Ubiquitylation of histone H2B controls RNA polymerase II transcription elongation independently of histone H3 methylation

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Transcription by RNA polymerase II (polII) is accompanied by dramatic changes in chromatin structure. Numerous enzymatic activities contribute to these changes, including ATP-dependent nucleosome remodeling enzymes and histone modifying enzymes. Recent studies in budding yeast document a histone modification pathway associated with polII transcription, whereby ubiquitylation of histone H2B leads to methylation of histone H3 on specific lysine residues. Although this series of events appears to be highly conserved among eukaryotes, its mechanistic function in transcription is unknown. Here we document a significant functional divergence between ubiquitylation of H2B and methylation of Lys 4 on histone H3 in the fission yeast Schizosaccharomyces pombe. Loss of H2B ubiquitylation results in defects in cell growth, septation, and nuclear structure, phenotypes not observed in cells lacking H3 Lys 4 methylation. Consistent with these results, gene expression microarray analysis reveals a greater role for H2B ubiquitylation in gene regulation than for H3 Lys 4 methylation. Chromatin immunoprecipitation (ChIP) experiments demonstrate that loss of H2B ubiquitylation alters the distribution of polII and histones in gene coding regions. We propose that ubiquitylation of H2B impacts transcription elongation and nuclear architecture through its effects on chromatin dynamics.

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Nucleosomes pose a structural barrier to RNA polymerase II (polII) transcription [Izban and Luse 1991; Sims et al. 2004]. The challenge posed by nucleosomes to transcribing polII is highlighted by recent genome-wide mapping studies that demonstrate extensive nucleosome occupancy throughout the coding regions of polII genes [Erkan and Simpson 2004; Lee et al. 2004; Bernstein et al. 2005; Yuan et al. 2005]. In keeping with these findings, significant evidence points to partial or complete nucleosome disassembly as an important component of the transcription elongation process [Kireeva et al. 2002; Belotserkovskaya et al. 2003; Schwabish and Struhl 2004]. Transcription-coupled nucleosome assembly has also been inferred from the observation that certain histone variants are selectively deposited within gene coding regions in a replication-independent manner, although their precise connection to transcription remains unclear [Ahmad and Henikoff 2002; Li et al. 2005; Raisner et al. 2005; H. Zhang et al. 2005]. Various conserved histone chaperone complexes are thought to facilitate either disassembly or reassembly of nucleosomes during transcription, including FACT, Spt6, Asf1, HIRA [for the variant H3.3], and SWR-C [for the variant H2A.Z] [Belotserkovskaya et al. 2003; Kaplan et al. 2003; Kobor et al. 2004; Mizuguchi et al. 2004; Tagami et al. 2004; Schwabish and Struhl 2006]. The molecular details of the assembly and disassembly process, as well as how it is coordinated with the polII elongation complex, are not understood.

The extent to which other chromatin-modifying activities function with histone chaperones to facilitate polII elongation is also poorly understood [Workman 2006]. Of particular interest in this regard is a highly conserved set of histone post-translational modifications that accompany the onset of polII transcription in vivo. These include monoubiquitylation of histone H2B (H2Bub), methylation of specific lysines on histone H3, and acetylation of numerous other lysines in all histone tails [Shilatifard 2006]. Chromatin immunoprecipitation
(ChIP) experiments have shown that at most polIII genes, methylation of histone H3 Lys 4 (H3K4me) and acetylation are concentrated in a relatively small region overlapping the transcription start site (Santos-Rosa et al. 2002; Ng et al. 2003; Bernstein et al. 2005; Guenther et al. 2005; Liu et al. 2005; Pokholok et al. 2005). H2Bub appears to be broadly concentrated in gene coding regions, although its localization pattern has only been determined at a handful of loci (Xiao et al. 2005). Interestingly, there is a strict dependency relationship between H2Bub and H3K4me, such that H2Bub must precede H3K4me (Dover et al. 2002; Sun and Allis 2002). Mutants that are defective in either H2Bub or H3K4me in Saccharomyces cerevisiae share some phenotypes, including loss of telomeric silencing, consistent with a causal relationship between the two modifications (Nislow et al. 1997; Sun and Allis 2002). Furthermore, components of certain histone acetyltransferase complexes can recognize methylated H3K4, consistent with the co-occupancy of these modifications seen by ChIP (Martin et al. 2006; Taverna et al. 2006). These results support a model of a histone modification pathway leading to a certain transcriptional output, the nature of which has yet to be determined (Taverna et al. 2006).

