

RSC regulates nucleosome positioning at Pol II genes and density at Pol III genes

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Nucleosomes can restrict the access of transcription factors to chromatin. RSC is a SWI/SNF-family chromatin-remodeling complex from yeast that repositions and ejects nucleosomes *in vitro*. Here, we examined these activities and their importance *in vivo*. We utilized array-based methods to examine nucleosome occupancy and positioning at more than 200 locations in the genome following the controlled destruction of the catalytic subunit of RSC, Sth1. Loss of RSC function caused pronounced and general reductions in new transcription from Pol I, II, and III genes. At Pol III genes, Sth1 loss conferred a general reduction in RNA Pol III occupancy and a gain in nucleosome density. Notably at the one Pol III gene examined, histone restoration was partly replication-dependent. In contrast, at Pol II promoters we observed primarily single nucleosome changes, including movement. Importantly, alterations near the transcription start site were more common at RSC-occupied promoters than at non-occupied promoters. Thus, RSC action affects both nucleosome density and positioning *in vivo*, but applies these remodeling modes differently at Pol II and Pol III genes.

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Introduction

The packaging of eukaryotic genomes into chromatin provides remarkable compaction and organization within the nucleus. Nucleosomes comprise the basic repeating units of chromatin and consist of an octamer of four histones. Periodically the DNA must be made accessible to multiple types of factors, including DNA-binding factors, polymerases, and repair enzymes. To accomplish this, cells remodel chromatin to allow DNA accessibility through a variety of enzymatic processes (reviewed in Groth *et al*, 2007; Li *et al*, 2007). While chromatin remodeling has been characterized *in vitro* and *in vivo* at specific loci, a large-scale analysis of remodel-

ing *in vivo* is needed to understand how these processes are applied and regulated during, for example, transcription.

Typical RNA polymerase II (Pol II) promoters have a chromatin structure that includes a small (~150 bp) nucleosome-free region (NFR) located ~200 bp upstream of the coding region (Yuan *et al*, 2005). The NFR often coincides with the transcriptional start site of transcription, suggesting it is the site of assembly for the Pol II initiation complex. Adjacent to either side of the NFR are well-positioned nucleosomes that often contain inherent nucleosome-positioning sequences (NPS; Ioshikhes *et al*, 2006; Segal *et al*, 2006). Furthermore, TATA-containing promoters are more likely to have the TATA box either within, or adjacent to, a strong NPS. Not surprisingly, these promoters are typically regulated by histone acetyltransferases, histone deacetylases, Mediator complex, and chromatin remodelers including SWI/SNF and ISWI, which may collaborate to move proximal nucleosomes. Access to a TATA box and other promoter elements that are occluded by nucleosomes may be accomplished either by nucleosome sliding (Lomvardas and Thanos, 2001; Inai *et al*, 2007) or by ejection (Adkins *et al*, 2004; Boeger *et al*, 2004). Hence, the emergent view of active transcription involves the recruitment of multiple chromatin-modifying complexes to post-translationally modify, move, and/or eject nucleosomes to help establish or expand the NFR and allow the pre-initiation complex to be assembled.

Chromatin remodelers are evolutionarily conserved, multi-subunit complexes that utilize ATP hydrolysis to alter the composition or arrangement of chromatin. Nucleosome remodeling is based on one of two simple strategies: disassembling (ejecting) or sliding the nucleosome (Langst *et al*, 1999; Lorch *et al*, 1999; Whitehouse *et al*, 1999; Boeger *et al*, 2004). Remodelers are comprised of subunits with specialized roles. The central subunit contains the ATPase engine that provides a DNA translocation force (Saha *et al*, 2002; Whitehouse *et al*, 2003). Additional subunits may regulate the ATPase activity (Sif *et al*, 1998; Yang *et al*, 2007). Targeting of the remodeler can be accomplished through several means, including DNA binding (Angus-Hill *et al*, 2001), direct protein–protein interactions with activators (Yudkovsky *et al*, 1999; Govind *et al*, 2005; Inai *et al*, 2007), recruitment by basal transcription machinery (Soutourina *et al*, 2006), or by recognition of acetylated histones through bromodomains (Hassan *et al*, 2002; Geng and Laurent, 2004; Kasten *et al*, 2004).

Remodelers regulate a number of chromatin- and DNA-based processes, including transcriptional activation and repression, transcriptional elongation, replication, and DNA repair (Saha *et al*, 2006). One well-characterized family of remodelers is the SWI/SNF family, of which multiple homologues exist in vertebrates. The RSC (Remodels Structure of Chromatin) complex is an abundant, essential yeast paralogue of the SWI/SNF complex (Cairns *et al*, 1996). RSC is comprised of 15 subunits, including several bromodomain-containing subunits. RSC is involved in numerous cell processes throughout the cell cycle, including DNA repair,

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chromosome segregation, and DNA ploidy maintenance (Hsu *et al*, 2003; Huang *et al*, 2004; Chai *et al*, 2005; Campsteijn *et al*, 2007; Shim *et al*, 2007). However, the primary role appears to be transcriptional activation (Cairns *et al*, 1999; Angus-Hill *et al*, 2001; Govind *et al*, 2005). Genome-wide localization of RSC places it at ~700 targets, nearly all of which are Pol II promoter regions or Pol III genes (Damelin *et al*, 2002; Ng *et al*, 2002). Thus, the prevailing hypothesis is that RSC alters the chromatin structure of promoters to assist in transcription.

RSC is recruited to promoters by transcription factors and other transcriptional coactivators to enable chromatin remodeling and Pol II promoter transcription (Govind *et al*, 2005; Inai *et al*, 2007). Additionally, recent experiments at Pol III genes show an attenuation of Pol III transcription in RSC mutants (Soutourina *et al*, 2006). Furthermore, alterations in chromatin structure have been reported at a few individual loci in RSC mutants (Tsuchiya *et al*, 1998; Moreira and Holmberg, 1999; Soutourina *et al*, 2006; Inai *et al*, 2007). However, as RSC can both eject and reposition nucleosomes, a large-scale analysis is required to determine the extent that RSC uses these different modes at different classes of targets. Here, we generated a strong conditional mutant that abolishes RSC function and examined its impact on transcription and promoter chromatin structure. We utilized a custom RSC-specific microarray to explore RSC function at a genomic scale. Surprisingly, loss of RSC had a profound impact on

genome-wide transcription. Interestingly, at RSC targets chromatin structure was altered in a class-dependent manner in the absence of RSC function: Pol II promoters primarily displayed single nucleosome events with alterations near the transcription start site (TSS), whereas Pol III genes primarily displayed gross nucleosome gain.

