

# Two-Color Cell Array Screen Reveals Interdependent Roles for Histone Chaperones and a Chromatin Boundary Regulator in Histone Gene Repression

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## SUMMARY

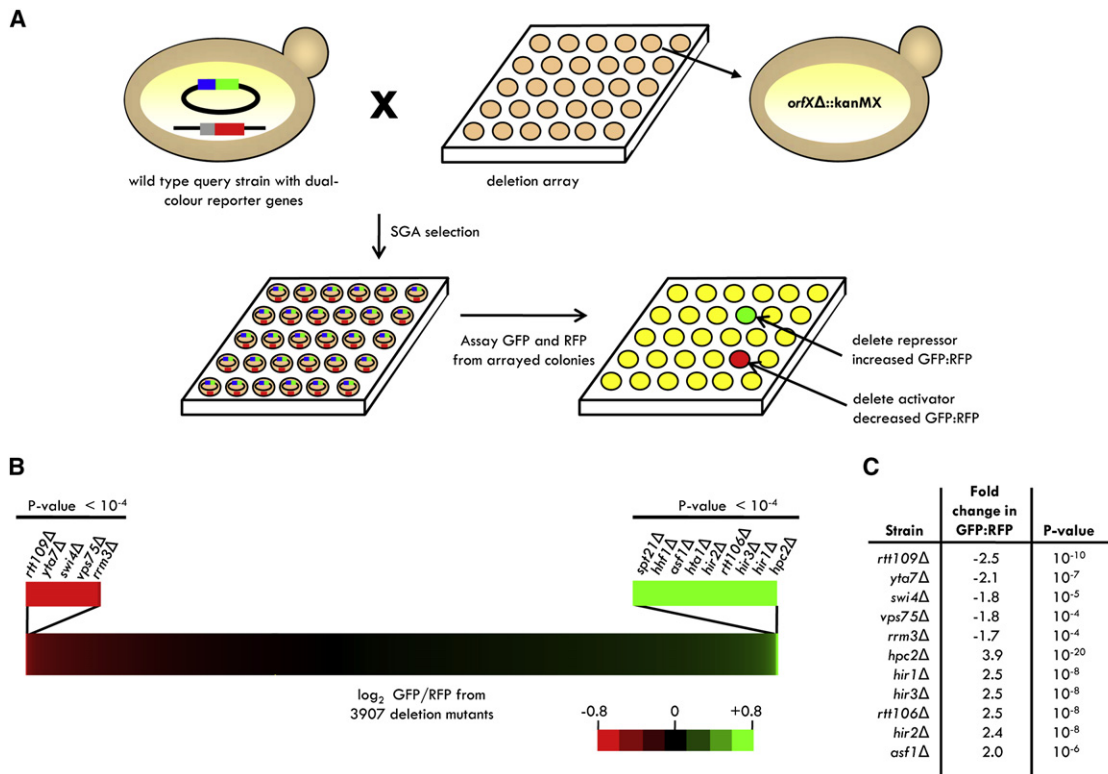
We describe a fluorescent reporter system that exploits the functional genomic tools available in budding yeast to systematically assess consequences of genetic perturbations on gene expression. We used our Reporter-Synthetic Genetic Array (R-SGA) method to screen for regulators of core histone gene expression. We discovered that the histone chaperone Rtt106 functions in a pathway with two other chaperones, Asf1 and the HIR complex, to create a repressive chromatin structure at core histone promoters. We found that activation of histone (*HTA1*) gene expression involves both relief of Rtt106-mediated repression by the activity of the histone acetyltransferase Rtt109 and restriction of Rtt106 to the promoter region by the bromodomain-containing protein Yta7. We propose that the maintenance of Asf1/HIR/Rtt106-mediated repressive chromatin domains is the primary mechanism of cell-cycle regulation of histone promoters. Our data suggest that this pathway may represent a chromatin regulatory mechanism that is broadly used across the genome.

## INTRODUCTION

Massive waves of cell-cycle-regulated transcription are a universal feature of eukaryotic cell cycles, yet our understanding of mechanisms linking gene expression to the cell cycle remains incomplete. Conventional genetic screens in yeast, and complementary experiments in many systems, have provided considerable insight, but it is clear that many regulators remain to be discovered. One important group of cell-cycle-regulated genes encode histones that form the nucleosome. Transcription of core histone genes is coordinated with the cell cycle to ensure

large amounts of new histones are available during DNA replication (Gunjan et al., 2005). The restriction of histone gene expression to S phase (DNA synthesis) is required not only to produce adequate histone pools but also to prevent toxicity that is associated with their inappropriate expression at other stages of the cell cycle (Gunjan and Verreault, 2003). The yeast *S. cerevisiae* (Sc) has proven a useful model to understand the mechanism of regulation of core histone transcription. Sc contains two copies of each core histone gene, each of which is arranged in opposite orientation to a gene encoding its dimer partner within the nucleosome: *HHT1-HHF1* and *HHT2-HHF2*, the two gene pairs that encode H3/H4, and *HTA1-HTB1* and *HTA2-HTB2*, the two gene pairs that encode H2A/H2B.

Four genes were identified in yeast genetic screens that encode transcriptional repressors of three of the four histone gene pairs, *HTA1-HTB1*, *HHT1-HHF1*, and *HHT2-HHF2*, both outside of S phase and in response to hydroxyurea (HU), a chemical that causes stalling of DNA replication forks (Osley and Lycan, 1987; Xu et al., 1992). These four proteins, Hir1, Hir2, Hir3, and Hpc2, were subsequently demonstrated to copurify as the HIR protein complex (HIR) (Green et al., 2005; Prochasson et al., 2005). A fifth protein, Asf1, also copurifies with HIR (Green et al., 2005) and is similarly required to repress transcription of *HTA1-HTB1*, *HHT1-HHF1*, and *HHT2-HHF2* (Sutton et al., 2001). Asf1 and HIR are both histone chaperones, proteins that bind to histones and assemble or disassemble chromatin (reviewed in De Koning et al., 2007). Asf1 and HIR are H3/H4-specific chaperones that together are able to deposit histones onto DNA in a replication-independent manner in vitro (Green et al., 2005). Asf1/HIR-mediated repression of transcription relies on a specific DNA sequence, the negative regulatory element (NEG), found in the promoters of the three HIR-regulated gene pairs but absent from that of *HTA2-HTB2* (Osley et al., 1986; Osley and Lycan, 1987). The only proteins known to regulate *HTA2-HTB2* are Spt10 and Spt21 (Dollard et al., 1994). Deletion of *SPT10* reduces levels of all core histone gene transcripts to some degree (Hess et al., 2004; Xu et al., 2005).



