Genome-wide function of H2B ubiquitylation in promoter and genic regions

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Nucleosomal organization in and around genes may contribute substantially to transcriptional regulation. The contribution of histone modifications to genome-wide nucleosomal organization has not been systematically evaluated. In the present study, we examine the role of H2BK123 ubiquitylation, a key regulator of several histone modifications, on nucleosomal organization at promoter, genic, and transcription termination regions in *Saccharomyces cerevisiae*. Using high-resolution MNase chromatin immunoprecipitation and sequencing (ChIP-seq), we map nucleosome positioning and occupancy in mutants of the H2BK123 ubiquitylation pathway. We found that H2B ubiquitylation-mediated nucleosome formation and/or stability inhibits the assembly of the transcription machinery at normally quiescent promoters, whereas ubiquitylation within highly active gene bodies promotes transcription elongation. This regulation does not proceed through ubiquitylation-regulated histone marks at H3K4, K36, and K79. Our findings suggest that mechanistically similar functions of H2B ubiquitylation (nucleosome assembly) elicit different functional outcomes on genes depending on its positional context in promoters (repressive) versus transcribed regions (activating).

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Transcription initiation and elongation are intricately linked to nucleosome remodeling and histone modifications. Several studies have demonstrated the contribution of nucleosome remodeling complexes to nucleosomal organization at model genes and across the genome (Whitehouse et al. 2007; Hartley and Madhani 2009; Tirosh et al. 2010). However, little is known about the contribution of transcription-linked histone modifications on genome-wide nucleosomal organization. Nucleosomes are highly organized across eukaryotic genes such that positioned nucleosomes flank a nucleosome-free promoter region (5' NFR) and termination region (3' NFR) (Yuan et al. 2005; Segal et al. 2006; Albert et al. 2007; W Lee et al. 2007; Schones et al. 2008). In *Saccharomyces*, an array of well-positioned genic nucleosomes begins at the transcriptional start site (TSS) (Mavrich et al. 2008). Thus, transcription initiation begins in yeast with the dismantling and/or displacement of the +1 nucleosome.

Upon transcription initiation, RNA polymerase II (pol II) is phosphorylated on Ser 5 of its C-terminal heptad repeat. This Ser5ph signals a series of events through the PAF

complex, by which the E2/E3 ligase complex containing Rad6 and Bre1 monoubiquitylates histone H2B (H2Bub) at Lys 123 (K123ub) in yeast and K120 in mammals (Robzyk et al. 2000; Wood et al. 2005; Xiao et al. 2005a; Pavri et al. 2006; Kim et al. 2009; for review, see Hartzog and Quan 2008; Weake and Workman 2008; Smith and Shilatifard 2010). Bre1-associated protein Lge1 stimulates H2Bub by assisting in the recruitment of Bre1 and inhibiting the recruitment of deubiquitylase Ubp8 (Song and Ahn 2010). H2Bub directs the Set1/COMPASS and Dot1 complexes to di- and trimethylate histone H3 at Lys 4 and Lys 79 (H3K4me2,3 and H3K79me2,3), respectively (Dover et al. 2002; Henry and Berger 2002; Sun and Allis 2002; JS Lee et al. 2007). The K4me3 mark may be recognized by histone acetylases such as NuA3 that acetylate and destabilize nucleosomes (Taverna et al. 2006). In contrast, the K4me2 mark is recognized by deacetylases like Set3C, which suppresses nucleosome acetylation and remodeling (Kim and Buratowski 2009). Yet, how these events globally impact chromatin structure in relation to transcription is still largely unknown.

A cycle of H2Bub ubiquitylation and deubiquitylation at the 5' end of at least the model *GAL1*, *SUC2*, and *ADH2* genes in yeast and *RARβ2* in mammals plays an important role in transcriptional activation (Henry et al. 2003;

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Pavri et al. 2006). Transcription-coupled H2Bub continues into the body of these genes and is known to impact the transcription cycle and histone occupancy (Pavri et al. 2006; Fleming et al. 2008). In this way, FACT may act on H2Bub to promote H2A/H2B dimer dissociation, followed by reassembly in the wake of pol II (Pavri et al. 2006; Fleming et al. 2008). However, it remains unclear as to whether disassembly, reassembly, or both are coupled to pol II elongation. Inasmuch as these mechanistic studies either used a defined biochemical system on a single gene having artificially positioned nucleosomes or involved low-resolution measurements of single-gene histone occupancy, a critical application of these models to gene regulation in general would be an *in vivo* analysis of all genes having naturally organized chromatin.