Results

Loss of *Sth1* protein results in loss of transcription

To study the impact of RSC on transcription and chromatin structure, we generated an *STH1* allele (*sth1^{td}*) tagged with an N-terminal temperature-sensitive degron to enable rapid and inducible loss of the Sth1 protein (Dohmen *et al*, 1994). We reasoned that the loss of the catalytic subunit of RSC should inactivate enzymatic activity. For our studies, we employed two isogenic yeast strains carrying the *sth1^{td}* allele that differed only at *UBR1*, which encodes the protein that recognizes the degron tag. One strain contained an inducible *UBR1* gene (degron strain) while the second was *ubr1Δ* (control strain; Labib *et al*, 2000). The degron strain failed to grow under degradation-inducing conditions (galactose media at 37°C), while full growth was observed for the control strain (Figure 1A). Furthermore, western blot analysis of cell lysates showed substantial loss of Sth1 protein selectively in the degron strain (Figure 1B). While the loss of Sth1 protein reduced cell growth, the effect was reversible; cells subjected

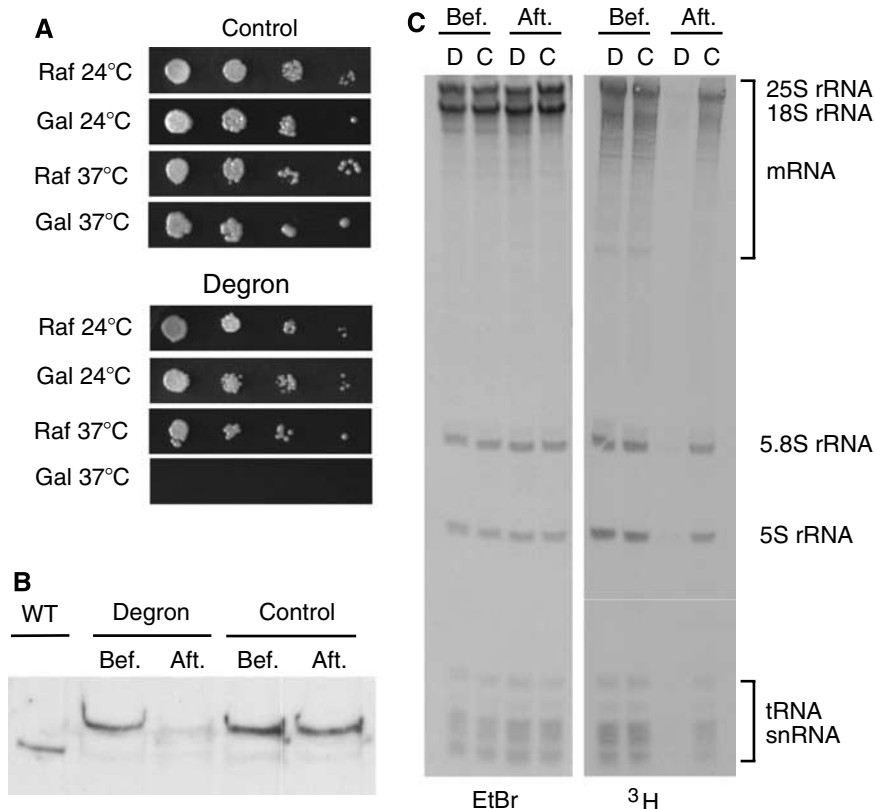


Figure 1 Sth1 loss results in inviability and general loss of transcription. (A) Control (*sth1^{td} ubr1Δ*) and degron (*sth1^{td} Gal-UBR1*) strains were serially diluted and spotted on media containing either raffinose (Raf) or galactose (Gal) and grown at either 24 or 37°C. (B) Whole-cell lysates from wild-type (WT), degron, or control strains were analyzed by western blot and probed with anti-Sth1. Lanes contain extracts from cells representing before (Bef.) or after (Aft.) Sth1 loss. (C) Degron and control strain cells either before or after Sth1 degradation were incubated with ³H-labeled uracil for 25 min before harvesting. RNA samples were separated by size on a denaturing polyacrylamide gel. Half of the gel was stained with ethidium bromide (EtBr) while the remaining half was exposed to film (³H).

to loss of Sth1 protein for 2 h and allowed to recover at room temperature showed >95% viability (data not shown). Similar results with another *sth1^{td}* allele were also recently reported (Campsteijn *et al*, 2007). Together, these results show the utility of the *sth1^{td}* allele in analyzing RSC function.

To determine the impact of the loss of RSC activity on new transcription, cells were grown in the presence of ³H-labeled uracil for 25 min either before or after Sth1 protein degradation. RNA was isolated and separated by PAGE. Ethidium bromide staining of the gel revealed uniform RNA levels, while autoradiograph exposure revealed the striking loss of new RNA transcription in the degon strain after Sth1 protein loss (Figure 1C). Importantly, transcription by all three RNA polymerases was affected, as tRNAs, rRNAs, and mRNAs were greatly diminished. Thus, the loss of Sth1 protein had a global impact on transcription. Whether these effects were direct or indirect presented a considerable challenge, which we addressed by multiple approaches.

Histone density increases at a Pol III locus with loss of Sth1 protein

RSC may directly impact transcription through alterations of the chromatin structure at RSC targets. If so, then direct targets might share a common chromatin feature. Notably, the largest class of RSC-occupied loci is Pol III genes, primarily tRNA genes (Ng *et al*, 2002). Furthermore, tRNA genes have the lowest histone occupancy genome-wide (Supplementary Figure 1; Rao *et al*, 2005). This inverse correlation between histone and RSC occupancy at tRNA genes raised the possibility that RSC helps to keep Pol III genes nucleosome sparse, and that the loss of RSC activity might result in a gain of histone occupancy over time.

To initially investigate RSC function at a Pol III locus, we examined *SCR1*, the longest Pol III gene in the genome and a known RSC target. *SCR1* was previously used as a model Pol III gene to map the distribution of Pol III components over the body of the gene (Roberts *et al*, 2006). Histone chromatin immunoprecipitation (ChIP) was performed in the degon and control strains both before and after Sth1 protein loss. The precipitated DNA was then analyzed by quantitative PCR using six amplicons distributed across the locus (Figure 2A and B). Histone occupancy was low over the body of the *SCR1* gene in both strains before Sth1 loss, consistent with our observations at tRNA genes genome-wide. After 1 h of Sth1 loss, however, we observed a small gain of histone density and a more robust gain after 2 h over the body of *SCR1* in the degon strain, but not in the control strain. As an alternative measure, the chromatin structure of *SCR1* was probed using micrococcal nuclease (MNase) digestion and analyzed by Southern blot. The body of the *SCR1* gene yielded a detectable nucleosomal ladder of hybridization in the degon strain but not the control strain (Figure 2C). Together, these results indicated that Sth1 protein loss results in a progressive gain of nucleosomes at the *SCR1* locus.

We next examined whether this progressive return of histone density was due to the loss of transcription, and whether DNA replication was additionally involved. To determine whether this nucleosomal gain was simply due to a lack of transcription, we performed histone ChIP with *STH1*⁺ cells during nutrient deprivation, which rapidly (within 10 min) represses Pol III transcription (Clarke *et al*, 1996; Harismendy *et al*, 2003; Roberts *et al*, 2003). Interestingly,

histone density was not restored to *SCR1* during Pol III repression (Figure 2D). Notably, the Pol III machinery is known to maintain residence at Pol III genes during Pol III repression (Roberts *et al*, 2006), prompting a test of whether RSC likewise maintains association. Here, we found that during repression RSC remained at the three Pol III loci tested: *SCR1* and two tRNA genes (Supplementary Figure 2).

To determine whether replication-dependent chromatin assembly contributes to the restoration of histone density in the absence of RSC, we first arrested *Sth1^{td}* strains in G1 phase with alpha-factor, degraded Sth1, and then examined histone density over the *SCR1* locus after 2 h (Figure 2E). Interestingly, the restoration of histone density was attenuated compared with cycling cells, indicating that replication-dependent chromatin assembly accounts for a large portion of the acquired histone density on Pol III genes lacking RSC. Taken together, our work suggests that RSC helps keep Pol III genes nucleosome-deficient, regardless of their transcriptional status, and that the absence of RSC makes Pol III loci susceptible to replication-dependent assembly into chromatin.