**Figure 1. Reporter-Synthetic Genetic Array Functional Genomic Screen for Regulators of *HTA1* Expression**

(A) Outline of R-SGA screening procedure describing construction of the output array having each deletion mutant combined with GFP and RFP reporter genes. Fluorescence is assayed directly from colonies arrayed on agar plates using a scanning fluorimeter, and GFP:RFP ratios are calculated to assess specific effects of gene deletions on the GFP reporter of interest (see the [Experimental Procedures](#)).

(B) Results of R-SGA screen for identification of regulators of the histone H2A gene *HTA1*. A reporter plasmid with the promoter normally driving *HTA1* expression fused to GFP was screened as described in (A). Gene expression measurements taken from 3907 yeast deletion mutants are displayed, and mutants causing differential GFP expression with P value < 10<sup>-4</sup> are highlighted.

(C) The fold change in GFP:RFP for each mutant is described along with corresponding P values for each gene expression measurement.

Although several regulators of histone gene expression are known, underlying molecular mechanisms remain unclear. To address this void, we sought to exploit the functional genomic tools available in yeast for performing rapid, saturating genetic screens. Specifically, we devised a two-color GFP-RFP reporter system called Reporter-Synthetic Genetic Array (R-SGA) to systematically assess the consequences of gene deletions on a promoter of interest. Our R-SGA screen revealed a previously unappreciated role for the H3/H4 histone chaperone Rtt106 in repression of histone gene expression. We demonstrate that Rtt106 functions downstream of Asf1 and the HIR complex to create a repressive chromatin structure at the *HTA1-HTB1* regulatory region. Our genomic screen also revealed an activating role for Yta7, an evolutionarily conserved protein containing both a bromodomain and an AAA ATPase domain. Our molecular analysis indicates that Yta7 acts as a boundary element within the *HTA1* locus, preventing the spread of Rtt106 and associated repressive chromatin into the histone gene coding regions. We also discovered that the cell-cycle-specific histone acetyltransferase (HAT) Rtt109 functions as an *HTA1* activator, and genetic tests suggest that it counters HIR/Rtt106 repressive chromatin to permit transcription in a cell-cycle-regulated manner. Finally, our

genome-wide analysis of nucleosome positioning suggests that comparable HIR/Rtt106-Yta7 domains may dictate regions of repressive chromatin throughout the genome.

## RESULTS

### A Dual-Reporter Functional Genomic Screen to Discover Regulators of Gene Expression

We devised a two-color GFP-RFP reporter system, R-SGA, to systematically assess the consequences of gene deletions on a promoter of interest (Figure 1A). Our system involves the creation of a wild-type yeast strain with the promoter sequence of a particular gene of interest fused to GFP along with a control reporter construct with the constitutively expressed *RPL39* promoter driving tdTomato (Shaner et al., 2004) (RFP) expression. The dual-reporter strain is compatible with SGA methodology, which enables marked genetic elements to be combined in a single haploid cell through standard yeast mating and meiotic recombination via an automated procedure (Tong et al., 2001, 2004). Here our goal was to survey the yeast deletion collection (Giaever et al., 2002), which contains the set of ~5000 viable *KanMX*-marked deletion mutants, for defects in gene expression.

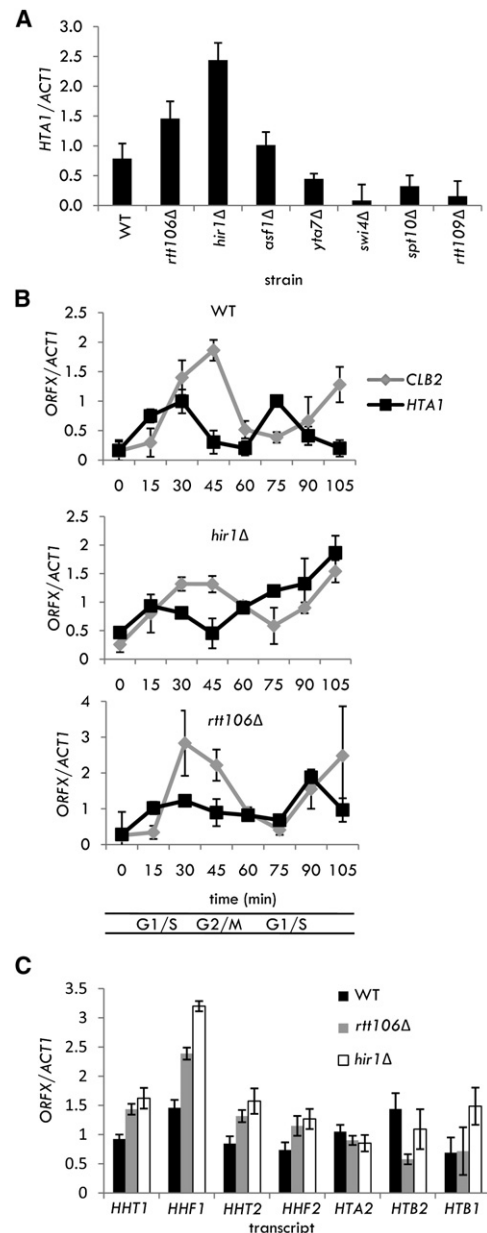
To do so, we apply the SGA approach to introduce both the test and control fluorescent reporters into the deletion collection. The resulting panel of yeast deletion mutants is then assayed for enhanced or diminished promoter-GFP expression by scanning both fluorescence intensities directly from colonies arrayed on agar plates using a scanning fluorimeter. The ratio of GFP to RFP fluorescence intensity for each yeast deletion mutant provides a genome-wide survey of the effect of viable deletion mutants on the promoter of interest. We expect decreased GFP:RFP when the deleted gene is a specific activator of the reporter gene, while deletion of a repressor will result in higher GFP:RFP.