Although this proposed “trans-tail” pathway explains the colocalization of these modifications on chromatin, their mechanistic functions in transcription remain unclear. To study this pathway in greater detail, we have focused on H2Bub, its most upstream component. Conjugation of monoubiquitin to H2B at a conserved C-terminal lysine (K119 in fission yeast, K123 in budding yeast, or K120 in mammals) has long been known to be associated with active transcription by polII (Jason et al. 1997; Sun and Allis 2002). Furthermore, K119R mutants lack H2Bub, with a single Flag epitope at its C terminus (htb1-Flag). In parallel, we constructed a similar strain in which the conserved ubiquitin acceptor site was mutated to arginine (htb1-K119R-Flag). Both strains were initially generated as heterozygous diploids, which were then sporulated to derive haploid tagged strains. We noticed that whereas the htb1-Flag strain grew at the same rate as the wild type, the htb1-K119R-Flag strain grew slowly at 30°C. Backcrossing of both strains to a wild-type parent confirmed the presence of a growth defect in the htb1-K119R-Flag strain that was linked to the kanamycin resistance marker used to integrate the Flag tag [Fig. 1A].

Anti-Flag Western blots on whole-cell extracts prepared from wild-type, htb1-Flag, and htb1-K119R-Flag strains confirmed the presence of Flag-tagged H2B at the expected size in both htb1-Flag and htb1-K119R-Flag (see Fig. 1B, lanes 2,3). Extracts from the htb1-Flag strain also showed a slower-migrating band at the size predicted for H2Bub. This band was absent from htb1-K119R-Flag extracts [Fig. 1B, cf. lanes 2 and 3]. We note the presence of a band at a slightly higher molecular weight than the putative H2Bub band in the htb1-K119R-Flag strain [Fig. 1A, asterisk]. This likely corresponds to a SUMO-conjugated form of H2B, as has been observed previously in S. cerevisiae (Nathan et al. 2006). Blotting against total histone H3 showed that the extracts were equally loaded [Fig. 1C].

Excision of the putative H2Bub band from a Coomassie-stained gel and analysis by tandem mass spectrometry confirmed its identification as a monoubiquitylated form of H2B, and confirmed the ubiquitin attachment site as H2B Lys 119 (Supplementary Fig. 1). Therefore, H2Bub is present in S. pombe and is required for normal growth, in contrast to what has been found in S. cerevisiae. However, both share a requirement for H2Bub in methylation of H3K4 (Fig. 1C), in agreement with previous results (Roguev et al. 2003; Maruyama et al. 2006).

Knockouts of conserved components of the H2Bub pathway were crossed to the htb1-Flag strain to monitor their effects on H2Bub. As expected from previous work, a deletion of rhp6+, encoding the S. pombe homolog of the RAD6 ubiquitin conjugating E2 enzyme, resulted in a loss of H2Bub and H3K4me [Fig. 2A; Supplementary Fig. 2; Roguev et al. 2003; Maruyama et al. 2006].
Ubiquitylation of H2B in *S. cerevisiae* and metazoan systems also requires the RING finger E3 ubiquitin ligase BRE1 (called RNF20 or hBRE1 in human cells) (Hwang et al. 2003; Wood et al. 2003; Kim et al. 2005; Zhu et al. 2005). Deletion of either of the two *S. pombe* genes similar to BRE1 also resulted in loss of H2Bub and H3K4me (Fig. 2A). We have named these genes brl1+ (SPCC1919.15) and brl2+ (SPCC970.10c) (for BRE1-like). Strains that were deleted for any of *rhp6*, *brl1+*, or *brl2+* showed slow-growth phenotypes, further arguing that H2Bub is required for normal growth (data not shown).

We were intrigued by the fact that both Brl1 and Brl2 proteins were required for H2Bub. To further characterize the functional connection between these proteins and Rhp6, *rhp6* was tagged at its endogenous locus with a 13-myc epitope and purified, and copurifying proteins were identified by Coomassie staining and tandem mass spectrometry (Fig. 2B; data not shown). We found that both Brl1 and Brl2 copurified with Rhp6, suggesting that Rhp6 acts in concert with both Brl1 and Brl2 to promote H2Bub (Brl1 and Brl2 have near-identical molecular masses and therefore were not resolved on the gel) (see Fig. 2B).

**Functional divergence of H2B ubiquitylation and H3K4 methylation**

Given that H2Bub is needed for methylation of H3K4, we wished to know whether the growth defect observed in the *htb1-K119R-Flag* strain was due to loss of downstream methylation. We constructed untagged, isogenic wild-type and *htb1-K119R* strains and compared them to a strain deleted for the *set1* gene, encoding the *S. pombe* homolog of the H3K4 methyltransferase (Noma and Grewal 2002). In keeping with previous results in *S. cerevisiae* and *S. pombe*, Western blots showed that the *set1Δ* strain lacked all H3K4me but was proficient in H2Bub (see Supplementary Fig. 2; data not shown). This strain also grew at the wild-type rate, in contrast to the *htb1-K119R* strain (Fig. 3A). Therefore, at least some functions of H2Bub are independent of H3K4me in *S. pombe*.

