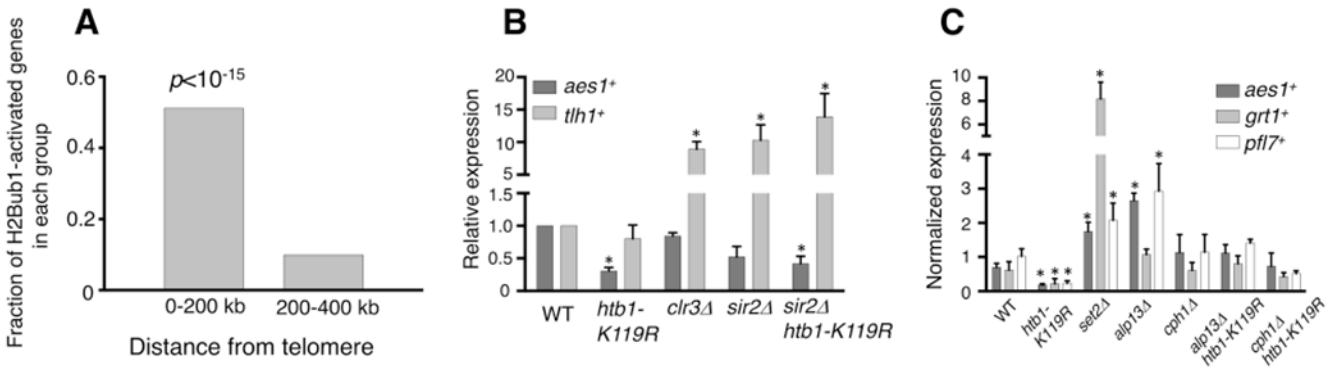

Fig. 3.

H2Bub1 preferentially activates genes in subtelomeric "knob" domains. **A** Fraction of H2Bub1-activated genes (defined by RNA-seq in ref 49) within the indicated genomic intervals. Significant enrichment within subtelomeric regions was assessed by hypergeometric test. **B** Expression of the knob gene *aes1*$^+$ or the heterochomatic gene *tlh1*$^+$ was determined by RT-qPCR as in ref 49 in the indicated strains; values in the wild-type strain were set to 1. Asterisks denote significant differences from wild-type ($n = 3$; unpaired t test with Bonferroni correction; $p < 0.05$). **C** Expression of the indicated knob genes was determined by RT-qPCR in the indicated strains and normalized to *act1*$^+$ expression as in ref 49. Asterisks denote significant differences from wild-type ($n = 4$; unpaired t-test with Bonferroni correction; $p < 0.05$). Primer sequences are available upon request