### Identification of Regulators of *HTA1* Expression

To uncover regulators of S phase-specific expression of histone genes, we fused the *HTA1* promoter to GFP (*HTA1pr-GFP*) and used the R-SGA-based screening approach described above (Figure 1A) to explore the genome for potential regulators of *HTA1* expression. We carried out this screen in duplicate using a yeast deletion array in which each mutant is represented twice, providing an average GFP:RFP intensity from two deletion mutant colonies for each replicate screen. Thus,  $\log_2$  GFP:RFP ratios are averaged from four replicate deletion mutants. As a test of significance of mutants causing differential GFP:RFP expression, we assigned P values to these  $\log_2$  ratios based on the normal distribution of Z scores transformed from average  $\log_2$  ratios from each screen. From replicate experiments, we observed a Pearson correlation of 0.81.

Since our screen relies on GFP under the control of the *HTA1* promoter, we expect candidate regulators to reflect bona fide promoter regulation rather than regulation of histone mRNA stability. As proof of the utility of our approach, we identified a number of previously characterized *HTA1* gene regulators in our screen, including *HPC2*, *HIR1*, *HIR2*, *HIR3*, *ASF1*, and *SWI4* (Figure 1B) (Green et al., 2005; Hess and Winston, 2005; Osley and Lycan, 1987; Prochasson et al., 2005; Simon et al., 2001; Xu et al., 1992). Specifically, deletion of *HPC2*, *HIR1*, *HIR3*, *HIR2*, or *ASF1* caused *HTA1pr-GFP* expression to increase between 2- and 3.9-fold (Figure 1C). Deletion of the known *HTA1* transcriptional activator *SWI4* caused a reduction in GFP levels by 1.8-fold (Figure 1C). These results validate the utility of our approach and led us to explore regulatory roles of other genes uncovered from our screen. These include the histone H3-H4 chaperone, *RTT106*, which upon deletion caused a 2.5-fold increase in *HTA1pr-GFP* expression, suggesting *Rtt106* has a repressive role in *HTA1* transcription. In contrast, deletion of the H3-specific histone acetyltransferase, *RTT109*, caused a 2.5-fold reduction in *HTA1* expression (Figure 1C), which links *Rtt109* to *HTA1* activation. Other genes uncovered by our screen that potentially encode activators of *HTA1* expression include *YTA7*, *VPS75*, and *RRM3* (Figure 1C). *Yta7* encodes a bromodomain-containing protein that was recently suggested to repress histone transcription (Gradolatto et al., 2008). However, in our R-SGA screen, deletion of *YTA7* resulted in decreased *HTA1pr-GFP* levels, indicating *Yta7* may have a more complex role at *HTA1* than previously appreciated (see below).

To confirm our screen results, we used quantitative real-time PCR (qPCR) to assess endogenous *HTA1* transcript levels in



**Figure 2. *Rtt106*, *Rtt109*, and *Yta7* Regulate Histone Gene Expression**

(A) cDNA was prepared from the indicated strains. The ratio of the indicated transcript to that of *ACT1* was determined using qPCR.

(B) *Rtt106* represses *HTA1* through the cell cycle. Each strain was blocked with 5  $\mu$ M  $\alpha$  factor and released, and samples were taken at the indicated times. For each time point, cDNA was prepared and analyzed by using qPCR. *CLB2* transcription was assayed in addition to show proper progression through the cell cycle.

(C) HIR-regulated histone genes are also regulated by *Rtt106*. Error bars represent standard deviations from the mean from at least three replicate qPCR reactions.

deletion mutants of potential *HTA1* regulators. Consistent with the results of our genomic screen, deletion of *RTT106* resulted in increased *HTA1* transcript levels (Figure 2A). The derepression

observed in an *RTT106* deletion strain was clear but slightly less pronounced than that observed in a strain lacking *HIR1*. Also, consistent with our R-SGA results, deletion of *YTA7* and *RTT109* caused decreased *HTA1* transcript levels relative to wild-type, similar to the deletion of the previously described histone activators *SPT10* and *SWI4* (Hess and Winston, 2005).

The HIR proteins and Asf1 repress *HTA1* expression outside of S phase. To determine if Rtt106 represses *HTA1* in a manner similar to that of the HIR proteins, we assessed *HTA1* expression during the cell cycle in cells deleted for *RTT106* and *HIR1*, which have no obvious cell-cycle defect by FACS analysis (data not shown). Wild-type, *rtt106Δ*, and *hir1Δ* strains were arrested in late G1 phase with  $\alpha$  factor, and *HTA1* transcripts were profiled every 15 min using qPCR after release into fresh medium. In wild-type cells, *HTA1* transcription fluctuated throughout the cell cycle, peaking in S phase before being repressed as cells progress past S phase into G2/M (Figure 2B). Consistent with previous results, deletion of *HIR1* caused a significant defect in *HTA1* expression, with obvious derepression of gene expression throughout the cell cycle (Figure 2B). Similarly, the absence of *RTT106* also caused constitutive *HTA1* expression through the cell cycle (Figure 2B). The *HTA1* transcription peak occurred at 15–30 min after  $\alpha$  factor release and was not completely repressed past S phase of the cell cycle (45 and 60 min postrelease [Figure 2B]). Cell-cycle regulation of *CLB2* transcripts, which peak at G2/M, was monitored to mark proper progression through the cell cycle. These results indicate that the observed increase in *HTA1* transcript levels in *rtt106Δ* log phase cells is due to a failure to repress transcription outside of S phase rather than overactivation during S phase of the cell cycle.

