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The Histone Chaperones FACT and Spt6 Restrict H2A.Z from Intragenic Locations

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SUMMARY

H2A.Z is a highly conserved histone variant involved in several key nuclear processes. It is incorporated into promoters by SWR-C-related chromatin remodeling complexes, but whether it is also actively excluded from non-promoter regions is not clear. Here, we provide genomic and biochemical evidence that RNA polymerase II (RNAPII) elongation-associated histone chaperones FACT and Spt6 both contribute to restricting H2A.Z from intragenic regions. In the absence of FACT or Spt6, the lack of efficient nucleosome reassembly coupled to pervasive incorporation of H2A.Z by mislocalized SWR-C alters chromatin composition and contributes to cryptic initiation. Thus, chaperone-mediated H2A.Z confinement is crucial for restricting the chromatin signature of gene promoters, which otherwise may license or promote cryptic transcription.

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ACCESSION NUMBERS

Datasets are available at the Gene Expression Omnibus (GEO) database with accession no. GSE62880. Reviewers can privately access the data following the link http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=czezqgqafbgdtkb&acc=GSE62880.

AUTHOR CONTRIBUTIONS

C.J. and F.R. designed the study with contributions from C.D.K. and C.L.P. C.J. conducted most of the experiments. S.W. performed the experiments described in Figure 3 and Figure S3. C.D.K. performed some yeast genetics, including the Spt- phenotype assay shown in Figure S4A. F.R. performed the bioinformatic analyses. F.R. and C.J. wrote the manuscript with input from C.D.K. All authors commented on the manuscript.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, Figures S1–S4 and Tables S1–S2 and can be found with this article online at XXX.

INTRODUCTION

Compaction and accessibility of eukaryotic DNA is controlled through protein-DNA complexes whose primary unit is the nucleosome. Four small, basic core histones (H2A, H2B, H3, H4) assemble into an octameric structure around which ~147 bp of DNA is wrapped ~1.7 turns to form a nucleosome. Although massive nucleosome assembly takes place in S-phase during DNA replication, nucleosomes are assembled and disassembled throughout the cell cycle, notably during transcription where RNA polymerase II (RNAPII) needs to translocate across nucleosomes (Avvakumov et al., 2011; Burgess and Zhang, 2013). Several histone chaperones facilitate nucleosome assembly/disassembly during transcription, the best characterized perhaps being the well-conserved FACT (Facilitates Chromatin Transcription) and Spt6. FACT is a protein complex composed of Spt16 and Pob3/SSRP1 (Belotserkovskaya et al., 2003; Brewster et al., 1998; Orphanides et al., 1998) while Spt6 interacts with Spn1/Iws1 (Krogan et al., 2002; McDonald et al., 2010; Yoh et al., 2007). *SPT16* and *SPT6* were both originally identified in genetic screens looking for suppressors of Ty element insertion within the *HIS4* gene (Clark-Adams et al., 1988; Winston et al., 1984) and subsequently shown to play roles in transcriptional elongation (Hartzog et al., 1998; Kaplan et al., 2000; Orphanides et al., 1998). Both FACT and Spt6 possess histone chaperone activity in vitro (Belotserkovskaya et al., 2003; Bortvin and Winston, 1996) and are required for proper chromatin organization in vivo (Bortvin and Winston, 1996; DeGennaro et al., 2013; Ivanovska et al., 2011; Jamai et al., 2009; Kaplan et al., 2003; Schwabish and Struhl, 2004; Voth et al., 2014).

Chromatin organization and composition are very plastic, as cells need to modulate DNA accessibility to several proteins in space and time for regulation of various nuclear transactions such as gene expression, DNA replication and DNA repair. This is achieved by at least three different mechanisms. First, histones in nucleosomes can be covalently modified, affecting internucleosomal interactions and/or regulating the interaction with effector proteins (Suganuma and Workman, 2011; Tessarz and Kouzarides, 2014). Second, nucleosomes can be mobilized through the action of ATP-dependent chromatin remodeling complexes, therefore modulating the access of DNA binding proteins to chromatin (Clapier and Cairns, 2009; Narlikar et al., 2013). Finally, replacing core histones with histone variants in specific nucleosomes is a strategy used by cells to regulate DNA-associated processes (Talbert and Henikoff, 2010; Weber and Henikoff, 2014).

H2A.Z is an H2A variant conserved from yeast to human (Malik and Henikoff, 2003). It occupies very well defined nucleosomes in promoters, enhancers and centromeres where it achieves different functions (Billon and Cote, 2013; Talbert and Henikoff, 2010; Weber and Henikoff, 2014; Zlatanova and Thakar, 2008). In addition, a hypoacetylated and ubiquitinated version of H2A.Z is thought to cover heterochromatic domains (Fan et al., 2004; Hanai et al., 2008; Sarcinella et al., 2007; Swaminathan et al., 2005). H2A.Z is loaded into promoter nucleosomes by ATP-dependent chromatin remodeling complexes related to the yeast SWR-C (Billon and Cote, 2013). More recently, we have shown that H2A.Z is depleted from transcribed regions in yeast and mammalian cells relative to intergenic regions (Hardy et al., 2009). Interestingly, the extent of H2A.Z depletion correlates with the level of transcription. In addition, and quite strikingly, we found that H2A.Z within ORFs is

depleted within minutes upon induction of transcription (Hardy et al., 2009). Conversely, reducing transcription leads to rapid accumulation of H2A.Z within genes. These results led us to propose that H2A.Z incorporation is not solely limited to SWR-C-targeted regions, but that random incorporation and transcription-coupled eviction contribute to shaping the H2A.Z genomic landscape (Hardy and Robert, 2010). This mechanism may also explain how H2A.Z accumulates into large heterochromatin domains which are poorly transcribed (Fan et al., 2004; Hanai et al., 2008; Hardy et al., 2009; Sarcinella et al., 2007; Swaminathan et al., 2005). The presence of H2A.Z in gene bodies has also been reported in plants and in mouse B-cell lymphoma, where it anti-correlates with DNA methylation (Coleman-Derr and Zilberman, 2012; Conerly et al., 2010; Zilberman et al., 2008).

Here we show that FACT and Spt6 play key roles in limiting H2A.Z association with gene bodies. We propose that these two histone chaperones selectively triage H2A.Z from chromatin during transcription. In addition, by preventing nucleosome loss, FACT and Spt6 impede SWR-C from invading gene bodies and loading additional H2A.Z. Importantly, we show that these mechanisms contribute to prevent the emergence of cryptic transcription from within genes.