A genome-scale microarray examines chromatin changes at numerous RSC targets

We then determined whether our observations of histone gain at *SCR1* were representative of Pol III genes in general, and whether this observation extended to Pol II genes. Toward this end, we generated a custom RSC-specific miniature microarray at relatively high resolution to interrogate a large number of target loci. The array covers 218 specific genomic locations selected by their RSC occupancy levels and genomic content (Figure 3A; Ng *et al*, 2002). Four general categories of loci were selected: 99 highly-occupied RSC target loci containing 155 Pol II promoters (including 56 divergent pairs), 43 loci containing Pol II promoters not occupied by RSC, 54 loci containing Pol III genes of varying RSC occupancies, and 22 other loci. Each locus, which typically consisted of an intergenic and a portion of the flanking ORFs, was tiled with 60-mer oligonucleotide probes at a mean resolution of 110 bp, which was sufficient for monitoring changes in individual nucleosomes.

As an initial test of the microarray, we performed ChIP against RSC. We assessed occupancies of two different RSC subunits, Rsc3 and Rsc8, in wild-type *STH1*⁺ cells, since RSC demonstrates low cross-linking efficiency (Ng *et al*, 2002). ChIP eluates were amplified, labeled, and hybridized to the arrays. We averaged the RSC occupancy of all probes within a 500-bp window at each Pol II promoter on the array, and plotted the distribution of RSC occupancies at all the Pol II promoters (Figure 3B). Under normal growth conditions (mock before), we observed a distinct enrichment at a large number of promoters, consistent with our expectations from our array design. Comparison of our results with published results (Ng *et al*, 2002) revealed a strong positive correlation (Pearson correlation coefficient = 0.87). Since the array represents a small fraction of the genome, the enrichment ratios are not absolute values but rather a relative representation of the RSC occupancy for the promoters on this array. Therefore, we divided promoters into classes representing the least, moderately, and most likely occupied by RSC; for simplicity, these classes are referred to as non-occupied, moderate, and RSC-occupied, respectively (Figure 3C). Based on these

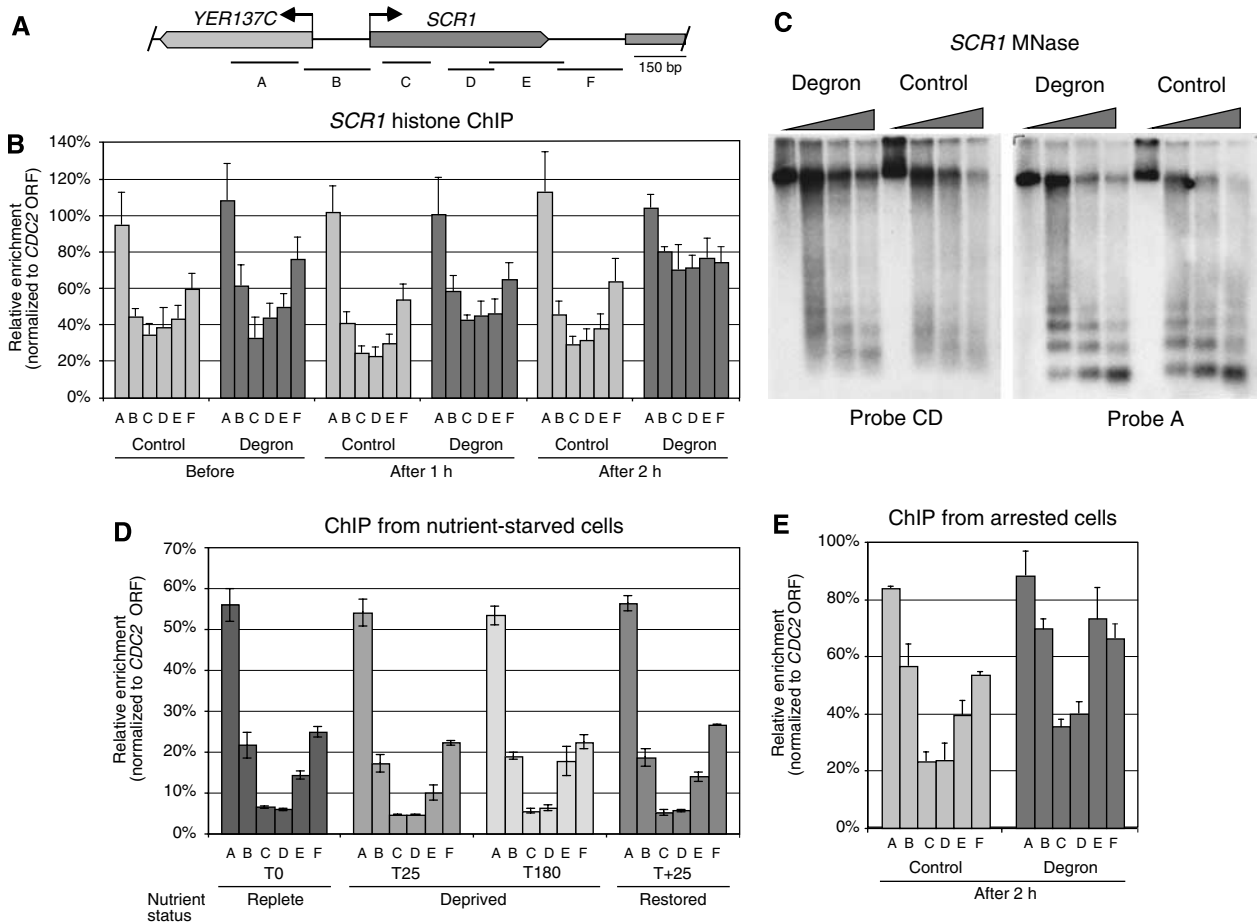


Figure 2 Changes in chromatin structure are detected at the Pol III gene *SCR1* upon loss of Sth1 protein. (A) The positions of PCR amplicons A–F are mapped onto a diagram of the *SCR1* locus drawn to scale. (B) ChIP of histone H4 was performed on chromatin extracts from degron and control strains before and after one or 2 h of Sth1 degradation. Quantitative PCR was performed on the ChIP DNA at six amplicons (labeled A–F) encompassing the *SCR1* locus. Enrichment values were normalized to an amplicon from the *CDC2* ORF. Error bars indicate standard deviation between three biological replicates. (C) The chromatin structure from degron and control strains following Sth1 degradation was probed with increasing amounts of MNase and analyzed by Southern blot with a probe comprised of amplicons C and D. The blot was stripped and reprobed with amplicon A. (D) ChIP against myc-tagged H4 was performed in *STH1*⁺ cells during transcriptional activation and repression of *SCR1*. Pol III expression is active during nutrient replete conditions (T0), repressed after 25 and 180 min of starvation (T25 and T180), and active during nutrient restoration (T + 25; Roberts *et al*, 2003). (E) Histone density over *SCR1* was measured as in panel B in strains that were arrested in G1 using alpha-factor before degrading Sth1.

criteria, 84% of the promoters predicted to be RSC occupied demonstrated occupancy, and 63% of the promoters predicted not to be occupied demonstrated lack of occupancy; the majority of the remaining promoters fell in the moderate class. These results indicate the validity of using this microarray in analyzing RSC function at its target sites *in vivo*.