### Rtt106 Represses HIR-Regulated Histone Genes

We next asked if Rtt106, like HIR, represses expression of other histone genes. First, we tested the effect of *RTT106* deletion on transcription of *HTA1*'s partner locus, *HTB1*. We did not detect a significant effect of *RTT106* deletion on transcription of *HTB1* (Figure 2C), possibly reflecting its demonstrated complex regulation by both transcriptional and posttranscriptional mechanisms (Lycan et al., 1987; Xu et al., 1992). Both Asf1 and HIR repress *HHT1*, *HHF1*, *HHT2*, and *HHF2* gene expression. Likewise, we saw that deletion of *RTT106* resulted in higher levels of *HHT1*, *HHF1*, *HHT2*, and *HHF2* transcripts (Figure 2C), indicating that the *HHT1-HHF1* and *HHT2-HHF2* loci are also regulated by Rtt106. Unlike the other histone loci, *HTA2-HTB2* is not subject to Asf1/HIR repression. Similarly, we found that Rtt106 did not repress transcription of *HTA2* and *HTB2* (Figure 2C). We conclude that Rtt106, like HIR and Asf1, represses transcription at three of the four histone gene pairs.

### Rtt106 Localizes to *HTA1-HTB1* in a Manner Dependent on Asf1 and the HIR Complex

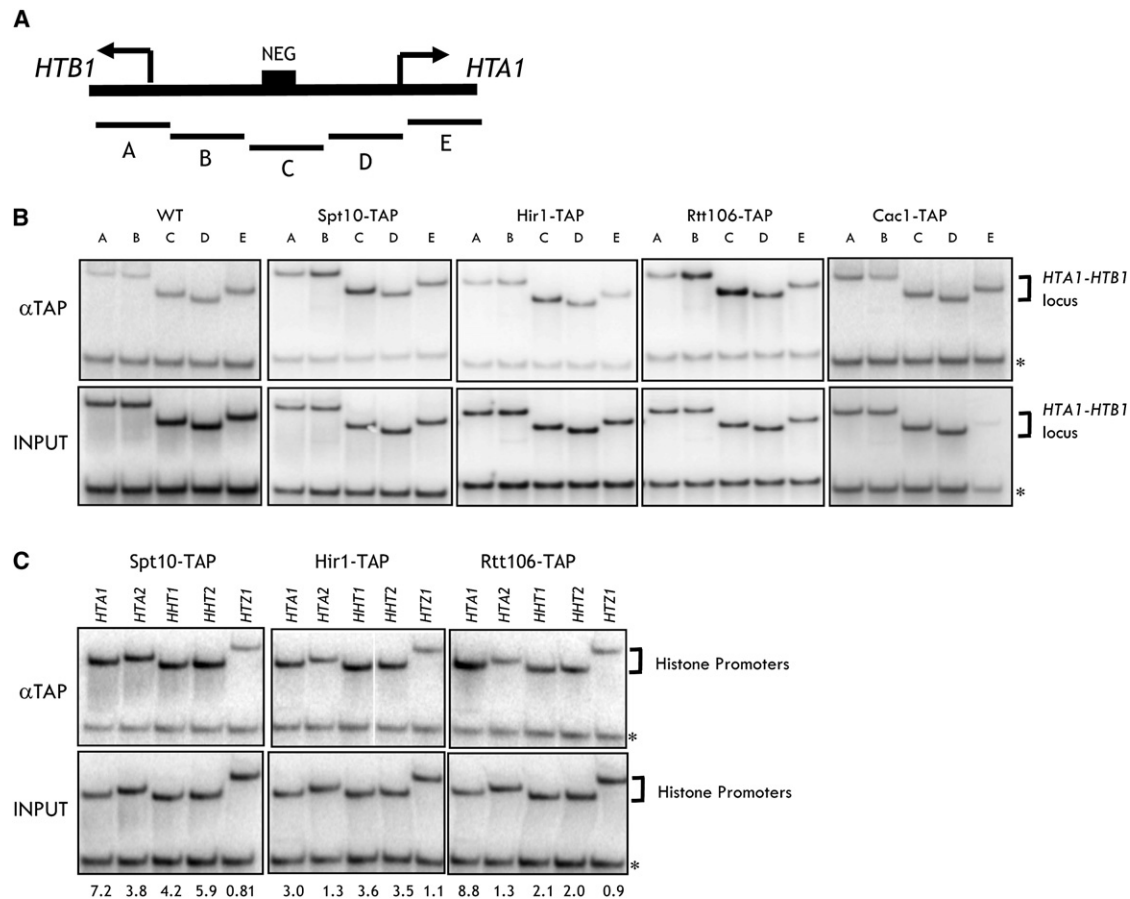
Our data link the histone chaperone Rtt106 to repression of *HTA1-HTB1* expression. To ask if Rtt106 acts directly on the *HTA1-HTB1* promoter, we used chromatin immunoprecipitation (ChIP) to assess whether Rtt106 and the four members of the HIR complex localize to the *HTA1-HTB1* region. Figure 3A shows the *HTA1-HTB1* locus with approximate locations of primer sets used in our ChIP analysis. As a control, we found that Spt10-TAP

crosslinked most effectively to the promoter region containing the NEG site as well as several upstream activating sequences (primer set "C," Figure 3B), consistent with previous results (Eriksson et al., 2005; Xu et al., 2005).

We next used ChIP to assay the crosslinking pattern of Rtt106 and the four members of the HIR complex at *HTA1-HTB1*. Hir2 is known to crosslink at the promoter region of *HTA1-HTB1* (Green et al., 2005). Consistent with this result, we found that all four members of the HIR complex, Hir1-TAP, Hir2-TAP, Hir3-TAP, and Hpc2-TAP, specifically localized to this region (Figure 3B and see Figure S1 available online). HIR binding was restricted to only background levels outside of region C and at the ORFs (Figure 3B and Figure S1). Importantly, like the HIR proteins, Rtt106-TAP specifically localized to the *HTA1-HTB1* promoter region and did not localize to the ORFs (Figure 3B).