During the early stage of transcription elongation, the deubiquitylated state of H2B signals Ctk1 to phosphorylate Ser 2 of the heptad repeats in pol II (Wyce et al. 2007). Ser2ph is recognized by Set2, which di- and trimethylates H3 on Lys 36 (K36me_{2,3}) (Li et al. 2002, 2003; Krogan et al. 2003; Xiao et al. 2003; Kizer et al. 2005). This methyl mark is then recognized by the Rpd3C histone deacetylase complex, which removes potentially destabilizing acetyl marks (Li et al. 2007). Thus, the H2Bub cycle represents a bifurcation point, where one pathway (ubiquitylation) leads to histone acetylation, whereas the other (deubiquitylation) leads to deacetylation. Thus, any effect that H2Bub might have on chromatin structure could, in principle, be occurring through altered acetylation states.

H2Bub is involved in suppression of cryptic initiation at certain genes, and this might occur by promoting nucleosome formation that blocks access of the transcription machinery to DNA (Fleming et al. 2008; Chandrasekharan et al. 2009). H2Bub might represent a transient state, in that bulk measurements of H2Bub levels by immunoblotting indicate that only a small fraction of H2B is ubiquitylated (Schulze et al. 2009). This low-level ubiquitylation appears to be spread across most genes and is augmented at both promoter and coding regions of *GAL* genes during transcription (Henry et al. 2003; Fleming et al. 2008).

An emerging view of transcription-linked histone modifications is that individual genic nucleosomes acquire an assortment of marks that help identify their position within an array of nucleosomes. Some of these marks may direct proteins to bind specific nucleosome positions (Koerber et al. 2009). Other marks may also alter nucleosome stability. However, for many marks, it is unclear what they contribute to genome-wide nucleosome organization, on the whole or in the context of transcription. To address the contribution of H2Bub at K123 and its downstream dependent methyl marks at H3K4, K36, and K79 across the yeast genome, we used high-resolution MNase chromatin immunoprecipitation and sequencing (ChIP-seq) mapping of nucleosome positions in histone point mutants that are unable to accept modifications at specific amino acid residues. Where appropriate, we also examined mutants that are impaired in catalyzing the deposition of such modifications. Our results suggest a widespread role of H2BK123ub in promoting nucleosome assembly

across the genome, with negative consequences on assembly of pol II at promoters and positive consequences on elongation of pol II into the body of genes. Our findings provide a clear correspondence between model *in vitro* biochemical systems and genome-wide physiological behavior.

Results

H2BK123 ubiquitylation promotes nucleosome reassembly in the wake of transcription

Since the H2B ubiquitylation/deubiquitylation cycle is a linchpin for multiple diverging *trans*-histone modification pathways, we examined genome-wide nucleosome organization in an H2BK123A mutant. We used alanine mutants to examine K123 and other histone modification sites because it represents the simplest mutant form. A mutation to any amino acid not only removes the modification, but also potentially creates altered interactions that may not be neutral. Since alanine removes the side chain beyond the α carbon, the results described below likely reflect both the loss of the modification and a loss of any wild-type lysine side chain interactions.

We cross-linked nucleosomes at their *in vivo* positions, and these positions were detected across the yeast genome at high resolution by MNase ChIP-seq. Since not all nucleosomes become cross-linked, we implemented an H3 immunoprecipitation under stringent conditions to ensure that only cross-linked nucleosomes were isolated. The cross-linking prevents any artifactual repositioning that could occur during sample workup. This may be particularly important for mutants that could destabilize nucleosomes.

We did not want to assume that total nucleosome occupancy levels were the same between mutant and wild-type strains, as such an assumption might skew interpretations of occupancy changes. Therefore, using immunoblotting, we measured bulk nucleosome levels by measuring the amount of H2B present in chromatin (which excludes soluble H2B) isolated from an equal number of cells (Supplemental Fig. S1). We used H2B, rather than H3, as a more accurate measure of bulk nucleosome levels because subnucleosomal particles like tetramers would be counted as nucleosomes in an H3 immunoblot. Our genome-wide maps of nucleosomes use size-selected mononucleosomal DNA that include predominantly full-length nucleosomes and exclude potential tetramers.

The total number of sequencing tags in each sample (reflecting occupancy levels) was then scaled such that they equaled the level of H2B measured by immunoblotting (determined by averaging at least six replicates). Occupancy levels were then reported as the summation of the number of extended tags covering genomic coordinates. Tags were extended to equal the measured nucleosomal DNA length. To facilitate data visualization at each gene and at all genes, occupancy levels (relative to the genomic average) were plotted as heat map colors along one-dimensional tracks (Fig. 1A). Tracks were then stacked and aligned by the midpoint of each genic nucleosomal

Figure 1. K123ub modulates genic nucleosomal occupancy. (A) Heat maps of nucleosomal occupancies in wild-type (WT) and H2BK123A strains. Each row represents a genic nucleosomal array; arrays are sorted by array length and aligned by array midpoint (illustrated above the plot). Blue areas are the 5' and 3' NFRs. The color bar represents the nucleosomal occupancy from dark blue (zero) to green (genomic average) to dark red (twice the genomic average). (B) Heat maps of log₂ fold changes in nucleosomal occupancies in K123A over wild type, aligned by TSS and sorted by gene length. Each column represents a consensus nucleosome position relative to the TSS. (C) Composite distribution of nucleosomal tags (shifted to represent dyads) in wild type (gray fill) and the K123A mutant (line trace), aligned by the wild-type consensus position of the first nucleosome dyad for all genes (black), highly expressed genes (red), and lowly expressed genes (blue).

array, then sorted by array length (Vaillant et al. 2010). Arrays extended from the first to the last nucleosome found between the TSS and transcription termination site (TTS). We found this to be an intuitive means of examining genomic nucleosome organization on a gene-by-gene basis.