Since analysis of our *sth1*^{td} allele requires growth in media containing galactose at 37°C, we needed to determine whether the RSC targets we identified under normal conditions remain targets under mock Sth1^{td} protein degradation growth conditions. Therefore, we also performed RSC ChIP under mock degradation conditions (mock after). Interestingly, we observed a redistribution of RSC occupancy (Figure 3B). Presumably, this redistribution of RSC reflects the transcriptional program changes associated with the new growth conditions. Surveying the promoters present on this array, we found fewer but still significant numbers of promoters that we could confidently identify as having RSC occupancy (Figure 3C). Notably, we observed bidirectional shifts, with some promoters gaining occupancy while others

losing occupancy. While shifts in RSC occupancy at Pol II promoters occurred, tRNA genes appeared remarkably stable in RSC occupancy during mock after conditions (Figure 3D). Together, these results indicated that RSC occupancy is dynamic at Pol II promoters and stable at Pol III genes under our conditions.

Nucleosomes return to Pol III genes upon loss of Sth1 protein

To examine chromatin changes at Pol III genes, we performed ChIP against tagged H4 in the degron and control strains and hybridized the eluates to the microarray. We first present microarray results from the *SCR1* locus (Figure 4A). As a measure of change in histone occupancy between the presence and absence of Sth1 protein, we plotted the ratio of the signal from the degron and control strains, such that a ratio of 1 indicates no change, > 1 indicates a gain of nucleosomes in the degron strain, and < 1 indicates a loss of nucleosomes. In agreement with our PCR results (Figure 2), we observed a large gain in nucleosome density over the *SCR1* gene after

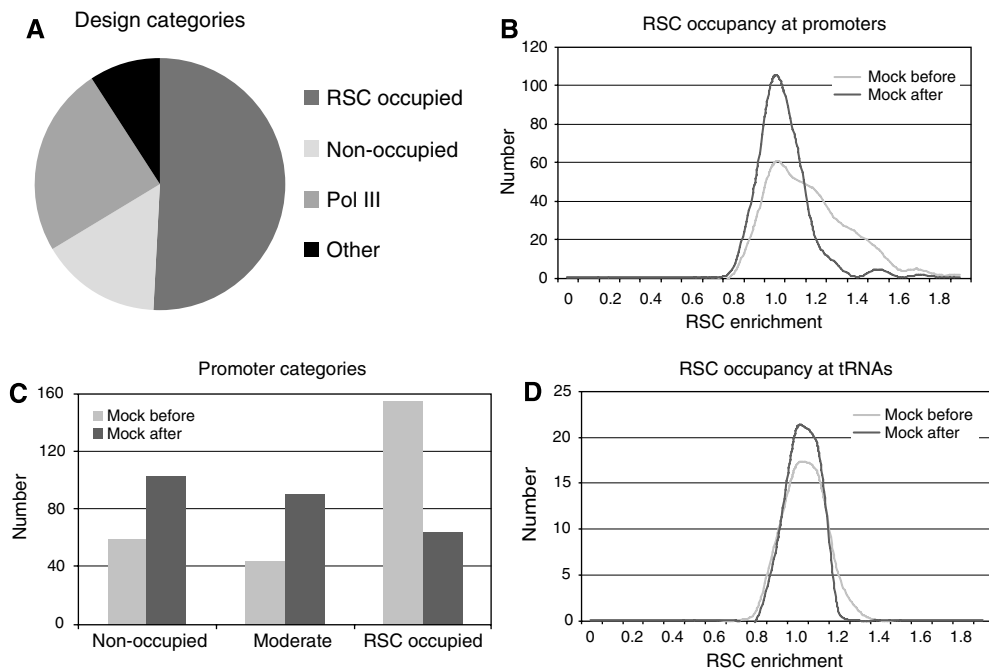


Figure 3 Genome-scale analysis of RSC occupancy using a custom microarray shows dynamic occupancy at Pol II promoters. **(A)** A pie chart shows the distribution of the 1900 oligo probes from the custom microarray among four general design categories. The categories reflect loci predicted or not predicted to have RSC occupancy based on published results (Ng *et al*, 2002). **(B)** ChIP was performed against Rsc3 and Rsc8 in wild-type cells during a mock degenon-inducing time course. The mean RSC occupancy values for 259 Pol II promoters (using a 500-bp window from -400 to $+100$ from the ATG) were determined and the distributions were plotted. The dynamic range of RSC fold enrichment of 0.8–1.2 is fully consistent with published observations (Damelin *et al*, 2002; Ng *et al*, 2002). **(C)** Promoters were assigned to three categories based on RSC occupancy before and after mock Sth1 loss. Non-occupied promoters had enrichment ratios of <0.95 ; moderate promoters had ratios between 0.95 and 1.05; and RSC-occupied promoters had ratios >1.05 . **(D)** The mean RSC occupancy values for 49 tRNA genes were determined using a 400-bp window encompassing the tRNA and the distributions were plotted.

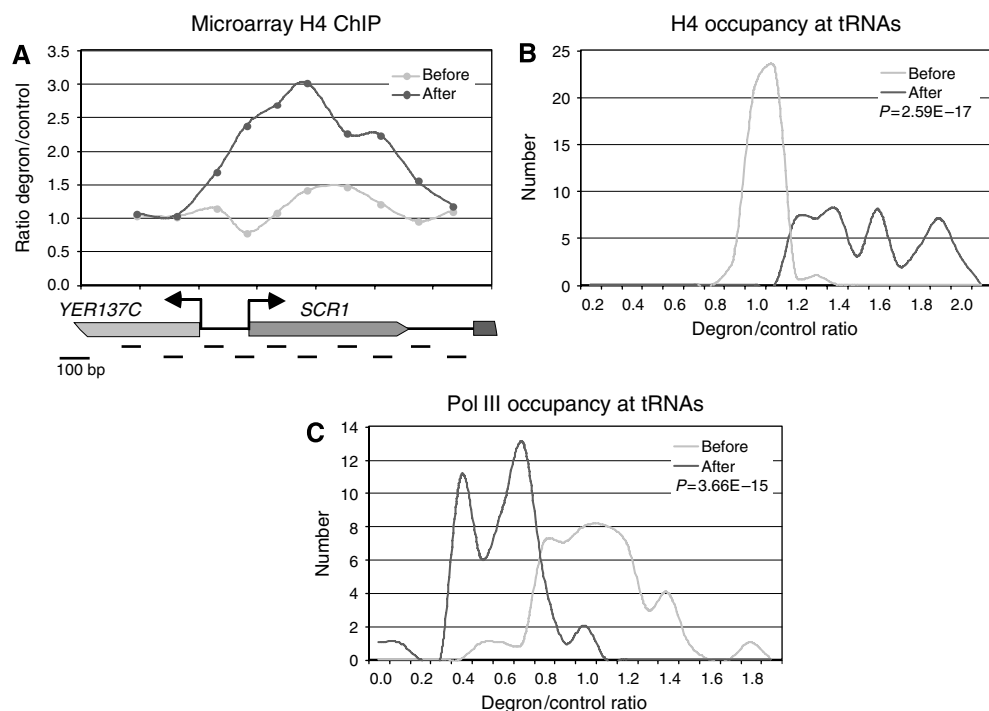


Figure 4 Pol III genes generally demonstrate nucleosome gain upon loss of Sth1 protein. **(A)** ChIP products against tagged histone H4 were applied to the RSC specific microarray. The results from the *SCR1* locus are shown. Values representing the ratio between degenon and control strains as an indication of histone gain (>1) or loss (<1) were plotted for before (gray) and after (black) Sth1 loss. The ChIP data were plotted against a physical map of the *SCR1* locus drawn to scale. The microarray oligo probes are indicated as thick black lines. **(B)** The mean histone H4 ratio between degenon and control strains representing histone gain or loss was determined at tRNA loci both before (gray) and after (black) Sth1 degradation and the distributions were plotted. **(C)** ChIP against the Pol III subunit Rpc82 was performed before and after Sth1 protein degradation and plotted as in panel B.

loss of Sth1 protein, indicating a gain in nucleosome occupancy.