Microscopic examination of *htb1-K119R* cells grown at 30°C showed an abnormal morphology: Cells were large and tended to associate in clumps. Staining with DAPI (diamino-phenylindole) and calcofluor revealed occasional cells with multiple compartments enclosed by septa, some containing a single nucleus (Fig. 3B, middle panel). Cells in which the nuclei were separated by multiple septa were also observed (Fig. 3B, middle panel).
These phenotypes are indicative of cell separation defects (Simanis 2003). Overall, mutants lacking H2Bub showed a two- to threefold increase in percentage of cells containing septa in an asynchronous culture (Fig. 3C). Importantly, set1Δ cells were morphologically normal, indicating that the htb1-K119R defects were not due to lack of H3K4me.

We also observed aberrant nuclear morphology in htb1-K119R cells. Wild-type, htb1-K119R, and set1Δ cells were stained with DAPI and an antibody recognizing the nuclear pore complex and examined using deconvolution microscopy. Nuclei in wild-type and set1Δ cells showed a crescent-shaped DAPI-staining region occupying roughly half of the area bounded by the nuclear pore staining [Fig. 4A,C,D,F]. However, among htb1-K119R cells, we found examples of nuclei containing fragmented DAPI-stained material inside the nucleus [Fig. 4B,E], indicating a defect in nuclear architecture.

We then turned to microarray analysis to determine whether the phenotypes observed in the mutants were correlated with specific defects in gene expression. In assigning the total number of genes that are affected by each of the mutations, we adopted a cutoff such that genes whose expression changed by at least twofold in two independent hybridizations were included. We consider this to be a stringent cutoff, since in a typical experiment ~90% of genes that satisfied this requirement experienced expression level changes of threefold or less (see Supplementary Tables 2–4). All analyses were conducted on asynchronous cultures grown in rich medium at 30°C.
Affected genes in all the mutants were clustered using hierarchical clustering analysis (a representative example of which is shown in Fig. 5A). The \textit{htb1-K119R} mutant had altered expression of 84 genes (43 increased and 41 decreased). Surprisingly, the expression of only 12 genes was found to reproducibly change in the \textit{set1\Delta} mutant (nine increased and three decreased). Of these, six were also found in the \textit{htb1-K119R} data sets (five of nine for increased, one of three for decreased), as expected from the loss of H3K4me in the \textit{htb1-K119R} mutant. These results corroborate the phenotypic data described above and demonstrate that H2Bub has gene regulatory functions that are independent of H3K4me.

We also determined the gene expression profiles of \textit{brl1}\Delta and \textit{brl2}\Delta mutants. Both of these mutants affected a broader spectrum of genes than the \textit{htb1-K119R} mutant [see Fig. 5A,B]. However, as illustrated in Figure 5B, highly significant overlaps were found between data sets for \textit{htb1-K119R} and \textit{brl1}\Delta (28 of 95 and 23 of 75; \(p = 1.36 \times 10^{-41}\) and \(1.6 \times 10^{-35}\) for increased and decreased genes, respectively), \textit{htb1-K119R} and \textit{brl2}\Delta (35 of 98 and 29 of 101; \(p = 1.06 \times 10^{-56}\) and \(2.87 \times 10^{-48}\)), and \textit{brl1}\Delta and \textit{brl2}\Delta (56 of 114 and 38 of 108; \(p = 9.97 \times 10^{-89}\) and \(2.22 \times 10^{-86}\)). In all, 49 of the 84 genes altered in the \textit{htb1-K119R} mutant were also altered in both \textit{brl1}\Delta and \textit{brl2}\Delta. These data argue that Br1 and Br2 proteins function together through the ubiquitylation of H2B. Together with the physical interaction data presented above [Fig. 2B], these data suggest that a Rhp6--Br1--Br2 complex mediates most H2B ubiquitylation in vivo. Also, since the functions of Br1 and Br2 cannot be accounted for completely by H2Bub, they likely have other ubiquitylation targets that are important for gene regulation.

The affected genes in our data sets overlapped significantly with two functional categories annotated in the \textit{S. pombe} genome database. These are the amino acid permeases, which are represented in genes repressed by \textit{brl2}\Delta [\(p = 0.00359\)], and membrane transporters, which are represented in genes repressed by \textit{htb1-K119R} [\(p = 0.00236\)] and \textit{brl2}\Delta [\(p = 0.0482\)]. Thus, H2Bub may have an especially important role in activating genes involved in membrane functions, which would be consistent with its role in septation. We also observed a broad and significant overlap between the expression profiles of mutants affecting H2Bub and genes whose expression is altered in response to various forms of stress (summarized in Supplementary Table 5; Chen et al. 2003). This could reflect a specific role of H2Bub in the stress response, or an indirect effect of other phenotypes in the mutants.