RESULTS

Histone Chaperones FACT and Spt6 Are Important for Proper H2A.Z Localization

Because our previous work showed that highly transcribed genes are depleted of H2A.Z to a greater extent than non-transcribed regions (Hardy et al., 2009), we hypothesized that an unknown activity assists elongating RNAPII in restricting H2A.Z to promoters. To identify this activity, we analyzed genome-wide H2A.Z localization by chromatin immunoprecipitation from various budding yeast *Saccharomyces cerevisiae* strains mutant for different chromatin remodelers followed by tiling microarrays (ChIP-chip). Since H2A can be exchanged for H2A.Z by the ATP-dependent remodeler SWR-C (Kobor et al., 2004; Krogan et al., 2003; Mizuguchi et al., 2004), and because the INO80 complex (another chromatin remodeler) has been shown to carry out the opposite reaction (Papamichos-Chronakis et al., 2011), we first profiled H2A.Z localization in mutants for subunits of all known ATP-dependent chromatin remodelers in yeast. As shown in Figures 1A and 1B, except for the *swr1*Δ strain, which –as expected (Kobor et al., 2004; Krogan et al., 2003; Mizuguchi et al., 2004)– dramatically affected H2A.Z levels, none of the tested mutants caused any detectable defect in H2A.Z occupancy. Unlike previously published (Papamichos-Chronakis et al., 2011), deletion of *INO80* did not affect H2A.Z occupancy in this study (Figure 1B, dark blue trace). While the reasons for this discrepancy are not clear, differences in strain background may be the cause. Indeed, the growth phenotype resulting from the deletion of *INO80* has been shown to vary depending on the genetic background. Also, we and others (Chambers et al., 2012) noted that *ino80*Δ cells become polyploid over time, and this is also background specific. For all these reasons, we think it is likely that differences in genetic background may explain the discrepancy between our data and that of (Papamichos-Chronakis et al., 2011).

Next, we assessed roles for histone chaperones, since these are likely candidates to assist RNAPII in the apparent specific depletion of H2A.Z during elongation. Among the eight

histone chaperone mutants tested, only two, namely temperature sensitive alleles of *SPT16* and *SPT6*, *spt16-197* and *spt6-1004*, affected H2A.Z occupancy (Figure 1C). Indeed, the dynamic range of H2A.Z occupancy across the genome was reduced in these strains. The importance of these two chaperones in H2A.Z distribution was confirmed by nuclear depletion of Spt16 and Spt6 using the anchor-away system (Haruki et al., 2008) (Figure S1A–C). Similar results were obtained whether H2A.Z ChIP data were normalized against H2B, Mock IPs or Input DNA (Figure S1D). Finally, and because *spt16-197* mutant cells have been shown to exhibit G1/S arrest (Prendergast et al., 1990, Morillo-Huesca, 2010), we repeated the H2A.Z ChIP experiments in *spt16-197*/*sic1*Δ cells. While deletion of *SIC1* releases the cell cycle arrest (Morillo-Huesca et al., 2010), it did not alter the H2A.Z localization defect of the *spt16-197* mutant (Figure S1E), ruling out the possibility of a cell cycle arrest-induced defect. Together, these results support roles for FACT and Spt6 in H2A.Z localization.

FACT and Spt6 Prevent Accumulation of H2A.Z in Gene Bodies

Because microarray data are normalized to the total signal from the experiment, the reduced dynamic range of H2A.Z observed in *spt16* and *spt6* mutants could reflect either decreased H2A.Z occupancy at promoters, increased occupancy in gene bodies, or both. In order to discriminate these possibilities, we performed three different experiments. First, we repeated the ChIP-chip experiments using a spike-in control strategy to correct for microarray normalization artifacts caused by global occupancy changes (Figure 2A). In these experiments, a fixed amount of foreign DNA (here, sonicated bacteriophage phiX174 genomic DNA) was spiked-in (added) to chromatin extracts prior to immunoprecipitation. Because the phiX174 DNA is added exogenously, a similar amount is pulled down irrespective of the genetic background (WT or mutants; data not shown), allowing for external normalization using spots corresponding to the phiX174 genome present on our tiling microarray. The ratios for the phiX174 controls from the *spt16-197* and *spt6-1004* mutants were therefore set equal to the ratios of their corresponding WT strain. Using this rescaling scheme, it became apparent that H2A.Z incorporation increases broadly throughout the genome in *spt16-197* or *spt6-1004* cells (Figure 2B). H2A.Z levels decrease at promoters and radically increase in gene bodies (Figure 2C). In order to confirm this data in an assay not involving any spiking strategy, we performed ChIP-qPCR for several genes. Expressed as percent of ChIP Input, the data show increases in H2A.Z occupancy within gene bodies in both *spt16-197* and *spt6-1004* mutants (Figure 2D). Reminiscent of the genomic data, the increases in gene bodies are often accompanied by decreased H2A.Z occupancy at promoters. Finally, we looked at bulk levels of H2A.Z within the chromatin of *spt16-197* and *spt6-1004* cells in order to assay for H2A.Z-chromatin association using a non-ChIP-based assay. As shown in Figure 2E, bulk levels of chromatin-associated H2A.Z are slightly elevated in *spt16-197* and *spt6-1004* mutants when normalized for histone H4. Taken together, these data suggest that despite a widespread loss of nucleosomes (Figure 2F and text below), FACT and Spt6 mutants accumulate H2A.Z-containing nucleosomes in gene bodies. In both mutants, the accumulation of H2A.Z in gene bodies correlates slightly but positively with transcription as measured by RNAPII occupancy (Pearson $r \sim 0.2$, Figure S2A), suggesting that the phenomenon is related to the transcription-associated function of FACT and Spt6. In essence, compromising these chaperones causes H2A.Z to lose its

relative specificity for promoters and to adopt a distribution profile similar to canonical histones.