We next asked whether the gain of nucleosomes at *SCR1* was representative of all Pol III genes. We examined as a class the general chromatin structure of loci containing a tRNA gene by identifying those microarray probes that were located within a 400-bp window centered over the tRNA gene (on average 4–5 probes). We then averaged the degenon/control ratios of the probes within this window and plotted the distribution of the resulting values for all the tRNA loci (Figure 4B). We observed a dramatic shift in nucleosome occupancy at tRNA loci following the loss of Sth1 protein ($P=2.59E-17$), similar to what we observed at *SCR1*. Examination of two additional Pol III-transcribed genes, *RPR1* and *SNR6*, also revealed general nucleosome gains, including both their coding and 5' flanking intergenic regions (data not shown). Together, these results suggest that RSC, directly or indirectly, helps to remove nucleosomes from these loci, and that nucleosome density increases in the absence of RSC function. Furthermore, this effect appears to be a general feature at all Pol III loci.

Since an increase in nucleosome density was observed at tRNA loci, we next asked whether this would impact Pol III occupancy. We performed ChIP against the tagged Rpc82 subunit of Pol III in our *sth1^{td}* strains and analyzed the eluates on our microarray (Figure 4C). Interestingly, we observed a striking, corresponding decrease in Pol III occupancy at tRNA loci in the degenon strain upon loss of Sth1 protein ($P=3.66E-15$). This result suggests a mutually exclusive relationship between histone and Pol III, and may help explain the loss of Pol III transcription upon loss of Sth1 protein.

Loss of Sth1 affects nucleosome positioning at Pol II promoters

We next turned our attention to Pol II promoters. Since the loss of Sth1 had a profound impact on transcription, our ability to attribute transcriptional defects directly to RSC action relied upon observing a more robust change in chromatin structure at RSC-occupied promoters than at non-occupied promoters. To test for this, we focused on those probes located within promoter regions, defined as –400 to +100 bp from the ATG. The mean ratios of the probes within each promoter region were then plotted as a distribution. At non-occupied promoters, we detected a slight histone gain upon loss of Sth1 protein (Figure 5A; $P=0.005$). The effects at these 'non-occupied' promoters could either be indirect or due to low or transient RSC occupancy. However, we also observed only modest changes at the vast majority of RSC-occupied promoters (Figure 5B; $P=0.014$), although there were a small percentage of promoters that did show a notable gain. Therefore, regardless of classification, the majority of Pol II promoters examined did not exhibit the large general gains in histone density observed at Pol III promoters.

We then considered that RSC-associated chromatin changes at Pol II promoters might be more subtle than the gross nucleosome density changes that are detected by conventional ChIP. To examine chromatin structure at a more detailed level, we performed MNase digestion on chromatin and hybridized the liberated mononucleosomal DNA (~150 bp) to our microarrays (Sekinger *et al*, 2005; Yuan *et al*, 2005). This method can detect either the gain or loss of

a single nucleosome centered over an individual probe, and thus monitor single nucleosome events. With traditional ChIP, the fractional gain or loss of a single nucleosome may not be easily detected due to the larger fragment size (~500 bp) and the associated summation of nucleosome occupancy. Hence, the mononucleosome technique can provide a more sensitive detection of chromatin alterations at the nucleosome level.

We anticipated that three types of chromatin events could be detected by mononucleosome hybridization: movement or sliding of a nucleosome, as inferred by the concordant gain and loss in signal of neighboring probes; single nucleosome gain; and single nucleosome loss. Examples of three candidate loci demonstrating each class of events are presented in Figure 6. In some cases, two separate events were identified within the same promoter or intergenic (Figure 6B and C). In many cases, the MNase procedure revealed a single location where nucleosome density changed, which was either largely (Figure 6A) or partly (Figure 6B) masked in the H4 ChIP data due to the nucleosome averaging effect of ChIP. In other cases, the MNase data correlated well with the H4 ChIP results (Figure 6C); these cases may represent promoters where more than one nucleosome was gained. Together, these examples illustrate the types of nucleosome events occurring in response to loss of RSC function.

As a simple measure of nucleosome events, we identified the probe in each promoter that gained the most nucleosome occupancy from the mononucleosome hybridization, termed the Most Enriched Probe (MEP), in the degenon strain relative to the control strain (Figure 5C and D). The MEP could arise either from a single nucleosome gain or through a nucleosome movement where the gain is associated with the repositioning of the nucleosome completely over a probe location. Examination of the distribution of MEP ratios in non-occupied promoters revealed a slight gain in the magnitude of the MEP ($P=0.001$), a change similar to what was observed with the ChIP result. However, examination of the RSC-occupied promoters revealed a prominent shift in the distribution ($P=2.5E-13$), indicating that loss of RSC function resulted in a gain of nucleosome occupancy over at least one probe. These results suggested that single nucleosome events are much more prevalent than changes in gross nucleosome occupancy and are the primary effects due to the loss of RSC function at Pol II promoters.

Single nucleosome events generated by the loss of RSC function may impact transcription, depending upon the location of the event relative to promoter sequence elements. The TSS usually resides within the NFR and may be subject to regulation by nucleosome positioning (Yuan *et al*, 2005). We identified those probes that overlap a 50-bp window centered over the TSS and examined the distribution of degenon/control ratios (Figure 5E and F). We observed no significant shift in the distribution of ratios for non-occupied promoters ($P=0.28$). However, a clear shift is noted at RSC-occupied promoters ($P=1.57E-4$), suggesting that one location of RSC action may be the NFR that encompasses the TSS.