Since HIR and Rtt106 repress three of the four histone loci (Figure 2C), we tested whether they also localize to other histone promoters to further correlate transcriptional effects with promoter binding. We used ChIP of Spt10-TAP to the promoter regions of the four core histone promoters as a control for the performance of our primers. We also assessed binding to the promoter region of the *HTZ1* gene, which encodes an H2A variant and serves here as a negative control since its transcription is not under cell-cycle control. As expected (Eriksson et al., 2005), Spt10-TAP effectively localized to the promoter regions of the four core histone promoters, but not to that of *HTZ1* (Figure 3C). Hir1-TAP also crosslinked to the promoters of *HTA1-HTB1*, *HHT1-HHF1*, and *HHT2-HHF2*, but not *HTA2-HTB2* (Figure 3C), consistent with the failure of the HIR complex to regulate *HTA2-HTB2*. Like Hir1, Rtt106-TAP also localized to the promoter region of *HTA1-HTB1* and was enriched at *HHT1-HHF1* and *HHT2-HHF2*, although not to the same degree as at *HTA1-HTB1* (Figure 3C). Like Hir1, Rtt106 did not crosslink above background levels to *HTA2-HTB2*. Thus, promoter localization of Hir1 and Rtt106 correlates with their ability to specifically repress transcription of *HTA1-HTB1*, *HHT1-HHF1*, and *HHT2-HHF2*, but not *HTA2-HTB2*.

So far, our experiments place HIR and Rtt106 in a common pathway that functions to repress histone transcription. To further explore the relationship between Rtt106 and other histone gene regulators, we used our ChIP assay to test the genetic requirements for specific crosslinking of Hir1 and Rtt106 to the promoter of *HTA1-HTB1*. Consistent with previous results (Green et al., 2005), deletion of *HPC2*, but not *ASF1*, prevented recruitment of Hir1-TAP to the *HTA1-HTB1* promoter (Figure 4A). Similar to *ASF1*, deletion of *RTT106* did not affect Hir1-TAP recruitment to *HTA1-HTB1* (Figure 4A). However, when we deleted either *HIR1* or *ASF1*, recruitment of Rtt106 to *HTA1-HTB1* was undetectable (Figure 4B). Rtt106-TAP recruitment to the *HTA1-HTB1* promoter was also prevented by deletion of *HIR2*, *HIR3*, or *HPC2* (Figure 4C). Asf1 and HIR form a protein complex in yeast (Green et al., 2005), and the interaction of Asf1 with the HIR complex is abolished when any of the four HIR subunits are deleted. These results suggest that an intact Asf1-HIR complex functions upstream of Rtt106 recruitment to *HTA1-HTB1*. By contrast, although Rtt106 physically interacts with the CAF-1 protein complex (Huang et al., 2005), localization of Rtt106 to *HTA1-HTB1* occurred independently of Cac2, a subunit of the



**Figure 3. Rtt106 and HIR Localize to the Promoter Region of *HTA1-HTB1***

(A) Schematic representation of the PCR products (A–E) used in ChIP to cover the *HTA1-HTB1* locus. (B) Rtt106 and members of the HIR complex (but not Cac1) crosslink to the promoter region of *HTA1-HTB1*. ChIP analysis was performed exactly as in Figure 3A. PCR results from IgG-Sepharose ChIP of an untagged negative control (WT) or positive control (Spt10-TAP) show that PCR products A–E effectively cover the locus. Precipitated chromatin was used for PCR amplification (upper panels). The top band is specific to the *HTA1-HTB1* locus, while the common lower band (marked by an asterisk) is an internal background control from a nontranscribed region on chromosome V. The bottom panels show the input control. (C) Rtt106 and members of HIR crosslink to the promoters of the same set of histone genes. A similar analysis was performed as in Figure 3A with primers directed against the promoters of the indicated histone genes.

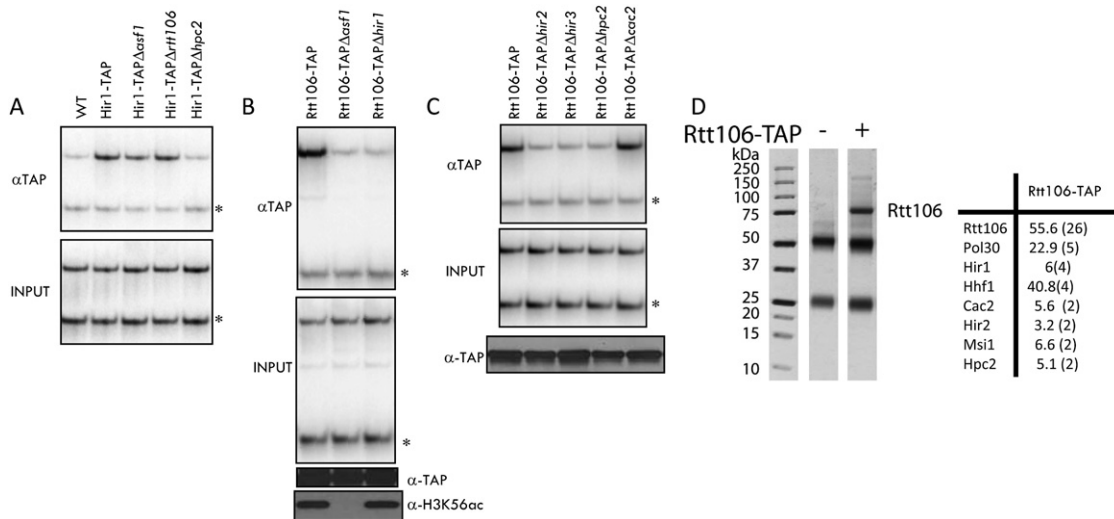
CAF-1 complex (Figure 4C). This result is consistent with the failure of Cac1-TAP to crosslink to the region (Figure 3B). In order to determine the molecular basis of the HIR requirement for Rtt106 recruitment, we copurified its associated proteins and identified them using mass spectrometry (Lambert et al., 2009). We copurified two of the three members of the CAF-1 complex (Figure 4D) along with Pol30/PCNA (which interacts with CAF-1), consistent with previous results (Huang et al., 2005). In addition, we copurified three of the four members of the HIR complex (Figure 4D). Our genetic experiments demonstrating Asf1/HIR-dependent localization of Rtt106 to *HTA1-HTB1*, coupled with these mCHIP data, suggest a direct physical interaction between these proteins at a core histone promoter.