In addition to side-by-side comparison of the K123A pattern with wild type, the two were compared by dividing one pattern by the other to produce nucleosome-by-nucleosome log₂ fold changes in occupancy in the mutant (Fig. 1B). Patterns related to particular conclusions drawn in this study are also shown as quantitative composite plots in which the average occupancy level and positioning of nucleosome midpoints were plotted around the consensus dyads of the first or last nucleosomes (as defined by the wild-type data set) (Fig. 1C; Supplemental Fig. S2). We opted for the start and end of nucleosome arrays as the reference point instead of the standard use of the TSS and TTS because some classes of genes (i.e., SAGA-dominated) lack a strong canonical relationship of nucleosomal positions with the TSS. For these genes, composite plots lacked well-defined peaks and valleys.

In comparing the K123A mutant with wild type, genome-wide decreases in nucleosomal occupancies were observed, particularly in genic regions downstream from the +1 nucleosome position (Fig. 1A,B; Supplemental Fig. S2). These defects were evident in both biological replicates. The changes in nucleosomal occupancies in the mutant

strains were not due to varied MNase digestion, since the sizes of the MNase-protected DNA were similar in all strains (Supplemental Table S1). Importantly, the loss of nucleosomal occupancy was highest at highly expressed genes (Fig. 1C). These observations together indicate that K123ub is of wide-spread importance for nucleosome reassembly across gene bodies in the wake of pol II transcription.

To address whether loss of ubiquitylation, rather than a loss of the lysine side chain at K123, was responsible for these defects, we mapped nucleosomes in strains lacking the H2BK123 ubiquitin ligase (*rad6Δ*) and its regulator (*lge1Δ*). In support of loss of ubiquitylation in creating the chromatin defect, these strains each recapitulated the same chromatin defect as seen with the K123A mutant (Fig. 2A,B; Supplemental Fig. S3A,B). Seemingly minor alterations in nucleosome positioning were seen in the mutants, where nucleosomes interior to genes were shifted upstream regardless of transcription frequency.

Since the PAF complex stimulates pol II transcription and activates the Rad6/Bre1 ubiquitin ligase (Ng et al. 2003; Wood et al. 2003; Xiao et al. 2005b), we therefore mapped nucleosome organization in a *paf1Δ* strain (Fig. 2A,B; Supplemental Fig. S3C). In contrast to what was observed with the ubiquitylation-specific mutants, the chromatin defect with *paf1Δ* was rather modest. Since this strain may be somewhat impaired at the very early stages of transcription, one possible interpretation is that the *paf1Δ* strain

Figure 2. Nucleosomal organization in K123ub-deficient mutants. (A) Heat maps of $\log_2$ fold changes in nucleosomal occupancies in wild type (WT) or the indicated mutants relative to an independently derived wild type. (B) Composite distribution of nucleosomal tags in wild type and indicated mutants for highly expressed genes (*bre1Δ* was not tested).

accumulates a combination of defects in which limited transcription has the consequence of limiting the penetrance of the transcription-coupled ubiquitylation defect.

Additionally, in the *paf1Δ* strain, nucleosomes in the middle of genes had their positions shifted downstream, and such a shift was transcription frequency-dependent (Supplemental Fig. S3C,D). Inactivation of pol II also shows a similar downstream shift of nucleosomes (Weiner et al. 2010). Since the PAF complex is important for transcriptional elongation, loss of Paf1 might be phenocopying defects in the pol II mutant. Collectively, these results indicate that the H2BK123 ubiquitylation pathway, which includes the PAF complex, plays an important role in regulating nucleosome occupancy levels and positioning in genic regions during transcription.