Transcriptional shutoff by *rpb1-1* results in nucleosome loss in the promoter

The observation of chromatin changes at the TSS prompted us to ask whether the changes we observed in our *sth1^{td}* mutant were in direct response to the loss of RSC activity or

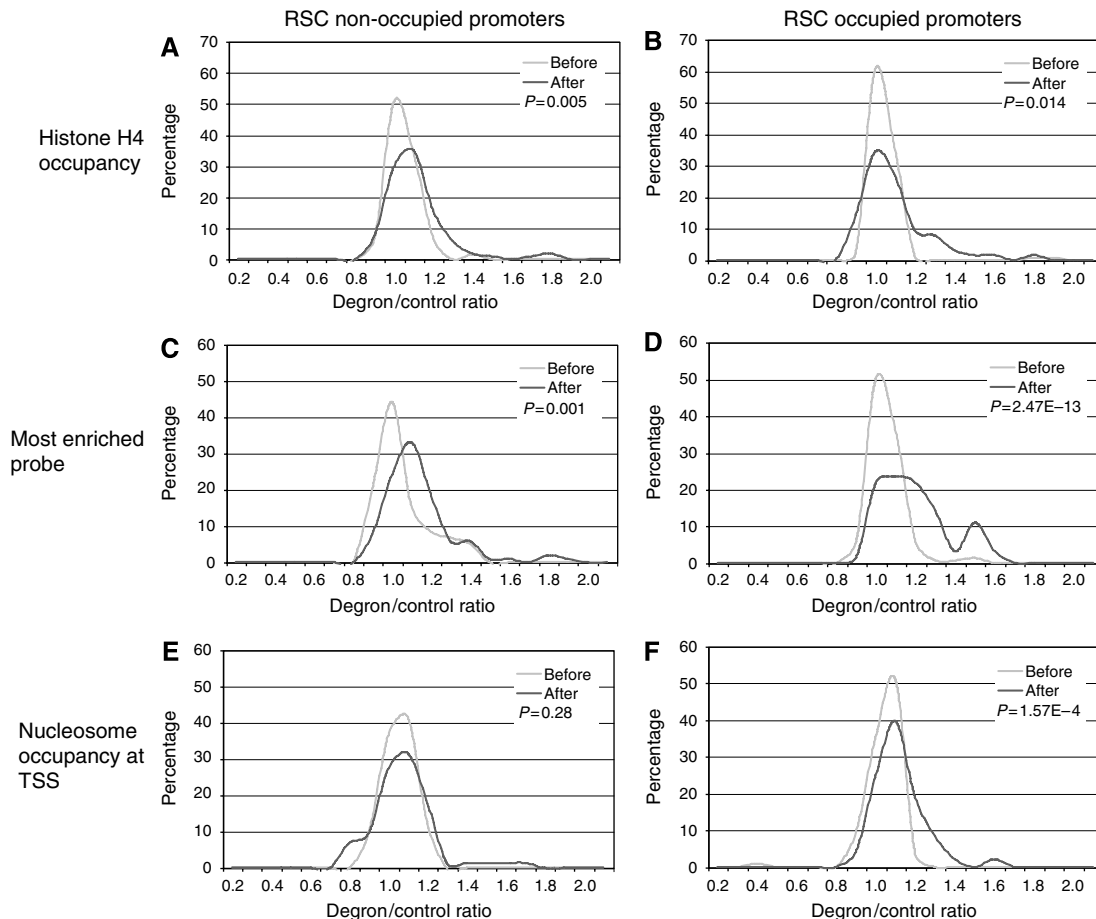


Figure 5 Pol II promoters demonstrate single nucleosome changes upon Sth1 loss. (A, B) The mean histone H4 ratio between degron and control strains representing histone gain or loss was determined at Pol II promoters both before (gray) and after (black) Sth1 degradation. Shown are the distributions for RSC non-occupied (A) and occupied (B) promoters. (C, D) Mononucleosomal DNA was isolated from degron and control strains by MNase digestion and hybridized to the microarray. The MEP represents the single probe within the promoter having the highest gain in nucleosome occupancy in the degron strain relative to the control strain. Shown are the distributions of MEP ratios at RSC non-occupied (C) and occupied (D) promoters. (E, F) The mononucleosome occupancy ratio between degron and control strains was determined for a 50-bp window encompassing the TSS. The distributions were plotted for RSC non-occupied (E) and occupied (F) promoters.

indirectly due to the loss of transcription. To help answer this question we prepared mononucleosomal DNA from an *rpb1-1* mutant both at permissive and non-permissive temperature (mimicking our degron time-course protocol). This temperature-sensitive Pol II mutant abrogates new transcription within minutes of shifting to a non-permissive temperature (Nonet *et al*, 1987). We determined the distribution of MEP ratios (non-permissive over permissive temperature) for both RSC-occupied and non-occupied promoters (Supplementary Figure 3). Surprisingly, we observed a lower mean ratio at RSC-occupied promoters compared with non-occupied promoters ($P=1.32E-6$), suggesting that RSC promoters generally lose mononucleosomes upon loss of transcription in the *rpb1-1* mutant. Furthermore, when the probes corresponding to the TSS were examined, no significant difference in the distribution was observed between the two promoter classes ($P=0.59$). These results suggested that loss of transcription in *rpb1-1* strains is associated with structural changes in promoter chromatin, including a general loss of nucleosome occupancy. The mechanism underlying the loss of nucleosomes in the *rpb1-1* strain is unknown, but the result nevertheless highlights the distinction between changes in

promoter chromatin structure due to loss of transcription versus loss of RSC activity.

Discussion

Here, we describe the effects on chromatin structure conferred by the loss of RSC activity. Our initial observation with the *sth1^{td}* mutant was a surprisingly large reduction in new transcription by all three RNA polymerases. Two possibilities are considered for this widespread effect. First, RSC could have a direct role in all transcription. There is some support for this notion, as a direct interaction between the subunit Rsc4 and Rpb5, a conserved subunit shared among all three RNA polymerases, was recently described (Soutourina *et al*, 2006). Furthermore, RSC appears to be generally recruited by transcriptional machinery at Pol III genes (Ng *et al*, 2002). This provides the potential for RSC as a direct coactivator at all gene classes in the genome. However, while the association of RSC with RNA polymerases likely occurs at some targets, the vast majority of RSC is not associated with polymerases (Cairns *et al*, 1996). Importantly, RSC is observed specifically at some promoters and not others,