The pronounced cell separation phenotypes of mutants affecting H2Bub prompted us to compare our data sets to those generated for known \textit{S. pombe} septation mutants [Lee et al. 2005]. Interestingly, we observed modest but significant overlaps between genes induced in \textit{brl1}\Delta and \textit{brl2}\Delta mutants and those induced in the \textit{sep15}\Delta mutant grown at the semipermissive temperature [\(p = 0.044\) for \textit{brl1}\Delta and \(p = 0.000239\) for \textit{brl2}\Delta]. For \textit{brl2}\Delta, overlap was also seen with \textit{sep10}\Delta [\(p = 0.038\)]. The \textit{sep10}\^+ and \textit{sep15}\^+ genes encode subunits of Mediator, a complex that facilitates communication between transcriptional activators and RNA polII (Zilahi et al. 2000; Szilagyi et al. 2002). These data implicate ubiquitylation by Br1 and Br2 in a repressive function of the Mediator complex.

Figure 4. Abnormal nuclear morphology in the \textit{htb1-K119R} mutant. Wild-type, \textit{htb1-K119R}, and \textit{set1\Delta} cells were stained with a nuclear pore antibody and DAPI. Shown are deconvolved images taken using a DeltaVision microscope equipped with a CCD camera. (A–C) DAPI stain alone. (D–F) DAPI stain merged with nuclear pore stain. The white arrow in B denotes a fragment of nuclear, DAPI-stained material in the \textit{htb1-K119R} mutant cell.
Ubiquitylation of H2B is required for normal transcription elongation in vivo

To arrive at a mechanistic understanding of how H2Bub and H3K4me regulate gene expression, we analyzed individual target loci using ChIP. We focused our analysis on two genes whose expression is repressed in the htb1-K119R and set1Δ mutants: hem2*, involved in heme biosynthesis, and sod2*, involved in sodium transport. These genes were chosen since they were reproducibly repressed in the microarray experiments (see Supplementary Tables 2–4). Quantitative PCR (Q-PCR) analysis and Northern blotting confirmed that these genes are bona fide targets (see Fig. 6A; Supplementary Fig. 3). As a control, we also analyzed the aro1* gene, whose expression is unaffected by either mutation (see Fig. 6A; Supplementary Fig. 3).

We monitored occupancy of polII and histone H3 trimethylated on K4 [H3K4me3] by ChIP at aro1*, hem2*, and sod2*, both at their 5′ ends and at a region downstream [Fig. 6B,C]. As has been observed in budding yeast and metazoan systems, H3K4me3 was greatly enriched at the 5′ ends of each gene and was completely dependent on both H2Bub and set1Δ [Fig. 6C]. We note that similar enrichment was observed at every active gene we tested, regardless of its dependence on H2Bub or H3K4me for expression [Supplementary Fig. 4]. In addition, very little enrichment was observed at inactive

Figure 5. Gene expression microarray analysis of mutants defective in H2Bub and H3K4me. (A) Transcripts whose levels were altered by at least twofold in one or more of the mutants were clustered using a hierarchical clustering algorithm. Shown is a representative cluster diagram from one of three independent experiments. Values from a wild-type control strain were subtracted from all data sets (WT column). (B) Venn diagrams showing overlap between htb1-K119R, brl1Δ, and brl2Δ data sets.

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gene between the different strains (Fig. 7A). For polII ChIP signals from the 5' end of active genes (data not shown), in agreement with the idea that H3K4me generally marks the 5' end of active genes (Bernstein et al. 2005; Guenther et al. 2005; Liu et al. 2005; Pokholok et al. 2005). Although only subtle differences in polII occupancy could be discerned between the wild-type and either mutant strain at a given location, we noticed that the htb1-K119R mutation caused a specific decrease in polIII occupancy at the 3' ends of hem2* and sod2* genes [Fig. 6B]. This is also evident when comparing the ratios of polII ChIP signals from the 5' and 3' probes within each gene between the different strains [Fig. 7A]. For hem2* and sod2*, the 5':3' ratios increase approximately threefold and approximately sixfold, respectively, in the htb1-K119R mutant relative to the wild type (Fig. 7A). Importantly, these effects correlate with the observed effects on gene expression, since they are much weaker in the set1Δ mutant and at the aro1* control gene. They are also absent in two additional genes that are not affected by the htb1-K119R mutation, cdc25* and psu1* (Supplementary Fig. 4). Since the occupancy of polIII at H2Bub target genes is specifically affected at their 3' ends in the htb1-K119R mutant [Fig. 6B], H2Bub likely plays a role in polII transcription elongation. Moreover, this function is independent of H3K4me.

Evidence linking H2B ubiquitylation to chromatin assembly

To explore the mechanism by which H2Bub enhances transcription elongation, we also derived 5':3' distribution ratios at the same genes for histone H3. At the sod2* gene, we found that H3 distribution is skewed toward the 5' end in htb1-K119R relative to wild type, similar to what we found for polII [Fig. 7B, right]. The set1Δ mutation caused a much smaller change in distribution. This shows that the transcription elongation defect at sod2* in the htb1-K119R mutant is accompanied by dramatic chromatin changes at the locus, and implies that H2Bub helps to alter nucleosome occupancy in some gene coding regions to accommodate elongating polII. Interestingly, histone H3 distribution at hem2* is unchanged in either mutant, suggesting that the elongation function of H2Bub is not strictly dependent on changes in nucleosome occupancy.