H2B ubiquitylation does not organize nucleosomes via H3K4, K79, or K36

K123ub is an effector of transcription-linked H3K4 and K79 methylation (Dover et al. 2002; Henry and Berger 2002; Sun and Allis 2002; JS Lee et al. 2007; Kim et al. 2009; Nakanishi et al. 2009). However, the role of ubiquitylation in transcription and other cellular phenotypes has been reported to be at least partially independent of H3 methylation (Hwang et al. 2003; Shukla and Bhaumik 2007; Tanny et al. 2007). To address whether loss of the K4 and K79 methyl marks, rather than loss of K123ub per se, might explain the chromatin defects, genome-wide nucleosome maps were generated in K4A and K79A mutants (Fig. 3; Supplemental Fig. S4). These mutants did not phenocopy the chromatin defects seen in

the K123A mutant and essentially behaved as wild type. Given the distinct genomic distribution of K4 and K79 methylation (Zhang et al. 2011), it seems unlikely that they function redundantly. Thus, the chromatin defects incurred upon loss of ubiquitylation were not due to loss of K4 or K79 methylation (or any other potential modification at those sites).

The deubiquitylated form of K123 is a positive effector of bulk H3K36 di- and trimethylation (by stimulating Ctk1 to phosphorylate Ser 2 of pol II, which allows Set2 to methylate K36) (Wyce et al. 2007). However, the dependency of K36 methylation on the deubiquitylated form of H2B has not been examined at individual nucleosome positions and on a genomic scale. We first examined the distribution of K36me2 and K36me3 marks, since the me2 mark had not been previously reported at single-nucleosome resolution (Rao et al. 2005; Zhang et al. 2011). The me2 mark tended to be more 5' than the me3 mark and was not linked to transcription frequency (Fig. 4A; Supplemental Fig. S5), both of which confirm a prior report (Rao et al. 2005). Consistent with the role of K123ub in inhibiting K36 methylation, the K123A mutant allowed for an increase in K36me2,3 (normalized to H3 levels) (Fig. 4B).

Conceivably, the transcription-linked underassembly of nucleosomes in the K123A mutant might be a consequence of elevated levels of K36 methylation. If true, we might expect that elimination of K36 methylation would cause transcription-linked overassembly of nucleosomes. However, we did not observe widespread increases in genome-wide nucleosomal occupancy levels in the K36A mutant when all or highly transcribed genes were examined (Fig. 4C; Supplemental Fig. S6). Thus, the chromatin

Figure 3. Nucleosomal organization in H3K4A and H3K79A mutants. Heat maps of nucleosomal occupancies in wild type (WT) and mutants are shown in the *top* panels. Heat maps of $\log_2$ fold changes in nucleosomal occupancies in the indicated mutants relative to wild type are shown in the *middle* panels. Composite distribution of nucleosomal tags in wild type (gray fill) and indicated mutants (black traces, no fill) for all genes are shown in the *bottom* panel.

defect in the K123A mutant is not through modification defects at H3K36.

A depletion of nucleosomes was observed in the more 3' region of genes in the K36A mutant. Moreover, this loss of nucleosomal occupancy was particular to those genes that were both long and lowly expressed (Supplemental Fig. S6A,B). This is consistent with an earlier report that such genes use H3K36 methylation to prevent cryptic transcription by recruiting Rpd3C deacetylase complex to promote nucleosome stability (Li et al. 2007). Possibly, loss of K36 methylation in this set of genes causes an increase in histone acetylation, which subsequently lowers nucleosomal occupancy. Overall, these results suggest that K123ub-dependent nucleosomal organization is independent of K123ub regulation of H3 methylation.

K123ub within genes stimulates transcription elongation, while K123ub within promoters inhibits pol II recruitment

K123ub might contribute to transcriptional regulation by acting as an effector of H3 methylation and pol II phosphorylation and/or by preventing cryptic transcription

(Kao et al. 2004; Shilatifard 2006; Wyce et al. 2007; Fleming et al. 2008). To understand the relationship between K123ub-dependent transcription and nucleosomal organization, microarray expression analyses were performed on a H2BK123A mutant strain and wild type. Using a 1.5-fold threshold, 345 genes were found to be negatively regulated and 302 genes were positively regulated by K123ub. The negatively regulated genes were normally lowly transcribed, and the positively regulated genes were normally highly transcribed (Fig. 5A), which demonstrates that K123ub functions in a highly selective manner, depending on the transcription status of its target genes. Thus, K123ub is inhibitory toward lowly expressed genes and activating toward highly expressed genes.

We next examined whether altered transcription in K123A was due to a direct effect on transcription, as opposed to an indirect effect arising from altered transcript stability or altered expression of regulatory factors that target the affected genes. To this end, genome-wide pol II occupancies in wild type and the K123A mutant were measured. Genes that were positively regulated by K123ub, which are normally highly transcribed, showed a decrease in pol II occupancy across the gene body, but displayed an

Figure 4. K123ub and K36me modulates nucleosomal organization through distinct pathways. (A) Composite nucleosomal H3 (filled gray), H3K36me2 (red), and K36me3 (blue) tag distribution for all genes. (B) Log₂ fold changes in K36 methylated nucleosomal occupancies in K123A relative to wild type (WT) and aligned by the first nucleosome dyad. (C) Heat maps of log₂ fold changes in nucleosomal occupancies in K36A relative to wild type.

increase in occupancy at their 5' ends (Fig. 5B). This result suggests that K123ub is a direct positive effector of pol II elongation at active genes, but does not appear to be affecting assembly of the transcription machinery at such genes. Consequently, pol II is recruited but has diminished capacity to move into gene bodies in the K123A mutant.