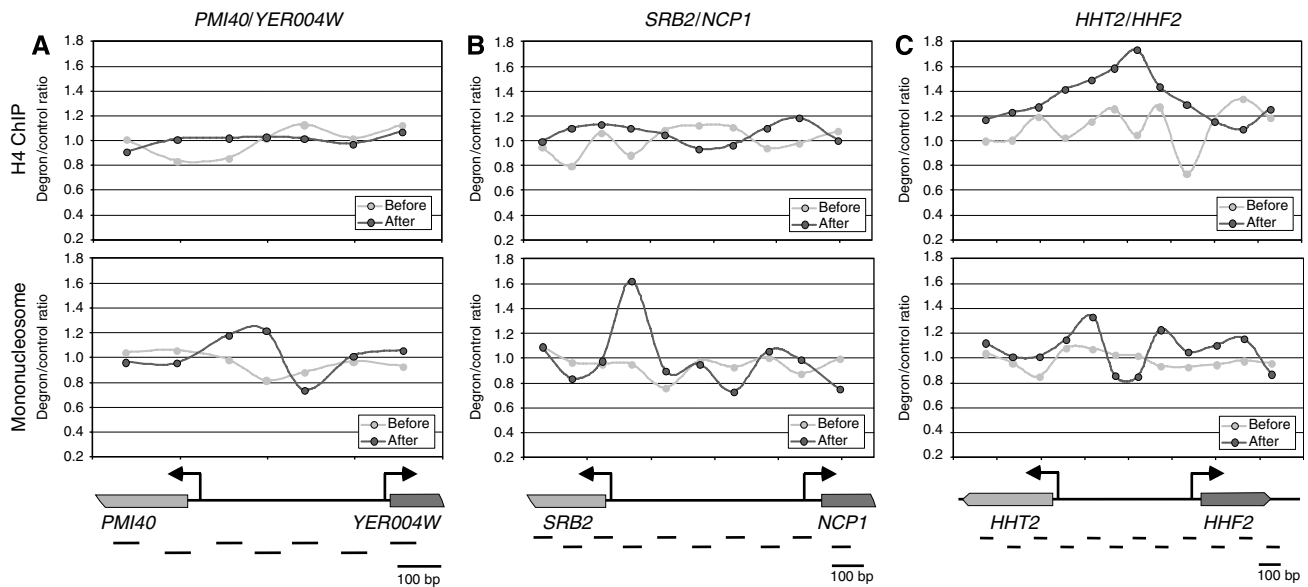


Figure 6 RSC-occupied loci demonstrate single nucleosome movement, gain, and loss. Three loci demonstrating single nucleosome events are illustrated. The upper graphs represent histone H4 ratios between degron and control strains. The middle graph represents the mononucleosome occupancy ratios between degron and control strains. The lower cartoon represents the physical map of the locus as described in Figure 4A. TSSs are represented by bent arrows and ORFs by rectangles. (A) The *PMI40/YER004W* locus demonstrates nucleosome movement. (B) The *SRB2/NCP1* locus demonstrates independent single nucleosome gain and loss. (C) The *HHT2/HHF2* locus demonstrates nucleosome movement and general nucleosome gain.

irrespective of transcriptional status (Damelin *et al*, 2002; Ng *et al*, 2002). Second, we considered that the profound loss of transcription might be a combination of both direct and indirect effects. Loss of products derived from direct target genes may induce a cascade of cell stresses, which could impact the remainder of the non-target genome. We note that RSC target genes are involved in a wide variety of processes including mitochondrial function, nitrogen and carbohydrate metabolism, and transcriptional regulation (Damelin *et al*, 2002; Ng *et al*, 2002). Additionally, RSC mutants affect the expression of cell cycle and ribosomal proteins, Gcn4 target genes, and others (Angus-Hill *et al*, 2001; Swanson *et al*, 2003; Soutourina *et al*, 2006). Our analysis identified key RSC target genes that demonstrated promoter chromatin effects, including *SRB2*, a mediator subunit; most tRNA genes; mitochondrial genes *MAS6*, *COX19*, and *OM14*; and both loci that encode H3 and H4 (Figures 4 and 6, and data not shown). Simultaneous loss of transcription of key RSC targets would likely have negative global consequences. Hence, we conclude that the global loss of transcription likely reflects both direct and indirect effects.

We observed two classes of chromatin effects due to the loss of RSC function: a change in general nucleosome density and specific single nucleosomal events. These effects are not necessarily mutually exclusive, as for example both nucleosome movement and gain were detected at the *HHT2/HHF2* locus. Furthermore, extensive sliding of one nucleosome toward a second neighboring nucleosome may result in the displacement and disassembly of the second nucleosome, a situation that would not be distinguished from simple ejection using our techniques. Gross nucleosome density changes were observed primarily at Pol III genes and at a limited number of Pol II promoters. This effect likely reflects the role of RSC to remove or eject one or more nucleosomes, perhaps

to allow room for transcriptional machinery that would normally be occluded by a nucleosome. Indeed, a loss of Pol III occupancy was correlated with an increase in histone density at tRNA loci (Figure 4); this is consistent with the requirement for RSC for full Pol III transcription (Soutourina *et al*, 2006). In contrast, single nucleosome events, including movement, may represent more subtle chromatin effects at Pol II genes. In this case, a nucleosome that partially or wholly occludes a transcription factor-binding site, such as a TATA box, could be shifted, rather than ejected, for access. In the absence of RSC, nucleosomes may either adopt a more energetically favorable position dictated by an underlying NPS, or be actively deposited and/or repositioned into a more regular, and presumably transcriptionally repressive, chromatin structure by remodelers such as ISWI. The determination of whether RSC will move or eject a nucleosome at a target locus may depend upon specific signals, either in the form of histone modifications on the target nucleosome or from direct interactions with transcriptional machinery. The numerous bromodomains present in RSC are candidates for this instructional mechanism.

The decision to eject nucleosomes (or to perform extensive sliding) may be general at Pol III genes, as we observed occupancy effects at all Pol III genes assayed. This localized gain in histone occupancy could be due to either *de novo* deposition or possibly a redistribution of nucleosomes from flanking regions. Regardless of the mechanism, the nucleosomes that return to these loci appear to do so in a non-positioned manner, as we were unable to detect gain by mononucleosome hybridization (data not shown); well-positioned nucleosomes should be detected by this technique, whereas randomly distributed nucleosomes may not be detected. This result is not unexpected considering the striking lack of detectable NPS signatures near tRNA genes (Ioshikhes

et al, 2006; Segal *et al*, 2006). In addition, Pol III genes, along with other highly transcribed genes, exhibit high rates of histone turnover (Dion *et al*, 2007). Together, the observations that tRNA genes normally demonstrate low nucleosome density, high histone turnover, high RSC occupancy, and gain of nucleosome density in the absence of RSC activity clearly suggest that RSC is required to maintain tRNA genes in a nucleosome-sparse environment. Here, the acquisition of nucleosomes is clearly not due to a lack of Pol III transcription, as repressing Pol III transcription by nutrient deprivation was not accompanied by a gain in nucleosomes at *SCR1*. Furthermore, our occupancy data show that both the Pol III machinery (Roberts *et al*, 2006) and RSC (Supplementary Figure 2) reside at Pol III genes during Pol III repression (nutrient deprivation for 25 min). This raises the possibility that acutely repressed Pol III genes are bound by a poised Pol III–RSC complex that helps keep these loci nucleosome-deficient, enabling rapid reactivation when environmental conditions improve. A similar role for RSC may also be required upon exit from S phase. Here, replication may displace the Pol III machinery, resulting in a competition for Pol III gene occupancy between histones (facilitated by replication-dependent chromatin assembly) and the Pol III machinery (facilitated by RSC). While RSC may be responsible for the ejection of nucleosomes at tRNA genes, other chromatin remodelers likely help deposit and position new nucleosomes. One candidate is ISW2, which is also localized near tRNA genes (Gelbart *et al*, 2005), raising the possibility that RSC and ISW2 have antagonistic functions at these loci.

Loss of transcription is often associated with changes in promoter chromatin. For example, repression of the *PHO5* promoter involves re-establishment of several promoter nucleosomes (Adkins and Tyler, 2006). In response to Sth1 degradation, we observed alterations in the promoter chromatin at a large number of promoters, which were likely associated with or contributed to the observed transcriptional repression. Interestingly, while a small fraction of promoters demonstrated a change in gross histone occupancy, a larger number exhibited single nucleosome events. In counter distinction to our observation, an interesting recent genome-wide study of CHD remodelers in *Schizosaccharomyces pombe* found general gains in nucleosome density at the promoters of Pol II genes in the absence of CHD activity (Walfridsson *et al*, 2007); Pol III genes were not examined. This distinction likely reflects the mode of action: CHD remodelers primarily catalyze the disassembly of nucleosomes at Pol II genes *in vivo* (Walfridsson *et al*, 2007), whereas our work suggests that SWI/SNF remodelers (RSC) generally slide nucleosomes at Pol II promoters. We observed two key differences between RSC-occupied and non-occupied Pol II promoters. First, a larger fraction of RSC-occupied promoters had high-value MEP ratios. Second, and more importantly, the change in nucleosome occupancy at the TSS was generally higher at RSC-occupied than non-occupied promoters. Since the TSS is often located within an NFR (Yuan *et al*, 2005), these observations may suggest that a key role of RSC function at target promoters is to help establish and/or maintain the NFR.