We sought genetic evidence that H2Bub acts at least in part through a chromatin assembly pathway coupled to polIII transcription. The highly conserved histone variant H2A.Z, encoded by the pht1* gene in S. pombe, is assembled into nucleosomes in an S-phase-independent manner and plays a role in transcription that remains poorly understood (Carr et al. 1994; Henikoff et al. 2004). Interestingly, its deposition pattern within polIII coding regions bears a striking resemblance to that of H2Bub and H3K4me, in that it is concentrated near the 5' end (Raisner et al. 2005). We examined the relationship between H2Bub and H2A.Z deposition by crossing the htb1-K119R mutant to a pht1Δ mutant. We found that double mutants could not be recovered from this cross, indicating that the two mutations are synthetically lethal (see Table 1). Double mutants between pht1Δ and set1Δ were readily recovered, indicating that the synthetic lethality is unrelated to H3K4me. This is consistent with previous work in budding yeast that identified rad6Δ and bre1Δ as synthetically lethal with htz1Δ (Hwang et al. 2003). The requirement for H2A.Z in the htb1-K119R mutant indicates that H2Bub function is related to S-phase-independent chromatin assembly.

We suspected that a specific histone chaperone complex may function through H2Bub to enhance polIII elongation. In mammalian cells, the histone chaperone HIRA has been implicated in replication-independent incorporation of histone H3 into chromatin (Tagami et al. 2004). Homologs of HIRA in budding yeast and fission yeast function in histone gene regulation, DNA repair, gene silencing, and chromosome segregation, but have not
been assigned a general role in transcription (Spector et al. 1997; Kaufman et al. 1998; Sharp et al. 2002; Blackwell et al. 2004). We attempted to combine the htb1-K119R mutant with a deletion of hip1+, the gene encoding the HIRA homolog in \textit{S. pombe}. We found that we were unable to recover double mutants from this cross, indicating that the two mutations are synthetically lethal. This was also true for a deletion of the \textit{slm9} gene, which encodes a protein that interacts with Hip1 and is similar to the budding yeast \textit{HIR2} gene (Blackwell et al. 2004). Thus, it is likely that H2Bub plays a role in chromatin assembly. Neither \textit{hip1} nor \textit{slm9} deletion was lethal in combination with \textit{set1}, indicating that the chromatin assembly function of H2Bub is independent of H3K4me (see Table 1). We note that all crosses involving \textit{hip1} and \textit{slm9} strains gave rise to \sim 10% inviable spores (see Table 1). These results suggest a role for H2Bub in chromatin assembly during transcription.

Recent studies have suggested a connection between H2Bub function in transcription elongation and the FACT chromatin assembly complex (Pavri et al. 2006). We tested whether FACT occupancy at H2Bub target genes was affected by the \textit{htb1-K119R} and \textit{set1} mutations using strains carrying an epitope-tagged FACT subunit (Supplementary Fig. 5). Western blots confirmed that Spt16-myc protein levels were unchanged in the mutant strains (Supplementary Fig. 5). Using ChIP, we found a modest (approximately twofold) decrease in Spt16-myc binding to the 3'-end of the H2Bub-regulated \textit{sod2} gene in the \textit{htb1-K119R} mutant (Supplementary Fig. 5A, middle panel), consistent with the transcription elongation defect noted previously [Figs. 6, 7]. However, FACT occupancy was unaffected at other loci tested (Supplementary Fig. 5A, data not shown), suggesting that H2Bub is not generally required for FACT binding to chromatin.

**Discussion**

Our data link the ubiquitylation of histone H2B to nucleosome dynamics and transcription elongation in vivo. Collectively, our results provide a mechanistic explanation for the correlation between H2Bub and polII transcription documented previously. Importantly, we demonstrate that at least part of the mechanistic role of H2Bub in polII transcription does not involve H3K4me, despite the causal link between the two modifications. Our data are consistent with the idea that H2Bub participates in at least two independent transcriptional regulatory pathways: one that has mechanistic consequences for transcription elongation and is required for normal cell growth and morphology, and another that leads to H3K4me and downstream events that have yet to be uncovered [Fig. 8].