To determine whether genes that were positively regulated by K123ub were associated with high levels of H2B ubiquitylation, we examined both pre-existing lower-resolution genome-wide K123ub levels (Schulze et al. 2009) and our own high-resolution MNase-derived genome-wide maps of K123ub-containing nucleosomes. In both cases, the K123ub maps were virtually identical to the overall nucleosomal pattern (i.e., H3-containing nucleosomes) (Fig. 5C), confirming that K123ub density, on average, is spread rather uniformly across genic nucleosome positions. Importantly, genes that are positively regulated by K123ub had their ubiquitylation levels enriched in the gene body compared with their promoter region, which contrasts to genes that are negatively regulated by ubiquitylation (Fig. 5D), as expected of a transcription-linked mark. Taken together, we interpret these observations to indicate that enhanced K123ub occurs in the body of many genes during transcription, and this ubiquitylation is directly and functionally important for properly organizing nucleosomes so that pol II can transit the gene.

Genes that were negatively regulated by K123ub and were generally quiescent also showed increased pol II occupancy at their 5' ends of the gene in the presence of a K123A mutant (Fig. 5B). Since these genes normally lack pol II, we interpret the increased occupancy as enhanced recruitment of pol II in the mutant. As such, K123ub would normally inhibit pol II binding at these genes. Consistent with this, these genes on average had a moderately higher density of K123ub in their promoter regions compared with the coding region (Fig. 5D).

Ubiquitylation imparts stability to nucleosomes across the genome

Our results (like that of others) support the notion that K123ub contributes positively to transcription elongation by promoting nucleosome formation in the wake of a transcribing polymerase. In contrast, K123ub contributes negatively to assembly of the transcription machinery in promoter regions by promoting nucleosome formation. We sought further evidence for a genome-wide stabilizing effect of K123ub by selecting those nucleosomes having the highest level of K123ub, then examining their turnover rate (histone exchange) using existing data (Dion et al. 2007). For comparison, turnover rates at highly expressed genes (high turnover rate) and lowly expressed genes (low turnover rate) are shown. In this regard, highly ubiquitylated nucleosomes had low turnover rates (Fig. 6A).

Next, we examined the nucleosome occupancy levels in a strain lacking the K123ub deubiquitylase Ubp8. A *ubp8Δ* mutant has elevated levels of K123ub (Henry et al. 2003; Daniel et al. 2004), particularly at the 5' ends of genes (Schulze et al. 2011). Whereas loss of K123ub leads to nucleosome loss, a gain of K123ub in the *ubp8Δ* mutant resulted in a widespread increase in nucleosomal occupancy toward the middle and 3' end of genes and partial elimination of 3' NFRs, compared with wild type (Fig. 6B). This result implies that nucleosome occupancy in wild-type cells is not at its maximal possible level, since the level can go even higher in the mutant. Since nucleosome spacing is unchanged, we are left to conclude that either more assembly or less disassembly is taking place at canonical nucleosome positions in the *ubp8Δ* strain.

Surprisingly, highly expressed genes, which normally have low nucleosome occupancy levels, did not show an increased nucleosome occupancy in the *ubp8Δ* mutant (Supplemental Fig. S7). Thus, for some reason, enhanced

Figure 5. K123ub differentially regulates transcription based on its genomic location. (A) Median transcription frequency for all genes (Holstege et al. 1998) and genes that are positively or negatively regulated by K123ub by >1.5-fold (see the Materials and Methods). (B) Log₂ fold changes in pol II occupancies in a K123A mutant strain over wild type (WT) for the indicated set of H2Bub-regulated genes, aligned by the TSS. (C) Composite nucleosomal H3 (filled gray) and K123ub (black trace) distribution for all genes. Similar plots were obtained using the data of Schulze et al. (2009; not shown). (D) Log₂ fold changes in ubiquitylation levels per base pair in promoter regions (−1 nucleosome) over coding regions (+1 to +5 nucleosomes) for the indicated set of genes.

ubiquitylation was unable to achieve higher nucleosome levels than what is normally seen in the wake of high levels of transcription. We first considered the possibility that Ubp8 does not function at highly transcribed genes, and therefore examined the other deubiquitylase, Ubp10. However, a *ubp10Δ* mutant also did not result in elevated nucleosome occupancy at highly transcribed genes or at most other genes across the genome (data not shown). Rather, we suspect that the physical presence of polymerase across highly transcribed gene bodies may be sterically blocking histones from reassembling onto the DNA, even under conditions of enhanced nucleosome formation in the *ubp8Δ* mutant.