FACT and Spt6 Prevent Nucleosome Loss in Transcribed Regions

While it is widely accepted that FACT plays a role in maintaining nucleosome occupancy during transcription, this model is based on data accumulated from specific genes and had –to our knowledge– never been investigated at the genomic scale. As shown in Figure 2F, *spt16-197* cells have a decreased dynamic range of histone H4 occupancy as measured by ChIP-chip. Our genomic data are therefore consistent with a previous report on specific genes (Schwabish and Struhl, 2004) showing that FACT is required for maintaining nucleosomes over genes. Figure 2F also shows the effect of inactivating Spt6 on histone occupancy. Interestingly, in *spt6-1004* cells, nucleosome loss is biased towards the 5′ part of gene bodies. This is consistent with a recent study in *S. cerevisiae* by the Bentley group (Perales et al., 2013) but differs from what was observed in *S. pombe* (DeGennaro et al., 2013) where the *spt6-1* mutant showed a uniform decrease in histone occupancy, similar to what we report here upon inactivation of FACT. Differences between fission and budding yeasts regarding Spt6 are not limited to nucleosome occupancy since *S. pombe* Spt6, but not *S. cerevisiae* Spt6, is important for histone H3K4 trimethylation (DeGennaro et al., 2013). Nevertheless, our data show that, in *S. cerevisiae*, FACT and Spt6 have different effects on nucleosome occupancy over genes. For both chaperones, however, –and as shown before for Spt6 (Perales et al., 2013)– the effect on histone occupancy correlated with transcription (Pearson $r \sim 0.44$, Figure S2B).

FACT and Spt6 Can Discriminate Between H2A.Z and H2A Dimers In Vitro

The data presented thus far argue for FACT and Spt6 assisting RNAPII in the eviction of H2A.Z from intragenic nucleosomes while preserving overall nucleosome integrity during transcription elongation. In order to further dissect the mechanism by which FACT and Spt6 confer discrimination between H2A.Z and H2A, we tested FACT and Spt6 for their ability to incorporate H2A.Z-H2B dimers (hereafter called H2A.Z dimers) into nucleosomes in vitro (Figure 3). Mononucleosomes were assembled on an immobilized DNA template using recombinant yeast canonical histones and challenged with H2A or H2A.Z dimers in the presence of highly purified FACT or Spt6 (Figure 3A). As a control for the system, we showed that H2A.Z dimers were efficiently incorporated by SWR-C in this assay (Figure 3B). While both FACT and Spt6 incorporated H2A dimers into nucleosomes, neither chaperone loaded H2A.Z dimers into nucleosomes (Figure 3C and Figure S3). This result is consistent with a previous study using human FACT and H2A.Z in a similar assay (Heo et al., 2008). Interestingly, when the immobilized nucleosome was prepared with H2A.Z, FACT and Spt6 could efficiently load H2A dimers but not H2A.Z dimers (Figure 3D), suggesting that the chaperones can evict H2A.Z from nucleosomes by exchanging them for H2A dimers (exchange is a necessary feature of incorporation). Collectively, these assays suggest that while FACT and Spt6 are able to replace H2A.Z in nucleosomes with H2A, these chaperones are unable to incorporate H2A.Z within nucleosomes. Combined with the genomic data shown above, these results support that FACT and Spt6 contribute to the eviction of H2A.Z from transcribed regions by virtue of their inability to re-incorporate the histone variant in the wake of RNAPII.

FACT and Spt6 Prevent Pervasive Recruitment of SWR-C to Gene Bodies

In addition to a defect in nucleosome reassembly, additional increase in H2A.Z incorporation (and not just failure to remove it) in non-promoter regions could contribute to the defect observed in the chaperone mutants. We therefore looked at genome-wide SWR-C occupancy in the Spt16 and Spt6 mutants. Strikingly, both mutants caused SWR-C to redistribute from promoters to gene bodies (Figure 4A). Elegant biochemical and genomic data from the Wu and Pugh labs recently showed that SWR-C is targeted to promoters by virtue of its affinity for free DNA in the nucleosome-depleted region (NDR) at gene promoters (Ranjan et al., 2013; Yen et al., 2013). SWR-C redistribution in the chaperone mutants may therefore be a consequence of their observed nucleosome loss (Figure 2F and (Ivanovska et al., 2011; Kaplan et al., 2003; Perales et al., 2013; Schwabish and Struhl, 2004)). Redistribution of SWR-C is likely to contribute to both the observed decrease in H2A.Z at promoters and its increase in gene bodies, representing a positive feedback loop for chromatin defects in the *spt16-197* and *spt6-1004* strains. In agreement with this model, we found that the accumulation of H2A.Z observed within gene bodies in these chaperone mutants is lost in *swr1*Δ cells for all genes tested (Figure 4B). Together, our data show that FACT and Spt6 impede H2A.Z accumulation outside promoters by at least two mechanisms: directly by selective reincorporation and indirectly by preventing supra-physiological *de novo* incorporation.

Mislocalization of H2A.Z Contributes to Cryptic Transcription

What are the consequences for inappropriate H2A.Z accumulation in gene bodies? Mutations in either *SPT16* or *SPT6* lead to cryptic transcription initiated from within genes (Cheung et al., 2008; Kaplan et al., 2003; Mason and Struhl, 2003), a phenomenon attributed to the inability of these mutants to maintain proper chromatin structure over genes during transcription. Because the presence of H2A.Z in promoter nucleosomes promotes RNAPII recruitment (Adam et al., 2001; Hardy et al., 2009), we hypothesized that the accumulation of H2A.Z outside of promoters might contribute to cryptic initiation in the chaperone mutants. In order to test this hypothesis, we used a *FLO8-HIS3* reporter system developed by the Winston group (Cheung et al., 2008). In this system, growth on plates lacking histidine is enabled by activation of a cryptic intragenic *FLO8* promoter driving *HIS3* expression (Figure 5A). As shown in Figure 5B, the ability of the *spt16-197* and *spt6-1004* strains to grow on plates lacking histidine is decreased upon the deletion of *HTZ1* (the gene coding for H2A.Z in *S. cerevisiae*) suggesting that H2A.Z contributes to the cryptic transcription phenotype of both FACT and Spt6 mutants. Consistent with suppression of *spt16-197* cryptic transcription at *FLO8*, the *spt16-197* Spt- phenotype for lys2-128∂, which is proposed to result from activation of a cryptic promoter within a ∂ element inserted into the 5′ end of *LYS2* (Malone et al., 1991), is also strongly suppressed by *htz1*Δ (Figure S4A). Also similar to the weaker suppression of *spt6-1004* cryptic transcription by *htz1*Δ at *FLO8*, *htz1*Δ suppression of the *spt6-1004* Spt- phenotype is also weaker than that for *spt16-197* (Figure S4A).