**The *HTA1-HTB1* Promoter Region Is Nucleosome-free in *asf1Δ*, *hir1Δ*, and *rtt106Δ* Mutants**

As described in the Introduction, Asf1, the HIR complex, and Rtt106 are histone chaperones/chromatin assembly factors. To

ask if their effects on *HTA1-HTB1* transcription are related to this function, we used an antibody generated against unmodified histone H3 to assess H3 levels at the *HTA1-HTB1* promoter in WT and several deletion strains (Figure 5A). Compared to a WT strain, or a strain deleted for *RTT109* that we also identified in our screen (see below), the amount of H3 that crosslinked to the *HTA1-HTB1* promoter in *asf1Δ*, *hir1Δ*, and *rtt106Δ* was low relative to a control locus (Figure 5A). A similar experiment with an antibody against unmodified histone H2B (Figure 5B) revealed that histone H2B levels were significantly lower again in *asf1Δ*, *hir1Δ*, and *rtt106Δ* mutants relative to a WT strain (Figure 5B).

To further explore the molecular defect in histone chaperone mutants, we assessed genome-wide nucleosome occupancy of promoter regions in wild-type cells as well as *hir1Δ* and *rtt106Δ* strains using a previously described method (Lee et al., 2007). Consistent with our ChIP results, we found that most of the *HTA1-HTB1* intergenic region was nucleosome-free when



**Figure 4. HIR and Asf1 Are Required for Rtt106 Localization to *HTA1-HTB1***

(A) Hir1 localization to the *HTA1-HTB1* promoter is independent of Asf1 and Rtt106. The analysis is the same as in Figure 3 with the exception that the top band represents PCR product C from *HTA1-HTB1*.  
 (B) Rtt106 localization to the *HTA1-HTB1* promoter requires Asf1 and Hir1. The analysis is the same as above except that the internal background control (indicated by an asterisk) is from the *ACT1* gene. Below the ChIP analysis, a western blot indicates that *RTT106* expression is not dependent on Asf1 or Hir1.  
 (C) Rtt106 localization to the *HTA1-HTB1* promoter requires all members of HIR, but not CAF-1. ChIP analysis is the same as in Figure 4A. Below the ChIP analysis, a western blot indicates that *RTT106* expression is not dependent on expression of HIR members.  
 (D) Affinity purification and identification of Rtt106-TAP-associated proteins. A silver-stained SDS-PAGE is shown with affinity-purified proteins from an untagged strain (–) and from Rtt106-TAP (+). Copurifying proteins were identified by LC-MS/MS as described in Lambert et al. (2009). The percent sequence coverage is indicated in the table, with the number of unique peptides shown in parentheses.

either *HIR1* or *RTT106* was deleted, while nucleosome positions at *HTA1-HTB1* coding regions remained unchanged (Figure 5C). Similarly, we found that, along with *HTA1-HTB1*, promoter regions of *HHT1-HHF1* and *HHT2-HHF2* were among the most nucleosome-depleted intergenic regions genome-wide in strains deleted for either *HIR1* or *RTT106* (Figure 5D, red). These results suggest that cell-cycle repression of *HTA1-HTB1*, *HHT1-HHF1*, and *HHT2-HHF2* is dependent on a nucleosome assembly pathway that relies on the coordinated actions of Asf1, the HIR complex, and Rtt106. A number of other promoters were also nucleosome-free in the *HIR1* and *RTT106* deletion strains, including several common to both (Figure 5D, cyan), suggesting HIR and Rtt106 may function together throughout the genome.

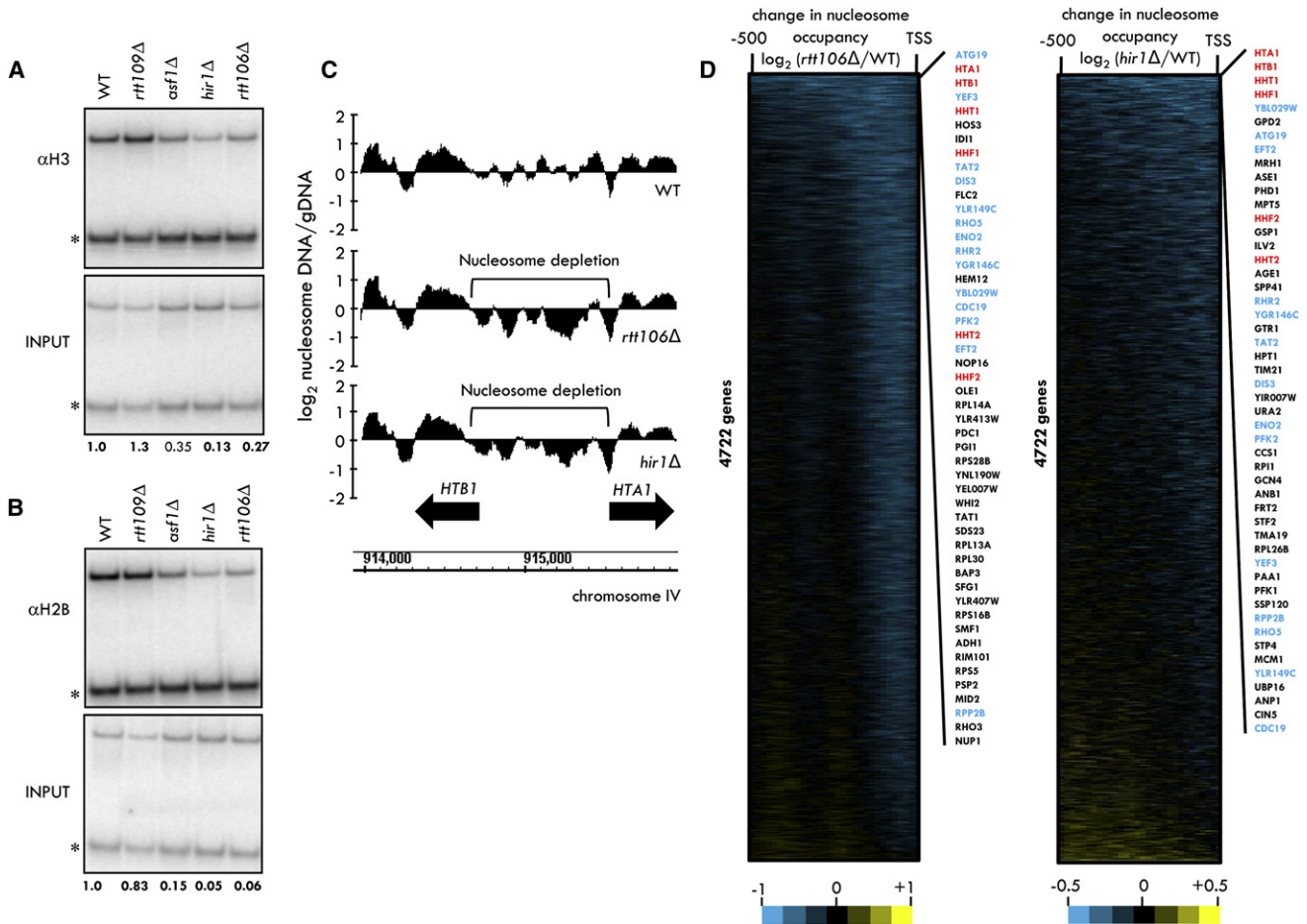
#### HIR/Rtt106 Repression at *HTA1-HTB1* Creates a Requirement for Rtt109