We also observed an increase in nucleosomal occupancy in promoter regions in the *ubp8Δ* strain. The increase was not due to partial 3' NFR filling of adjacent upstream genes, in that promoter regions shared by two adjacent divergently transcribed genes also showed an increase in nucleosomal occupancy (Fig. 6C). Thus, Ubp8 may play a more predominant role at deubiquitylating nucleosomes in a transcription-independent manner, and in doing so may help keep nucleosomes below their maximum occupancy levels. Together, these results provide multiple lines of evidence that K123ub promotes nucleosome formation and stability across the yeast genome.

Discussion

A global transcription-independent role for H2B ubiquitylation in promoting nucleosome formation

Nucleosomes occupy fixed positions around the promoter and termination regions of genes. Across the genome,

nucleosomes are dynamic (Dion et al. 2007; Rufiange et al. 2007), being evicted and deposited at rates that may be specific to their locations and functions. The dynamic nature of nucleosomes ensures that within a population of cells, a given nucleosome position will be occupied at some level below 100%. The data presented here lead us to conclude that ubiquitylation of H2BK123 promotes nucleosome assembly across much of the yeast genome. Loss of ubiquitylation through mutation of K123 or impairment of the ubiquitylation pathway (in *rad6Δ* or *lge1Δ* strains) leads to lower levels of nucleosomes genome-wide. Reciprocally, higher levels of ubiquitylation achieved by deleting the K123 deubiquitylase (*ubp8Δ* strain) results in increased nucleosome occupancy genome-wide. The ability to detect increased nucleosome occupancy genome-wide supports the notion that genomic nucleosome positions are less than fully occupied.

Given that only a small fraction of all H2B is ubiquitylated and that H2B ubiquitylation density is more or less similar across genes (Schulze et al. 2009), albeit with selective regions having some enrichment, K123ub may be rather transient. Thus, K123ub is not needed for nucleosome stability, but instead may be transiently coupled to assembly. Our findings do not exclude a role for K123ub in disassembly (Pavri et al. 2006), although our results indicate that such a role would not likely be rate-limiting in a nucleosome assembly/disassembly cycle. This is most evident during transcription, where we found that a defective ubiquitylation pathway results in defective nucleosome reassembly across entire genes in the wake of pol II. Since highly ubiquitylated nucleosomes have lower turnover, we surmise that ubiquitylation provides additional

Figure 6. Increased nucleosome occupancy in a $\Delta ubp8$ strain. (A) The median nucleosome turnover rate from Dion et al. (2007) for all nucleosomes that belong to highly or lowly expressed genes or ubiquitylation-enriched nucleosomes (top 500 nucleosomes ranked according to H2Bub/H3 ratio) was divided by the genome-wide median for all nucleosomes, then $\log_{10}$ -transformed. (B) Heat maps of nucleosomal occupancies in wild-type (WT) and $\Delta ubp8$ strains. (C) Composite nucleosomal distribution in wild-type (filled gray) and $\Delta ubp8$ (black trace) strains for divergently transcribed genes.

stability that is already intrinsic to nucleosomes. Further support for K123ub-enhanced nucleosomal stability comes from in vitro studies that showed an impaired rate of DNase digestion on chromatin reconstituted with H2B ubiquitylated on K123 compared with nonubiquitylated K123 (Davies and Lindsey 1994).

A picture that emerges is of a basal transcription-independent cycle of genome-wide nucleosome eviction and deposition, in which K123 ubiquitylation is coupled to nucleosome assembly. This contributes to nucleosome formation in and around promoter regions, which helps to keep lowly transcribed genes quiescent by preventing pol II from accessing promoters. As such, loss of ubiquitylation in a variety of mutants results in elevated pol II occupancy at quiescent promoters, and this results in elevated transcription.

K123ub-dependent nucleosome reassembly in the wake of RNA polymerase

A cycle of ubiquitylation and deubiquitylation of H2B at K123 is important for transcription of several model genes in yeast and mammals (Henry et al. 2003; Pavri et al. 2006; Fleming et al. 2008). Yet, the degree to which this cycle contributes to nucleosome organization and transcription across most active genes throughout the genome had not been known. Genes that are highly expressed may have already removed much of the inhibitory processes associated with promoters, including K123 ubiquitylated nu-

cleosomes. Such promoter regions therefore would not be directly affected by mutations in the ubiquitylation/deubiquitylation cycle.