We then used Northern blots to confirm the suppression of the cryptic phenotype of FACT and Spt6 mutants. These experiments showed that the cryptic transcription phenotype of *spt16-197* is partially suppressed by the deletion of *HTZ1* when cells are shifted at 33°C, but

not at 37°C (Figure 5C and Figure S4B). Because histone loss in *spt16-197* is much more pronounced at 37°C than at 33°C (data not shown), the contribution of H2A.Z mislocalization to cryptic transcription may be obscured by overwhelming histone loss at high temperature. At 33°C, however, the moderate histone loss in *spt16-197* cells appears to confer a requirement for H2A.Z for cryptic transcription. To further characterize the role of H2A.Z in cryptic transcription, we measured the 3′/5′ expression ratio by RT-qPCR as described before (Rossetto et al., 2014) at eight genes previously known to experience cryptic transcription in both Spt16 and Spt6 mutants (Cheung et al., 2008; Kaplan et al., 2003). Note that while changes in the 3′/5′ expression ratio could in principle be attributed to events other than cryptic transcription such as antisense transcripts, our unpublished strand-specific RNA-Seq data show that antisense transcripts in *spt6-1004* and *spt16-197* mutants are typically one order of magnitude less abundant than cryptic transcripts from the sense strand (not shown). At all genes tested, deletion of *HTZ1* led to a partial but reproducible suppression of cryptic transcription in *spt16-197* cells (Figure 5D and Figure S4C). The cryptic phenotype of *spt6-1004*, however, was not reproducibly suppressed by the loss of H2A.Z as measured by Northern blot (Figure S4B) or by RT-qPCR (data not shown). This may be due to the fact that *spt6-1004* abrogates multiple cryptic transcription suppression pathways, *e.g. spt6-1004* is defective for Set2-dependent H3K36 trimethylation (Chu et al., 2006; Youdell et al., 2008). Altogether, our data suggest that aberrant H2A.Z localization to gene bodies promotes cryptic transcription, and that cryptic initiation might require attributes of normal promoters such as the presence of H2A.Z.

Other Phenotypes of FACT and Spt6 Mutant Cells Are Not Suppressed by Deletion of *HTZ1*

In order to address whether the accumulation of H2A.Z in gene bodies is a direct cause of cryptic transcription in FACT and Spt6 mutants, we tested whether other phenotypes associated with these mutants could be suppressed by the deletion of *HTZ1*. As shown in Figure 5E–G, none of the phenotypes tested, namely loss of H3K36 trimethylation in *spt6-1004* as well as inviability at 37°C and nucleosome loss in both *spt16-197* and *spt6-1004*, were suppressed by deletion of *HTZ1*. It therefore appears likely that accumulation of H2A.Z in gene bodies directly contributes to cryptic transcription in these mutants. It is clear that H2A.Z is not essential for cryptic transcription, but instead appears to promote cryptic transcription under conditions where cryptic initiation is moderate (*i.e. spt16-197* strains at 33°C). Importantly, although *htz1*Δ partially suppresses *spt6-1004* cryptic transcription for the *FLO8-HIS3* reporter, *spt6-1004/htz1*Δ double mutants show synthetic growth defects (data not shown), suggesting that H2A.Z and Spt6 collaborate in some roles though they are antagonistic for cryptic transcription. Supporting this observation, we noticed that *spt6-1004/htz1*Δ cells spontaneously diploidize at high frequency (data not shown). This is consistent with the previously described role of H2A.Z in genomic instability in several organisms (Ahmed et al., 2007; Krogan et al., 2004; Rangasamy et al., 2004) and suggests that it might be exacerbated in *spt6-1004*. Nevertheless, both presumptive diploid and haploid *spt6-1004*/*htz1*Δ cells show similar suppression of the cryptic transcription of *spt6-1004* cells (Figure S4D).

DISCUSSION

Here we have shown that proper H2A.Z localization requires not only its targeted deposition at promoters by SWR-C but also its active restriction from gene bodies promoted by histone chaperone activity. When either FACT or Spt6 is compromised, failure to selectively reincorporate H2A from transcribed regions leads to increased H2A.Z occupancy. This also occurs concomitantly with overall nucleosome loss, which subsequently promotes further H2A.Z loading via the pervasive recruitment of SWR-C to free DNA. This self-promoting cycle leads to redistribution of H2A.Z-containing nucleosomes –normally a signature of promoters– into gene bodies, promoting cryptic transcription (Figure 6). In the broader sense, our work suggests that the chromatin signature of promoters depends not only on specific deposition at promoters but also on selective reincorporation during transcription. Therefore, promoter signature is intimately linked to the state of chromatin at distal sites. While this was demonstrated here for the histone variant H2A.Z, the phenomenon may also apply to other chromatin/epigenetic marks. It should be possible to test whether histone chaperones can recognize and evict histones carrying inappropriate histone modifications for example. Preventing nucleosome loss may also be important for restricting pervasive recruitment of other chromatin regulators as we showed here for SWR-C.

Although our study shows that an excess of H2A.Z in gene bodies (as observed here in FACT and Spt6 mutants) can be harmful, it also questions whether some appropriately constrained levels of H2A.Z play positive roles. It is indeed tempting to speculate that a cycle of H2A.Z deposition/eviction exists in gene bodies and contributes positively to gene expression. For example, some level of transcription, initiated from within genes may uphold chromatin plasticity. Also, maintaining some level of H2A.Z in gene bodies may promote transcription elongation. Consistent with this model, elongation rate is decreased in *htz1*Δ yeast cells (Santisteban et al., 2011) and elongation through the +1 nucleosome in *Drosophila* is facilitated by the presence of H2A.Z (Weber et al., 2014).

While, to our knowledge, our work is the first to show that Spt6 can discriminate between histone variants, growing evidence points toward FACT being a highly discriminative chaperone. Indeed, FACT can discriminate between H2A.Z and H2A ((Heo et al., 2008) and this work) but does not differentiate between H2A.X and H2A (Heo et al., 2008). More recently, FACT was shown to prevent ectopic localization of the centromeric H3 variant CENP-A (Deyter and Biggins, 2014). Collectively, this and our work argue for a role for FACT in preserving epigenetic identity by preventing pervasive incorporation of promoter (H2A.Z) and centromeric (CENP-A) specific variants at distal sites. Interestingly, the exclusion of CENP-A from euchromatin by FACT involves the ubiquitin-mediated degradation of the centromeric histone (Deyter and Biggins, 2014). While we cannot completely rule out such a mechanism for H2A.Z, our data suggest otherwise. Indeed, FACT (and Spt6) can selectively remove H2A.Z from nucleosomes in vitro in the absence of additional factors. Also, the pervasive recruitment of SWR-C in FACT and Spt6 mutants is unlikely to require protein degradation and can itself explain excessive H2A.Z loading in gene bodies.