Our analysis of *HIR* and *RTT106* requirements for histone gene expression and promoter binding suggest that activation of *HTA1* may reflect relief of repression, rather than the function of specific activators, as seen at other cell-cycle-regulated promoters (reviewed in Wittenberg and Reed, 2005). To test this idea further, we first used a synchronized cell culture and our ChIP assay to assess Rtt106 localization at the promoter of *HTA1-HTB1* throughout the cell cycle. We found that the proportion of *HTA1-HTB1* promoter that bound Rtt106 did not change significantly during the cell cycle (Figure 6A). In addition, consistent with our previous results, Rtt106 did not crosslink to the ORF region of *HTA1* at any time in the cell cycle (Figure S2A). qPCR analysis of *HTA1* expression confirmed that the cells progressed

synchronously through the cell cycle in our experiment (Figure S2B). Thus, temporal changes in Rtt106 localization to the promoter cannot account for repression of cell-cycle transcription of the histone genes. Rather, we reasoned that an activating factor may serve to relieve Rtt106-mediated repression during S phase. As noted earlier, our functional genomic screen uncovered *RTT109* as an activator of *HTA1* expression (Figures 1B and 2A). The Rtt109 histone acetyltransferase has specificity for K56 on histone H3 (H3K56ac) (Collins et al., 2007; Driscoll et al., 2007; Han et al., 2007), and acetylation of H3K56 is required for the cell-cycle-dependent transcription of the histone genes (Xu et al., 2005). We therefore examined *HTA1* expression in a series of double mutants by qPCR. As expected, *HTA1* expression was derepressed in *hir1Δ* and *rtt106Δ* single mutants (Figure 6B). *HTA1* expression was not considerably different in a *hir1Δ rtt106Δ* double mutant than the respective single mutants (Figure 6B), consistent with them functioning together in the same pathway. As we showed previously, *rtt109Δ* caused a reduction in *HTA1* transcript levels (Figures 2A and 6B). However, when either *hir1Δ* or *rtt106Δ* was combined with *rtt109Δ*, the inhibitory effect of *rtt109Δ* on *HTA1* expression was significantly reduced (Figure 6B). These genetic results suggest that Rtt109 functions to activate histone gene expression by antagonizing the repressive effect of Rtt106 and the HIR complex.

#### Yta7 Is a Boundary Element within the *HTA1-HTB1* Locus

Because Rtt106 and members of the HIR complex have recently been identified as transcriptional elongation factors (Imbeault

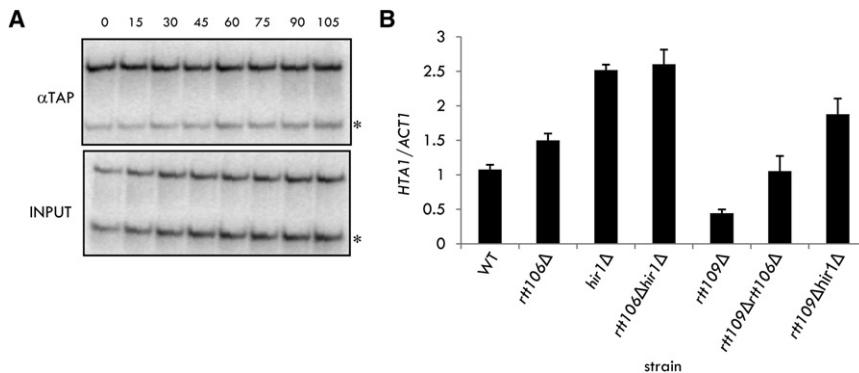


**Figure 5. *Asf1*, *HIR*, and *Rtt106* Collaborate to Assemble Chromatin at the *HTA1-HTB1* Promoter**

(A and B) Chromatin was prepared from the indicated strains and immunoprecipitated with an antibody against histone H3 (A) or H2B (B). The top band in the ChIP analysis represents PCR product C from *HTA1-HTB1*, and the internal background control (indicated by an asterisk) is from the *ACT1* gene. (C and D) A genome-wide nucleosome positioning assay was used to identify regions of depleted nucleosomes in *rtt106Δ* and *hir1Δ* strains. The *HTA1-HTB1* intergenic region is nucleosome-free when *RTT106* or *HIR1* is deleted (C). In (D), nucleosome profiles at promoter regions genome-wide are sorted in ascending order based on the average nucleosome occupancy up to 500 base pairs upstream of the transcriptional start site (TSS) for each ORF. The 50 top-ranking nucleosome-depleted promoter regions are shown. Histone genes are highlighted in red, while other nucleosome-depleted regions overlapping in *rtt106Δ* and *hir1Δ* strains are highlighted in cyan.