However, a different picture emerges in the transcribed region. Loss of K123ub results in a diminished capacity to assemble nucleosomes in gene bodies, an accumulation of polymerase at the start of these genes, and a decrease in transcription. Given that nucleosome traversal by RNA polymerase involves partial or complete disassembly/assembly of nucleosomes (Kireeva et al. 2002; Belotserkovskaya et al. 2003; Schwabish and Struhl 2004), we surmise that defects in transcription-coupled nucleosome reassembly in the wake of polymerase produces the low nucleosome occupancy level in the K123A mutant. This defect appears to negatively affect the ability of RNA polymerase to clear the promoter, possibly in subsequent rounds of transcription. As such, the contribution of K123ub to proper nucleosome positioning and/or formation may be important in allowing RNA polymerase to escape the promoter region and enter into an elongation state. Evidence that H2B ubiquitylation plays an important role in transcription elongation has also been reported in *Schizosaccharomyces pombe*, where loss of ubiquitylation caused a decrease in pol II occupancy at the 3' end of two tested genes (Tanny et al. 2007).

During transcription, at what point does H2B become ubiquitylated? In vitro evidence suggests that H2Bub is important for the pioneering round of transcription (Pavri et al. 2006). This would suggest that H2B becomes

ubiquitylated ahead of the transcribing polymerase, thereby promoting FACT-mediated dissociation of an H2A/H2B dimer. Importantly, though, our results demonstrate that H2B is coupled to reassembly, and this reassembly is important for elongation by pol II. Consistent with this, a FACT mutant (*nhp6Δ*) displays a decrease in genic nucleosomal occupancy (Celona et al. 2011), as seen with the K123A, *rad6Δ*, and *Ige1Δ* mutants. Thus, while H2B ubiquitylation might precede transcription, its rate-limiting function (and FACT as well) may lie in the reassembly of H2A/H2B immediately following pol II passage.

K123 ubiquitylation is a direct positive effector of H3K4 and K79 methylation (Dover et al. 2002; Sun and Allis 2002; JS Lee et al. 2007) and an indirect negative effector of H3K36 methylation (Wyce et al. 2007). Our studies exclude the likelihood that K123ub mediates its function through methylation changes at these residues, as mutation of these residues had little effect on the positioning or occupancy of individual nucleosomes across the genome. Consistent with this observation, transcriptional elongation is known to be independent of K4/K79me3 in *Saccharomyces cerevisiae* and *S. pombe* (Shukla and Bhaumik 2007; Tanny et al. 2007).

H3K36 was not entirely inert in its contribution to nucleosome organization. We found that genes that were both long and infrequently transcribed had lower nucleosome occupancy in the K36A mutant. These observations are fully consistent with other studies linking K36 methylation to enhanced nucleosome stability at this class of genes (Li et al. 2007), where the methylated form of K36 recruits the Rpd3C histone deacetylase to remove acetyl marks (Carrozza et al. 2005; Joshi and Struhl 2005). Acetyl marks are thought to destabilize nucleosomes (Carrozza et al. 2005), and thus need to be removed to prevent cryptic transcription. Thus, both K123ub and K36me contribute to nucleosome formation/stability in transcribed regions across the genome, but appear to do so by distinct mechanisms. The fact that loss of K123ub and K36me leads to cryptic initiation further supports the role of K123ub and K36me in nucleosome formation/stability (Carrozza et al. 2005; Fleming et al. 2008). Presumably, more open chromatin in these mutants is responsible for initiation of cryptic transcription.

Buffering the 5' ends of genes against defects in nucleosome assembly

Nucleosome organization around the 5' ends of genes is imparted by two major contributors. First, the underlying DNA sequence defines, to a large extent, nucleosome occupancy levels (Segal et al. 2006). By favoring or disfavoring nucleosome formation, the underlying sequence places nucleosomes in approximately the correct positions. This includes contributions toward nucleosome exclusion at NFRs. However, proper positioning requires ATP-dependent chromatin remodelers. As such, remodelers use the energy of ATP hydrolysis to pack nucleosomes against the 5' ends of genes (Zhang et al. 2011). When genes are impaired in nucleosome formation, this model suggests that chromatin

remodelers will continue to pack available nucleosomes against the 5' end of genes at the expense of nucleosomes toward the middle of genes. The data reported here support this prediction, in that the K123A and *rad6Δ* or *Ige1Δ* mutants, all of which have low genic nucleosome occupancy, nevertheless maintain proper nucleosome occupancy and positioning at the +1 position. As predicted by the packing model, occupancy dissipates toward the middle of the gene, although consensus positioning remains largely unchanged. Thus, placing our K123ub studies within this framework, the failure to ubiquitylate and assemble nucleosomes may start as early as the first nucleosome. However, the presence of remodelers may scavenge more distal nucleosomes, moving them to the +1 position, thereby buffering against chromatin defects at +1.

Materials and methods

Strain information

Wild-type H3/H4: YBL574 [MATa, leu2Δ1, his3Δ200, ura3-52, trp1Δ63, lys2-1288, [hht1-hhf1]ΔLEU2 [hht2-hhf2]Δ::HIS3 Ty912Δ35-lacZ::his4, [pDM9-HHT1-HHF1-URA3]]. Wild-type H2A/H2B: Y131: MATa hta1-htb1::LEU2 hta2-htb2::[with URA3 and selected for strong FOA-r] leu2-3,-112 his3-11,-15 trp1-1 ura3-1 ade2-1 can1-100 <pRS426-HTA1-HTB. All H3 and H2B point mutants were compared with their isogenic wild-type strains. Rpo21 was TAP-tagged in wild-type H2A/H2B and H2BK123A mutants using standard genetic methods. All deletion strains were obtained from Research Genetics (kind gift from Joseph C. Reese).