While our observations were made in budding yeast, several reasons suggest that our findings translate into higher eukaryotes, including humans. First, all the factors involved (H2A.Z, SWR-C, FACT and Spt6) have orthologs throughout eukaryotes, and human FACT can discriminate H2A.Z from H2A in vitro (Heo et al., 2008). Second, the localization of H2A.Z to promoters has been observed in all organisms examined to date (Billon and Cote, 2013; Talbert and Henikoff, 2010; Weber and Henikoff, 2014; Zlatanova and Thakar, 2008). Third, the relative depletion of H2A.Z in gene bodies is also a conserved phenomenon (Hardy et al., 2009). Fourth, two very recent studies identified a histone chaperone in human cells (Anp32e) that can mediate H2A.Z removal (Mao et al., 2014; Obri et al., 2014). Fifth, and quite interestingly, H2A.Z has been shown to redistribute to active gene bodies upon B-cell lymphomagenesis (Conerly et al., 2010). Given the established role of H2A.Z in cancer (Dryhurst and Ausio, 2014; Monteiro et al., 2014; Rangasamy, 2010), this raises the possibility that H2A.Z-mediated cryptic transcription may contribute to carcinogenesis. Also in favor of such a model, a recent study established that FACT acts as an “accelerator” of tumor formation (Garcia et al., 2013). Since high H2A.Z levels have been associated with cancer progression and a negative prognosis (Dryhurst and Ausio, 2014; Monteiro et al., 2014; Rangasamy, 2010), and because aberrant transcription is a well-recognized hallmark of cancer, activation of cryptic transcription by H2A.Z represents a new model for pathological changes to gene expression that merits scrutiny.

EXPERIMENTAL PROCEDURES

For detailed Experimental Procedures, see Supplemental Experimental Procedures.

Yeast Strains

Genotypes for the yeast strains used in this study are listed in Table S1. All deletions and tagging were performed using transformation and homologous recombination of appropriate PCR cassettes or by the *Delitto perfetto* method. The *spt6-1004*/*htz1*Δ and *spt16-197*/*htz1*Δ phenotypes were validated additionally by backcrossing of double mutants to wild type and showed identical phenotypes to those generated by homologous recombination (data not shown).

ChIP and ChIP-chip

ChIP experiments were performed at least in duplicates as previously described (Jeronimo and Robert, 2014). H2A.Z ChIP DNA was hybridized in competition with H2B ChIP DNA (performed on the same extract) except for experiments shown in Figure S1D (middle and right panels) where H2A.Z ChIP DNA was hybridized in competition with a Mock IP (using rabbit IgG) and Input DNA. H4 ChIP DNA was hybridized in competition with Input DNA. Swr1-HA ChIP samples were hybridized against similar ChIP samples performed from an isogenic strain where the *SWR1* gene is not tagged. In some experiments, 300 ng of sonicated bacteriophage phiX174 DNA (Thermo Scientific) was added to the extract prior to immunoprecipitation. The microarrays were custom designed by Agilent Technologies and contain a total of about 180,000 T_m-adjusted 60-mer probes covering the entire yeast genome with virtually no gaps between probes as well as several hundreds probes covering the phiX174 genome (Jeronimo and Robert, 2014). For the initial screen testing chromatin

remodeler mutants shown in Figures 1A and 1B, microarrays containg about 44,000 probes were used (Bataille et al., 2012).

ChIP-chip Data Analysis

The ChIP-chip data were normalized using the Limma Loess method and replicates were combined as described previously (Ren et al., 2000). The data were subjected to one round of smoothing using a Gaussian sliding window with a standard deviation of 100 bp to generate data points in 10 bp intervals as described before (Guillemette et al., 2005). In Figures 2B and 2C, the H2A.Z/H2B $\log_2$ ratios from the *spt6-1004* and *spt16-197* mutants were rescaled by setting the average $\log_2$ ratio of the control phiX174 spots to the same value as the one observed in experiments performed in WT cells.

Aggregate Profiles

Aggregate profiles were generated using the Versatile Aggregate Profiler (VAP) (Coulombe et al., 2014).

In Vitro Histone Incorporation Assay

Recombinant yeast histones were expressed and purified from *Escherichia coli*, and octamers were reconstituted as described (Luger et al., 1999a, b). In order to monitor histone deposition by FACT or Spt6, H2A or H2A.Z-containing mononucleosomes were reconstituted by salt dialysis onto a biotinylated DNA fragment (200 bp) containing the 601 nucleosome-positioning sequence (Lowary and Widom, 1998). FACT or Spt6 were preincubated with an equimolar amount of free histone dimers (either HA-H2A.Z/H2B or Flag-H2A/H2B dimers) in exchange buffer (70 mM NaCl, 25 mM HEPES [pH 7.5], 5 mM $MgCl_2$, 0.5 mM EGTA, 0.02% NP40, 0.1 mg/ml BSA and 1 mM DTT). Mononucleosomes (20 nM) were incubated with streptavidin-conjugated magnetic beads (Dynabeads M-280) in exchange buffer for 30 min at 30 °C. Subsequently, 30 nM of FACT or Spt6/histone dimers were added and incubated for another 60 min at 30 °C. Beads were washed three times with exchange buffer containing 0.1 mg/ml sonicated salmon sperm DNA. Bound fractions were subjected to 18% SDS-polyacrylamide gel electrophoresis and transferred onto PVDF membranes for Western blotting. Western blotting was performed using commercially available monoclonal antibodies against Flag (Sigma, 3165) or HA (Sigma, H9658), or polyclonal antibody against yeast histone H3 (Abcam, ab1791).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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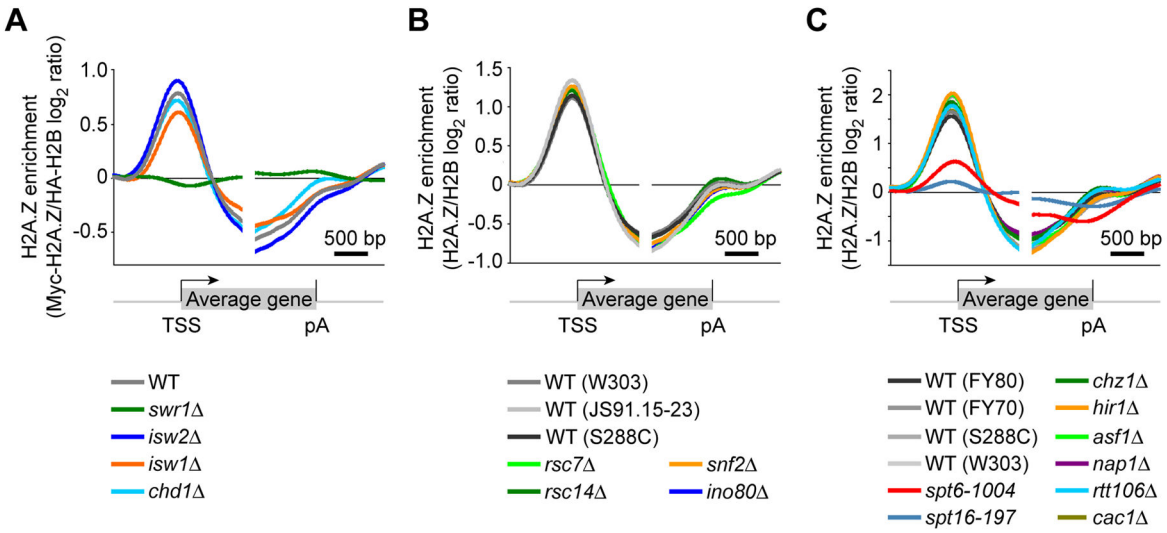