Much of the enzymatic machinery responsible for H2Bub appears to be conserved from yeast to human cells. Our study provides the first characterization of the \textit{S. pombe} homologs of budding yeast \textit{BRE1}, encoding the ubiquitin ligase for H2B. We found that knockouts of either \textit{brl1} or \textit{brl2} abolish H2Bub and result in similar phenotypes to those observed in the \textit{htb1-K119R} mutant [Figs. 2A, 3C]. This means that both of these RING finger proteins are individually required for H2Bub. Further, our physical interaction results, combined with the highly overlapping microarray profiles for both knockouts, strongly suggest that these proteins function together in the same complex [Figs. 2B, 5B; summarized in Fig. 8]. Interestingly, the two human proteins most similar to Bre1, RNF20/hBRE1 and RNF40, are also found in a complex, although whether both are required for H2Bub in vivo has yet to be determined [Zhu et al. 2005]. Direct association of functionally related RING finger proteins may be a general feature of ubiquitin ligase complexes that act on chromatin substrates, since this is

**Table 1.** Genetic interactions between \textit{htb1-K119R}, \textit{set1}, and chromatin assembly factor mutants

|          | \textit{pht1}\textsuperscript{Δ} | \textit{hip1}\textsuperscript{Δ} | \textit{slm9}\textsuperscript{Δ} |
|----------|--------------------------------|--------------------------------|--------------------------------|
| \textit{Wild type} | 21/21 | 18/22 | 13/16 |
| \textit{htb1-K119R} | 0/25 | 0/11 | 0/11 |
| \textit{set1}\textsuperscript{Δ} | 16/16 | 9/11 | 13/15 |

Pairwise crosses were carried out between the indicated strains. Data are presented as observed/expected numbers of viable progeny carrying both parental markers, as judged from dissection of tetratype and nonparental ditype tetrads.
also true of the Ring1b–Bmi1 and BRCA1–BARD1 complexes involved in H2A ubiquitylation in human cells (Brzovic et al. 2001; Buchwald et al. 2006). Detailed biochemical analyses of the activity of the Ring1b–Bmi1 complex have shown that Ring1b is primarily responsible for ubiquitin transfer to H2A, whereas Bmi1 functions to stimulate Ring1b activity (Buchwald et al. 2006). It remains to be seen whether the dual RING finger complexes involved in H2Bub have a similar functional organization.

In S. cerevisiae, the conserved ubiquitin-conjugating enzyme Rad6 mediates H2Bub (Robzyk et al. 2000). We have found that the S. pombe Rad6 homolog Rhp6 plays a similar role, in agreement with the findings of others (Roguev et al. 2003; Maruyama et al. 2006). In addition, we have found that Rhp6 physically interacts with both Brl1 and Brl2 (Fig. 2B). The human genome encodes two Rad6 homologs, hRAD6A and hRAD6B, but a recent report identified a different ubiquitin-conjugating enzyme, UbcH6, as the functional complement of the RNF20–RNF40 complex (Zhu et al. 2005). This may suggest a divergence between yeast and mammalian cells with respect to the H2Bub pathway, although the role of hRAD6 proteins in this pathway has yet to be extensively tested in vivo.

Our microarray analysis shows that only a small subset of genes in the S. pombe genome is significantly dependent on H2Bub or H3K4me for normal expression. This raises the question of what characteristics render a gene sensitive to regulation by H2Bub. One possibility is that certain gene-specific transcriptional regulators have a particular requirement for H2Bub for their function. This model is consistent with the finding that functionally related genes were enriched among the htb1-K119R-repressed genes, and is further supported by a recent study demonstrating that a mutation in the Drosophila BRE1 homolog dBRE causes specific defects related to Notch signaling (Bray et al. 2005). Another possibility is that the chromatin structure at certain loci, perhaps dictated by their underlying DNA sequence, is inherently unfavorable for polII transcription elongation, and thus would require the added fluidity conferred by H2Bub.

Although our analysis has focused on genes that are positively regulated by H2Bub, we found that equivalent numbers of genes were negatively regulated in our mutants. ChIP experiments at some of these genes showed that H3K4me is not enriched at these loci, even in the wild-type strain (data not shown). This negative regulation could either result from instances of H2Bub that are not associated with H3K4me, or from indirect effects. A handful of the genes that were induced in the H2Bub mutants were also induced in clr6-1 and ago1Δ mutants that are defective in heterochromatic gene silencing (Grewal and Moazed 2003). Importantly, the overlap also held for the set1Δ mutant, arguing that some of these effects are related to loss of H3K4me. The set1Δ and htb1-K119R mutants have been shown previously to be proficient in heterochromatic gene silencing (Noma and Grewal 2002; Jia et al. 2005; E. Hong and D. Moazed, pers. comm.), suggesting that the overlap is unlikely to result from a direct role for H3K4me in gene silencing. Instead, it likely reflects a heterochromatin “spreading” effect, as has been proposed to explain the loss of gene silencing in H3K4me-defective mutants in S. cerevisiae (Katan-Khaykovich and Struhl 2005).

Our experiments suggest that H3K4me has very little importance for gene regulation on its own in S. pombe. We emphasize, however, that the experiments presented here were conducted with cells grown in rich medium at 30°C, and that H3K4me may have specialized functions in gene regulation that are not apparent under these conditions.