Isolation of mononucleosomes

Mononucleosomes were prepared as described previously (Albert et al. 2007). Briefly, cells were cross-linked, harvested, and lysed, and the crude chromatin was solubilized using a concentration of MNase that produced ~80% mononucleosomes. The mononucleosomes were immunoprecipitated using anti-H3 antibody (Abcam 1791), and the eluted DNA samples were ligated with sequencing adapters followed by LM-PCR. Amplified mononucleosomal DNA was gel-purified and subjected to massively parallel DNA sequencing on the ABI SOLiD or Illumina GAII platforms.

H3K36me2-specific antibodies (Ab9049), H3K36me3-specific antibodies (Ab9050), and H2BK123Ub-specific antibodies (gift from Ali Shilatifard) were used to study genome-wide nucleosomal patterns for the respective modifications. Post-translationally modified mononucleosomes were immunoprecipitated using the specific antibodies, and the eluted DNA was processed as described above.

Immunoblot detection of bulk nucleosome levels

Cells were harvested and lysed, and crude insoluble chromatin was prepared. The insoluble chromatin was washed to remove unbound histones. DNA was then isolated from a portion of each sample and quantified. H2B immunoblots (ActiveMotif 39237) were then performed on equivalent portions of chromatin samples, based on the DNA quantification. H2B band intensities were quantified using Image J software. At least six independent replicates were used for calculations of histone levels.

Sequencing and data analysis

Sequencing tags were mapped to the reference yeast genome using SHRiMP (version 1.3.2)/Bowtie (version 0.12.7) software. Sequencing data are available at NCBI Sequence Read Archive (<http://www.ncbi.nlm.nih.gov/Traces/sra/sra.cgi>) under accession number SRA043952.1. Sequencing tags of at least 35 base pairs (bp), which were uniquely aligned to the yeast genome with maximum six nucleotide mismatches, were retrieved and used for further analysis. Using GeneTrack browser, nucleosome calls were made based on the coordinates of sequencing tags (Albert et al. 2008). Unique coordinates were shifted by half of the mode C-W distance, and the resulting dyad coordinates were used for further analysis. Nucleosomal arrays, from the first nucleosome downstream from TSS to the last nucleosome upstream of TTS, for all genes were determined as described previously (Zhang et al. 2011). Nucleosomal array plots represent the scaled nucleosomal occupancies (tag counts) for all genes sorted by array length and aligned by array midpoints. Nucleosomal occupancies were color-coded from dark blue (zero occupancy) to dark red (representing twice the genome-wide mean of the wild-type data set). The same color scale was used for the mutants, but the occupancy levels for individual nucleosomes were proportionally scaled such that the ratio of total tag counts in mutant versus wild type was the same as that measured in bulk chromatin by H2B immunoblots (reflecting total nucleosome content).

For the composite plots, nucleosomal dyads (shifted nucleosomal tags) were mapped from 2000 bp upstream of and downstream from the dyads of the reference features (the first or the terminal nucleosomes in a nucleosomal array). Scaled nucleosomal tag counts (as described above) were summed across all genes or subsets of genes at single-base-pair resolution from the reference feature.

Composite plots were generated by binning adjacent tag counts with respect to dyads of the first or last nucleosomes. Genes were categorized into specific subsets based on transcription rate (under normal conditions) or genes that are up-regulated or down-regulated in K123A mutant. Highly expressed and lowly expressed genes include the genes that were categorized as the top 10 and bottom 10 percentile of transcription frequency (Holstege et al. 1998). The top 500 genes with the enriched levels of modification marks (after normalization to H3) are considered as modification-enriched genes. Genes that are >2000 bp in gene length and mRNA rate less than five per hour are considered as long and lowly expressed genes.

RNA isolation and expression analysis

Total RNA isolation and mRNA isolation, reverse transcription, and Cy5 and Cy3 labeling were performed as described previously (Huisinga and Pugh 2004). Data analysis was performed as described previously (Zanton and Pugh 2004; Irvin and Pugh 2006). Genes that showed a change of 1.5-fold or more in their expression were considered as up-regulated or down-regulated genes.

ChIP-seq for pol II

ChIP assays were performed using IgG sepharose to capture TAP-tagged Rpo21-bound DNA in wild-type and H2BK123A mutant strains as described previously (Zanton and Pugh 2004). The eluted DNA was amplified using LM-PCR and subjected to paired-end sequencing on the Illumina GAII platform.

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