Figure 1. A Survey of Chromatin Regulator Mutants Identified Histone Chaperones FACT and Spt6 as Important Regulators of H2A.Z Occupancy

(A–B) Aggregate profiles of H2A.Z/H2B $\log_2$ enrichment ratios over all yeast genes longer than 1 kb (*n*=3439 genes) in various ATP-dependent chromatin remodeler mutants. ChIP experiments were either performed using epitope tagged H2A.Z (Myc) and H2B (HA) (A) or rabbit polyclonal antibodies against H2A.Z and H2B (B). (C) Aggregate profiles of H2A.Z/H2B $\log_2$ enrichment ratios over all yeast genes longer than 1 kb in various histone chaperone and chromatin assembly factor mutants. The experiments for the *spt16-197* and spt6-1004 strains, as well as their respective wild type, were performed after an 80 minute switch to non-permissive temperature (37°C). See also Figure S1.

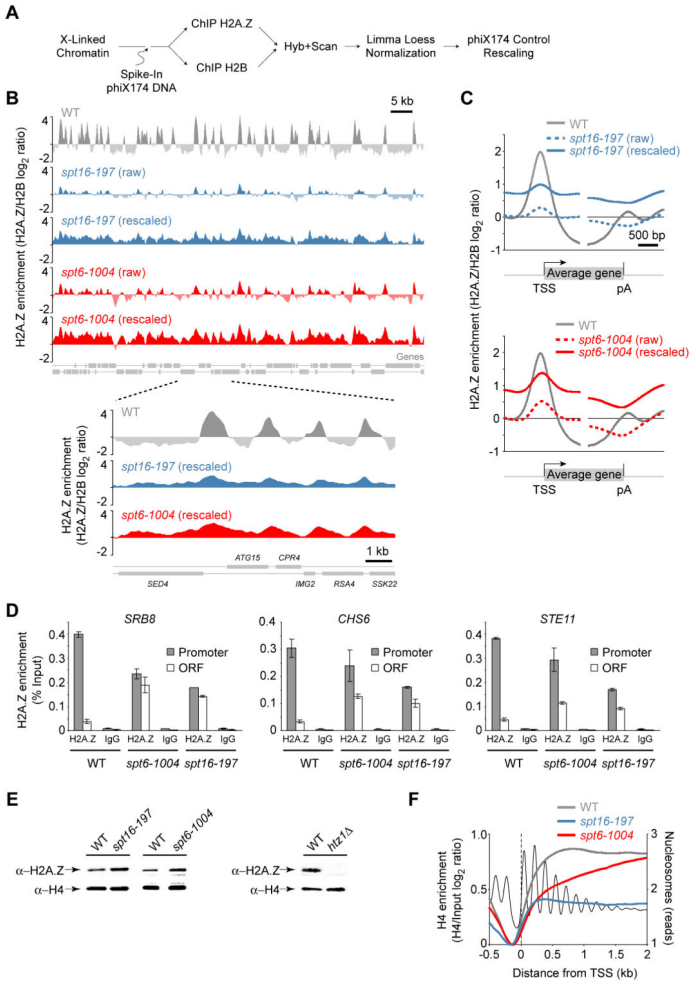

Figure 2. Inappropriate Accumulation of H2A.Z in Gene Bodies in *spt16* and *spt6* Mutants

(A) A schematic representation of the spike-in strategy used to rescale H2A.Z ChIP-chip data in *spt16-197* and *spt6-1004* cells. (B) H2A.Z/H2B $\log_2$ enrichment ratio along a 90 kb fragment of chromosome III is shown for WT (grey), *spt16-197* (blue) and *spt6-1004* (red) cells. The data from mutant cells are shown prior to ("raw") and after ("rescaled") rescaling using phiX174 exogenous spike-in controls. A zoom in around the *ATG15* locus is shown at the bottom. (C) Aggregate profiles of H2A.Z/H2B $\log_2$ enrichment ratios over all yeast genes longer than 1 kb in *spt16-197* (blue) and *spt6-1004* (red) cells, together with their respective WT (grey). The data from mutant cells are shown prior to (dashed trace) and after (solid trace) rescaling using phiX174 exogenous spike-in controls. (D) Absolute H2A.Z occupancy (expressed in % of Input) over the promoter (grey) and coding region (white, ORF) of selected genes, as determined by ChIP-qPCR. Control experiments using IgG antibodies are also shown. (E) Western blot showing bulk levels of H2A.Z in chromatin extracts prepared from *spt16-197* and *spt6-1004* cells, together with their respective WT. Loading was normalized using histone H4. The right panel shows bulk H2A.Z and H4 levels from chromatin extracts prepared from WT and *htz1*Δ cells, demonstrating the specificity of the H2A.Z antibody. (F) Aggregate profiles of H4 $\log_2$ enrichment ratios over all yeast

genes longer than 1 kb in WT (grey), *spt16-197* (blue) and *spt6-1004* (red) cells, which were shifted to 37 °C for 80 min. All traces were normalized by setting the minima (representing the NDR) to "0". Reads density from an MNase-Seq experiment (black) from WT cells (Jiang and Pugh, 2009) is shown as a guide for the position of nucleosomes. See also Figure S2.