Two lines of evidence suggest that the changes in promoter chromatin structure at RSC target genes were not simply due to the loss of transcription. First, we observed specific

chromatin alterations at RSC-occupied promoters relative to non-occupied promoters, even though new transcription at all promoters was reduced. Second, abolishing transcription in the *rpb1-1* mutant at the non-permissive temperature resulted in chromatin changes, although distinct from those in the *sth1^{td}* mutant. The nature of the nucleosome loss in the *rpb1-1* strain is not known, although we speculate that the continued action of transcriptional coactivators, including chromatin remodelers such as RSC, results in an apparent decrease in nucleosome occupancy within the promoter. In addition, continued abortive initiation and the lack of productive elongation by Pol II may prevent the restoration of promoter architecture to a non-active state, perhaps by the stalled transcriptional machinery inhibiting nucleosome reassembly.

Together, these results demonstrate a clear role for RSC in the process of transcription *in vivo*. Our work is consistent with RSC remodeling nucleosomes to allow establishment of RNA polymerase machinery. However, the mode of remodeling is clearly biased; at Pol II promoters single nucleosome events consistent with sliding are more often observed, whereas at Pol III genes the events are consistent with ejection. Future work will explore the nature of this bias.

Materials and methods

Media, genetic methods, and strains

Standard procedures were used for media preparation, transformations, integrations, and genetic analyses. Plasmid and strain constructions will be provided upon request. Strain genotypes (S288C derivatives) are listed in Table I. For degenon inductions, cultures were grown in liquid YP containing 2% raffinose and 0.05 mM Cu(II)SO₄ at 27°C until OD₆₀₀ 0.4–0.6. Galactose was then added to 2% final concentration and the culture incubated for 60 min to induce *UBR1*. Afterwards, the temperature was shifted to 37°C for 120 min to induce Sth1 degradation. Samples were cross-linked in 1% formaldehyde for 5 min for mononucleosome analysis, 15 min for histone ChIP, and 30 min for other proteins. For G1 arrest studies, alpha-factor was added to *bar1Δ* degenon and control strains simultaneously with galactose and the time course followed as above. Loss of *BAR1* did not affect Sth1 degradation. The nutrient deprivation time-course experiment was performed as described (Roberts *et al*, 2003).

In vivo RNA labeling

Cells were grown following the degenon time course above. Aliquots (2 ml) of culture were incubated with 165 μCi of ³H-labeled uracil for 25 min at each time point. Total RNA was immediately harvested using acid phenol extraction. Messenger RNA was enriched by one round of poly-A selection on oligo-dT beads. Enriched RNA was separated by denaturing polyacrylamide gel electrophoresis, followed by either staining with ethidium bromide or electrophoretic transfer to nylon membrane. The membrane was soaked in Amplify (Amersham) and exposed to film.

Microarray design and analyses

The microarray format was based on Agilent Technologies' 8-pack 22K format. Details on the custom microarray design, ChIP, mononucleosome preparation, hybridization, and data processing are available in Supplementary data. Microarray data are provided as Supplementary data and are present at the NCBI GEO repository under experiment GSE8862.

Genomic and statistical analyses

Promoters were defined as –400 to +100 relative to the translation start site. Regions containing tRNA genes were defined as –150 to +250 relative to the start site. Probes within or overlapping these regions were selected for analysis. TSSs were collected from published sources (Zhang and Dietrich, 2005; Miura *et al*, 2006).

Table 1 Yeast strains

Strain	Genotype	Source
YBC2191	<i>MATa ura3-52 trp1Δ63 his3Δ200 leu2::PET56 ubr1Δ::pGAL1-UBR1::HIS3 sth1Δ::pCUP1-sth1^{td}::URA3</i>	This work
YBC2192	<i>MATa ura3-52 trp1Δ63 his3Δ200 leu2::PET56 ubr1Δ::HIS3 sth1Δ::pCUP1-sth1^{td}::URA3</i>	This work
YBC2193	<i>MATa ura3-52 trp1Δ63 his3Δ200 leu2::PET56 ubr1Δ::pGAL1-UBR1::HIS3 sth1Δ::pCUP1-sth1^{td}::URA3 RPC82-13myc::TRP1</i>	This work
YBC2194	<i>MATa ura3-52 trp1Δ63 his3Δ200 leu2::PET56 ubr1Δ::HIS3 sth1Δ::pCUP1-sth1^{td}::URA3 RPC82-13myc::TRP1</i>	This work
MX1-4C	<i>MATα ura3-52 leu2-3,112 trp1-289 his3Δ1 (hht1-hhf1)Δ (hht2-hhf2)Δ pMS329 [CEN, ARS, URA3, HHT1, HHF1]</i>	Morgan <i>et al</i> (1991)
YBC2205	<i>MATα ura3-52 leu2-3,112 trp1-289 his3Δ1 (hht1-hhf1)Δ (hht2-hhf2)Δ pNOY436 [CEN, ARS, TRP1, HHT2, myc-HHF2]</i>	This work
YBC2449	<i>MATα ura3-52 leu2-3,112 trp1-289 his3Δ1 (hht1-hhf1)Δ (hht2-hhf2)Δ ubr1Δ::pGAL1-UBR1::HIS3 sth1Δ::pCUP1-sth1^{td}::URA3 pNOY436 [CEN, ARS, TRP1, HHT2, myc-HHF2]</i>	This work
YBC2451	<i>MATα ura3-52 leu2-3,112 trp1-289 his3Δ1 (hht1-hhf1)Δ (hht2-hhf2)Δ ubr1Δ::HIS3 sth1Δ::pCUP1-sth1^{td}::URA3 pNOY436 [CEN, ARS, TRP1, HHT2, myc-HHF2]</i>	This work
YBC3135	<i>MATa ura3-52 trp1Δ63 his3Δ200 leu2::PET56 ubr1Δ::pGAL1-UBR1::HIS3 sth1Δ::pCUP1-sth1^{td}::URA3 bar1Δ::KanMX</i>	This work
YBC3136	<i>MATa ura3-52 trp1Δ63 his3Δ200 leu2::PET56 ubr1Δ::HIS3 sth1Δ::pCUP1-sth1^{td}::URA3 bar1Δ::KanMX</i>	This work
yHN3	<i>MATa ura3-52 trp1Δ63 his3Δ200 leu2::PET56 Rsc3-9myc::TRP1</i>	Ng <i>et al</i> (2002)
yHN4	<i>MATa ura3-52 trp1Δ63 his3Δ200 leu2::PET56 Rsc8-9myc::TRP1</i>	Ng <i>et al</i> (2002)
Y260	<i>MATa ura3-52 rpb1-1</i>	Nonet <i>et al</i> (1987)

The most commonly used TSS position or, failing that, the position closest to the ATG was selected and used in the analysis. *P*-values between data sets before and after Sth1 degradation were determined by two-tailed, equal-variance Student's *t*-test. Distribution histograms for H4 ChIP, MEP, and Pol III ChIP were normalized so that the mean for the Before curve was 1.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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