It is possible that methylation of another histone lysine is affected in the absence of H2Bub in fission yeast. We note, however, that H3 Lys 79, which is methylated in an H2Bub-dependent manner in budding yeast and human cells (Briggs et al. 2002; Ng et al. 2002; Kim et al. 2005), is not detectably methylated in S. pombe, and a homolog of the H3K79 methyltransferase Dot1 is not encoded in its genome (J.C. Tanny and C.D. Allis, unpubl.). Also, no change in methylation of H3K36 or H4K20 is detected in htb1-K119R or set1Δ strains (data not shown).

Mutants defective in H2Bub showed defects in cell separation (Fig. 3B). These phenotypes are reminiscent of the septation defects observed in some known sep mutants, including sep10Δ and sep15Δ, and further, we observed some overlap between the microarray data sets for brl1Δ and brl2Δ and those for sep10Δ and sep15Δ. It is interesting that a number of gene products thought to be generally involved in polII transcription, such as Mediator components and histone ubiquitylation enzymes, appear to preferentially regulate specific sets of genes involved in cell separation. This is also true for the kinases Mcs6 and Cdk9 and the RING finger protein Pmh1, all involved in phosphorylation of the polII C-terminal domain (CTD) (Pei and Shuman 2003; Lee et al. 2005). In budding yeast, CTD phosphorylation has been shown to be required for H2Bub and subsequent H3K4me [Laribee et al. 2005; Wood et al. 2005], and phospho-CTD forms of polII colocalize with H3K4me by ChIP in human cells [Bernstein et al. 2005]. These data could indicate a conserved pathway that links some functions of Mediator and polII CTD phosphorylation with H2Bub and chromatin assembly.

In addition to the septation defects observed in htb1-K119R mutants, some nuclei in these cells showed a
fragmented pattern of DAPI staining (Fig. 4B,E). It is unlikely that this pattern is related to mitotic chromosome segregation defects, since previous studies failed to detect effects on integrity of centromeric chromatin in a rhp6Δ mutant [Maruyama et al. 2006]. We also did not observe fragmented DAPI staining patterns in nuclei of a hip1Δ mutant, arguing against their being a result of defective chromatin assembly per se [data not shown]. The crystal structure of the nucleosome predicts that the C terminus of H2B is exposed on the nucleosome surface [White et al. 2001], raising the intriguing possibility that the htb1-K119R mutant may cause global defects in internucleosome or other interactions that contribute to chromatin architecture.

What is the molecular mechanism by which H2Bub exerts its biological effects? Our ChIP experiments implicate H2B in control of transcription elongation and chromatin structure at a subset of genes [Fig. 6]. The htb1-K119R mutant also showed lethal genetic interactions with other mutants known to affect chromatin assembly [Table 1]. Thus, we propose that H2Bub directly promotes chromatin assembly and/or disassembly processes that accompany polIII transcription elongation. Recent in vitro studies also support a direct role for ubiquitylation in transcription on chromatin templates. First, transcriptional activation by LEF-1–β-catenin on chromatin templates, but not on naked DNA, could be blocked by addition of a strong ubiquitin-binding protein. This effect was reversed upon addition of free ubiquitin, arguing that ubiquitin is specifically needed for activated transcription on chromatin templates [Sierra et al. 2006]. More recently, a highly purified polIII in vitro transcription system was used to reconstitute activation by the retinoic acid receptor on a chromatin template [Pavri et al. 2006]. Importantly, addition of FACT, RNF20–RNF40 complex, and ubiquitin had a cooperative affect on transcription activation. Since our ChIP studies did not detect a general defect in chromatin association of the FACT complex in the absence of H2Bub [Supplementary Fig. 5A], this cooperativity could be due either to locus-specific effects on binding or to a different effect on FACT activity.

Protein monoubiquitylation plays well-documented roles in molecular recognition in a number of biological processes, notably endosomal protein sorting and DNA damage repair [Hoege et al. 2002; Alam et al. 2006]. Our results suggest an additional role in transcription-coupled chromatin assembly, and lay the groundwork for study of potentially new modes of ubiquitin recognition.

Materials and methods

Yeast strains and media

Yeast strains used in this study are listed in Supplementary Table 1. All knockout strains and tagged strains were constructed using standard techniques [Bahler et al. 1998; Sato et al. 2005]. Strains bearing the Flag epitope used plasmid pDM713 as template to generate the transforming PCR products [Tanny et al. 2004]. To generate the htb1-K119R-Flag strain, an A-to-G substitution was introduced at codon 119 in the forward primer used in the transforming PCR reactions. To construct strains with combinations of markers, haploid strains with the individual markers were crossed and tetrads were dissected to isolate haploid progeny with both parental markers. Rich media was YE-supplemented with adenine, uracil, leucine, and histidine [YES] [Moreno et al. 1991].