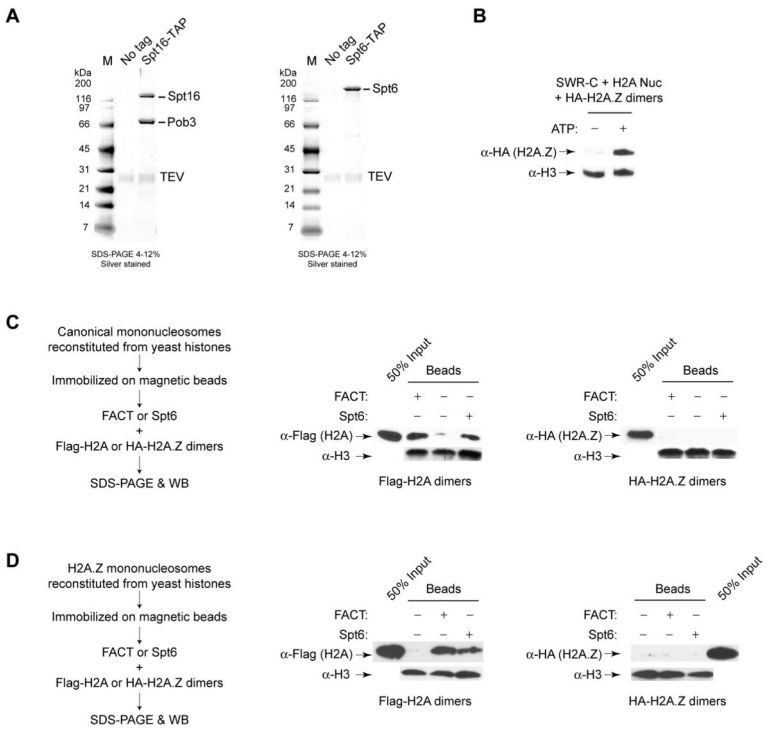

Figure 3. FACT and Spt6 Can Discriminate Between H2A and H2A.Z Dimers In Vitro

(A) Silver stained SDS-PAGE of the FACT (Spt16-TAP) and Spt6 (Spt6-TAP) protein complexes used. Traces of TEV protease remaining after purification are indicated. (B) Western blot showing the amount of HA-H2A.Z/H2B dimers incorporated within canonical nucleosomes (H2A Nuc) by purified SWR-C in the absence (−) or presence (+) of ATP. Histone H3 is shown as a loading control. (C) A scheme of the in vitro histone incorporation assay (left) and Western blots showing the amount of Flag-H2A (middle) or HA-H2A.Z (right) dimers incorporated within canonical nucleosomes in the absence (−) or presence (+) of purified FACT or Spt6 complexes. Inputs (50%) were loaded as controls. Blotting beads with an anti-H3 antibody shows that equivalent amount of mononucleosomes were used in both assays. (D) A scheme of the in vitro histone incorporation assay (left) and Western blots showing the amount of Flag-H2A (middle) or HA-H2A.Z (right) dimers incorporated within H2A.Z nucleosomes in the absence (−) or presence (+) of purified FACT or Spt6 complexes. The blots are also probed using an anti-H3 antibody. See also Figure S3.

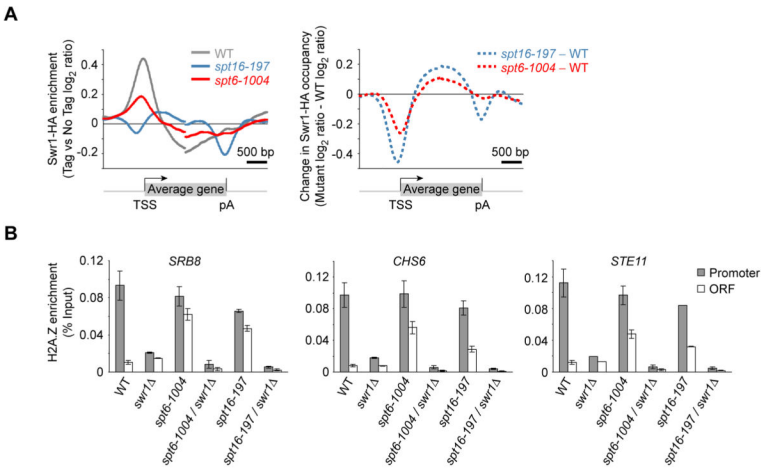

Figure 4. FACT and Spt6 Prevent Pervasive SWR-C Recruitment In Vivo

(A) Left panel shows Aggregate profiles of Swr1-HA $\log_2$ enrichment ratios (Tag vs No Tag) over all yeast genes longer than 1 kb in WT (grey), *spt16-197* (blue) and *spt6-1004* (red) cells. The right panel shows the difference in Swr1-HA levels between *spt16-197* and WT cells (dashed blue) or *spt6-1004* and WT cells (dashed red). (B) Absolute H2A.Z occupancy (expressed in % of Input) over the promoter (grey) and coding region (white, ORF) of selected genes, as determined by ChIP-qPCR in WT, *swr1*Δ, *spt6-1004*, *spt6-1004*/*swr1*Δ, *spt16-197* and *spt16-197*/*swr1*Δ cells.

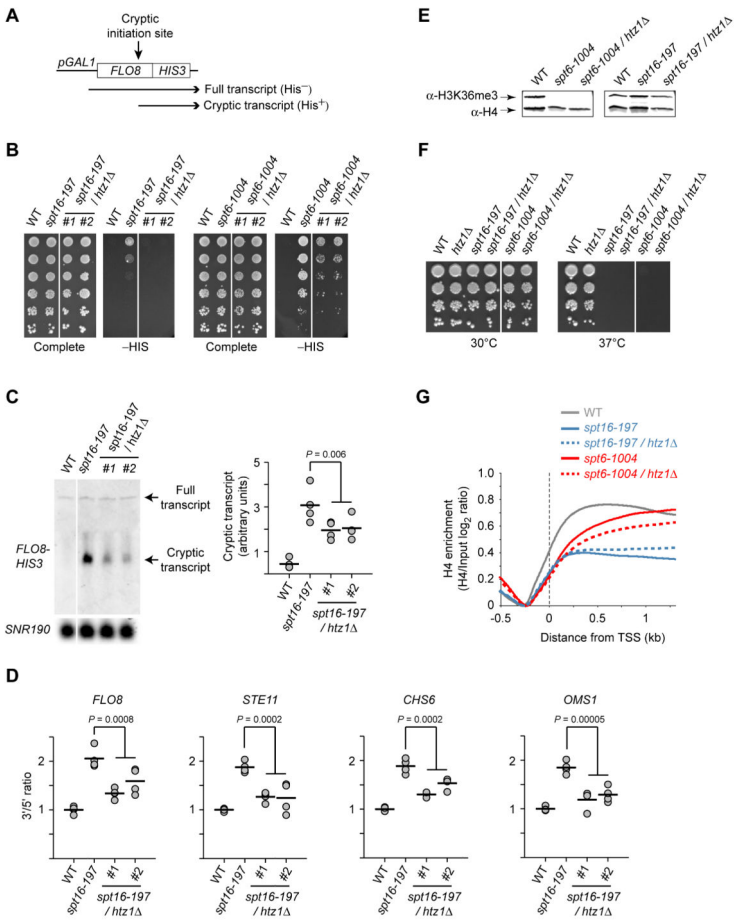

Figure 5. Deletion of *HTZ1* Partially Suppresses Cryptic Transcription From *spt16-197* and *spt6-1004* Cells

(A) A schematic representation of the *FLO8-HIS3* system used to detect cryptic transcription from the *FLO8* gene (Cheung et al., 2008). (B) The indicated yeast strains were grown to saturation in YNB-complete medium, washed, resuspended at the same density in water, serial diluted (5 fold series) and spotted on YNB-complete (Complete) and YNB medium lacking histidine (−HIS). Plates were incubated at 33°C. (C) Levels of cryptic transcript transcribed from the *FLO8-HIS3* locus, as determined by Northern blot, are shown after an 80 minute shift from 30°C to 33°C. *SNR190* was used as a loading control. The experiments were performed four times. A representative example is shown on the left and quantification for four independent biological replicates (grey circles) together with the average (black bars) is shown on the right. Indicated *P* value is from *T*-test. (D) RT-qPCR was used to measure cryptic transcription at four genes in the indicated strains (four additional genes are shown in Figure S4C). Expression was measured in the 5− and 3− regions of each gene and the 3−/5− ratio was used as a measure of cryptic transcription. Values for four independent biological replicates are shown (grey circles) together with the average (black bars). Indicated *P* values are from *T*-tests. (E) Western blots showing the amount of H3K36me3 at permissive temperature (30 °C) in WT, *spt6-1004* and *spt6-1004/ htz1*Δ cells (left) and in WT, *spt16-197* and *spt16-197/htz1*Δ cells (right). Histone H4 is shown as a loading control. (F) The indicated yeast strains were grown to saturation in YPD

medium, washed, resuspended at the same density in water, serial diluted (10 fold series), spotted on YPD plates and incubated at 30 °C or 37 °C. (G) Aggregate profiles of H4 $\log_2$ enrichment ratios over all yeast genes longer than 1 kb in WT (grey), *spt16-197* (solid blue), *spt16-197*/*htz1*Δ (dashed blue), *spt6-1004* (solid red) and *spt6-1004/htz1*Δ (dashed red) cells, which were shifted to 37 °C for 80 min. All traces were normalized by setting the minima (representing the NDR) to "0". See also Figure S4.

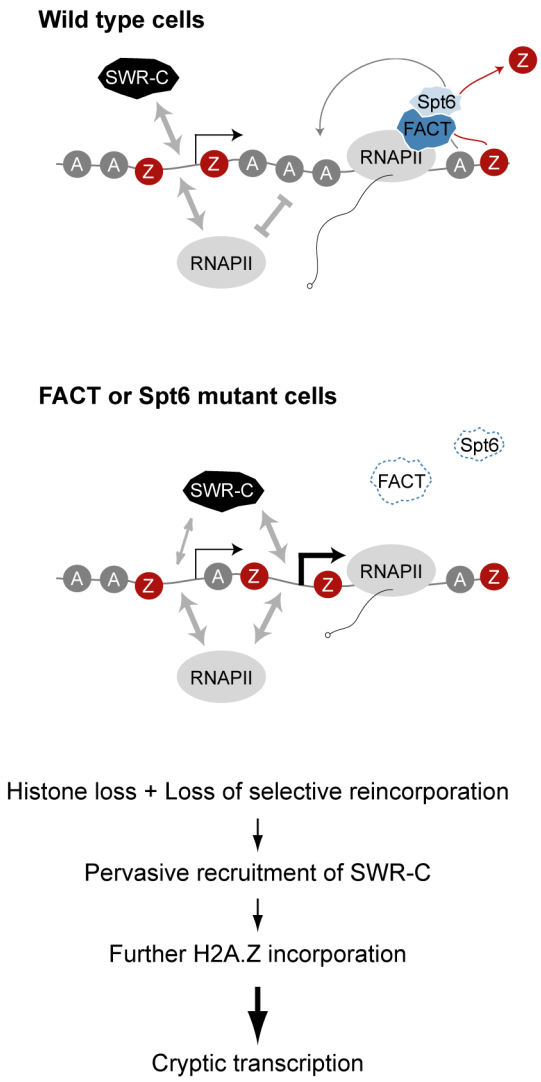

Figure 6. A Schematic Model Describing the Activities of FACT and Spt6 in Preserving the Epigenetic Landscape and Guarding Against Cryptic Transcription

FACT and Spt6 prevent nucleosome loss and selectively reincorporate H2A within gene bodies during transcription elongation. This ensures proper chromatin structure over genes and prevents cryptic transcription. When either FACT or Spt6 is compromised, nucleosome loss occurs and H2A.Z is not efficiently removed from gene bodies. The paucity of nucleosomes in gene bodies leads to pervasive recruitment of SWR-C, which exacerbates H2A.Z accumulation in these regions. This nucleosome-poor/H2A.Z-rich chromatin promotes cryptic transcription.