Immunoprecipitations and Western blots

Large-scale immunoprecipitation of Rhp6-myc was performed as described with minor modifications [Tackett et al. 2005]. Briefly, 3 L of culture grown in YES to 5 × 10⁶ cells/mL was harvested and flash-frozen in small chunks. Cells were lysed under cryogenic conditions as described [Tackett et al. 2005], and frozen cell powder was resuspended in 7 mL of buffer L [20 mM HEPES at pH 7.6, 250 mM KCl, 5% glycerol, 2 mM MgCl₂, 1 mM EDTA, 10 mM β-glycerophosphate, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride [PMSF], protease inhibitor cocktail [Roche]]. All subsequent steps were carried out at 4°C. Extracts were centrifuged for 15 min at 15,000g, and supernatants were incubated with 0.2 mL of anti-myc resin [Sigma] that was prewashed in buffer L and then eluted with 1 mL of 0.5 M NH₄OH/0.5 mM EDTA for 10 min at room temperature. Eluates were lyophilized, resuspended in 1× sample buffer, and analyzed by electrophoresis on 4%–12% gradient acrylamide gels [Novex] followed by Coomassie staining.

Extracts for Western blots were prepared using the TCA lysis method as described [Kao and Osley 2003]. SDS-PAGE, transfer to PVDF, and blotting were carried out as described [Cheung et al. 2005]. Anti-Flag antibody was purchased from Sigma, anti-H3K4me antibodies and anti-myc (clone 9E10) were from Millipore, anti-HA (clone 12CA5) was from the Sloan Kettering Monoclonal Antibody Core Facility, and anti-H3 was from Abcam. The tubulin antibody TAT1 was kindly provided by P. Nurse.

Microscopy and immunofluorescence

For DAPI/calcofluor staining, cells were fixed by addition of 1/10th culture volume of 37% formaldehyde and incubated with shaking for 15 min. Cells were pelleted, washed twice with dH₂O, and mixed with an equal volume of 10 µg/mL DAPI+ calcofluor on a glass slide. Photographs were taken using a Zeiss Axioscop 2 microscope.

Immunofluorescence with the nuclear pore antibody (mAb414, kindly provided by G. Blobel) was performed as described [Hagan and Ayscough 1999]. A 1:1000 dilution of primary antibody and 1:250 dilution of FITC-conjugated secondary antibody [Jackson Laboratories] were employed. Images were captured on a DeltaVision microscope [Applied Precision/Olympus] using a cooled-charged device [CCD] camera and deconvolved using SoftWorx software.

Microarrays and Q-RT-PCR analysis

Total RNA was extracted from 50 mL of YES cultures using hot phenol and purified further using Qiagen RNeasy columns. For Q-RT–PCR, 0.1 µg of total RNA was reverse-transcribed with an oligo-dT primer using the Invitrogen SuperScript III first-strand synthesis kit. Resulting cDNA was quantified by real-time PCR using a SYBR Green mix [Applied Biosystems] in a Stratagene Mx3000P instrument.
Gene expression microarray analysis was performed in the laboratory of Bruce Futcher and Janet Leatherwood at State University of New York at Stony Brook (Stony Brook, NY) as previously described (Oliva et al. 2005). In each experiment, samples from wild-type [htb1-kanMX6] (see Supplementary Table 1) or mutant strains were cohybridized with a sample prepared from a reference wild-type strain (no antibiotic resistance marker). Clustering analysis used a hierarchical clustering algorithm. Genespring software (Agilent) was used to derive p-values (hypergeometric probabilities).

ChIP

ChIP was performed as described [Huang and Moazed 2003] with modifications. Briefly, 50 µL of YES cultures were fixed with 1% formaldehyde, harvested, and flash frozen. Cells were lysed by bead-beating in 0.4 mL of immunoprecipitation buffer (50 mM HEPES at pH 7.6, 500 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate, 1 mM PMSF, protease inhibitor cocktail [Roche]). Extracts were incubated with 1 µL of antibody overnight at 4°C. After clearing by centrifugation, 100 µL of immunoprecipitation buffer and sonicated for seven cycles [30 sec on, 60 sec off] in a water bath sonicator (Diagenode) at 4°C. After clearing by centrifugation, 100 µL of antibody overnight at 4°C. Anti-myc, anti-HA, anti-H3K4me3, and anti-H3 were described above. Anti-RNApolyII (clone 8WG16) was purchased from Milipore. Immune complexes were recovered with 15 µL of protein G agarose beads (Pierce), then washed, eluted, and purified as described [Huang and Moazed 2003]. ChIP DNA (1.5 µL) and input DNA (1.5 µL of a 1/100 dilution) samples were analyzed by real-time PCR with specific primer pairs. Enrichment of a particular sequence in the immunoprecipitations was calculated relative to input after subtraction of enrichment obtained from a negative control immunoprecipitation (no antibody). Details regarding primers used for PCR are provided in the Supplemental Material.

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Ubiquitylation of histone H2B controls RNA polymerase II transcription elongation independently of histone H3 methylation

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