et al., 2008; Nourani et al., 2006), we compared their localization pattern at *HTA1-HTB1* to that of several other elongation factors implicated in chromatin assembly. We used ChIP to assess the

localization of the functionally related proteins Spt4-TAP, Spt5-TAP, and Spt6-TAP, as well as the two subunits of FACT, Pob3-TAP and Spt16-TAP, at the *HTA1* portion of the *HTA1-HTB1*



**Figure 6. Constitutive Repression at *HTA1-HTB1* Creates a Requirement for *RTT109***

(A) A *Rtt106*-TAP strain was blocked with  $\alpha$  factor and released, and samples for ChIP and qPCR (Figure S2B) were taken at the indicated times. The top band of the ChIP analysis represents PCR product C from *HTA1-HTB1*, while the common lower one (marked by an asterisk) is an internal background control from a nontranscribed region on chromosome V.

(B) The cDNA was prepared from the indicated strains. The ratio of the indicated transcript to that of *ACT1* was determined using qPCR. Error bars represent standard deviations from the mean from at least three replicate qPCR reactions.

region. All five factors crosslinked at *HTA1* in a pattern distinct from HIR/Rtt106. While HIR/Rtt106 crosslinked primarily to the promoter region of *HTA1* (Figure 3B and Figure S1), Spt4, Spt5, Spt6, Spt16, and Pob3 associated mainly with the coding regions of *HTA1* (Figure S3) and *HTB1* (data not shown). Thus, consistent with our other experiments, HIR-Rtt106 likely has a role at the *HTA1* locus distinct from the elongation factors that we tested.

We next assayed the relationship between *YTA7* and the various chromatin assembly factors and chaperones that are present at histone core promoters. As noted earlier, *YTA7* encodes a bromodomain-containing protein and was identified as an *HTA1* activator in our R-SGA screen (Figure 1B). *Yta7* is known to function as a boundary element within chromatin at *HMR*, a locus that is typically transcriptionally silent (Tackett et al., 2005). To ask if *Yta7* might perform a similar function at a transcribed locus, we assayed crosslinking of *Yta7* at *HTA1-HTB1*. We observed strong enrichment of *Yta7* at regions occupied by HIR/Rtt106 (region “C”), Spt4/5/6, and FACT (region “E”) and also to the region between them (region “D”) (Figure 7A). Similar to the Hir1 and Rtt106 proteins, we found that *Yta7* crosslinked to the promoter region of *HHT1-HHF1* and *HHT2-HHF2*, but not *HTA2-HTB2* (Figure 7B). Localization of *Yta7* was dependent on *HIR1* but not *RTT109* (Figure 7A) at all three histone promoter regions (Figure 7B). Because *Yta7* localized efficiently to the region bounded by HIR/Rtt106 and Spt4, Spt5, Spt6, and FACT, we asked whether HIR or Rtt106 localization to *HTA1-HTB1* was affected by deletion of *YTA7*. We did not observe a difference in the crosslinking pattern of Hir1-TAP at *HTA1-HTB1* in a *yta7* $\Delta$  strain (Figure 7C). Instead, we found that Rtt106 crosslinked throughout the entire *HTA1-HTB1* region in the absence of *YTA7*, including the transcribed regions where it is normally not present (Figure 7D). Thus *Yta7* influences Rtt106 but not Hir1 localization at *HTA1-HTB1*. We also saw increased Rtt106 crosslinking to the *HHT1-HHF1* and *HHT2-HHF2* promoters in the absence of *YTA7*, indicating the same relationships among Hir1, Rtt106, and *Yta7* at three of the four histone gene pairs (Figure S4). We conclude that *Yta7* may contribute to the proper activation of histone gene expression by preventing Rtt106 from spreading from the *HTA1-HTB1* regulatory region into the transcribed regions (Figure 7E).

## DISCUSSION

Cell-cycle-dependent regulation of histone gene expression is a universal feature of eukaryotic cell cycles, yet the mechanisms of activation and repression of histone genes have remained obscure. We developed a reporter-based functional genomic screen using SGA technology in *Sc* and identified several regulators of *HTA1* expression, demonstrating that the H3-H4 histone chaperone Rtt106 functions along with known *HTA1* regulators, Asf1 and the HIR complex, to repress *HTA1* transcription. We found that the *HTA1-HTB1* promoter region is mostly nucleosome-free in the absence of *ASF1*, *HIR1*, or *RTT106*. This result provides a possible underlying explanation for reduced recruitment to *HTA1-HTB1* of Snf5 (Dimova et al., 1999) and Rsc8 (Ng et al., 2002) when the NEG sequence required for HIR repression is absent. When nucleosomes are absent in the region, chromatin-binding factors like SWI/SNF (of which Snf5

is a member), the RSC complex (of which Rsc8 is a member), and *Yta7* (which contains a bromodomain, some of which specifically interact with acetylsine on chromatin proteins such as histones) cannot bind. Whether or not Asf1/HIR/Rtt106-mediated nucleosome formation is associated with a specific histone posttranslational modification pattern remains unknown. We also discovered that *Yta7* bound the *HTA1-HTB1* promoter region and that loss of *Yta7* results in a defect in activation of *HTA1* transcription. *Yta7* appears to mediate histone gene activation by restraining repressive chromatin formed by Asf1/HIR/Rtt106 as well as preventing the encroachment of several chromatin assembly/disassembly factors onto the *HTA1-HTB1* promoter.

Our genetic and biochemical experiments suggest that a primary role of the HIR complex and Asf1 in histone gene regulation is to recruit the histone H3/H4 chaperone Rtt106 to promoter regions. We found that Rtt106 is present at the *HTA1-HTB1* regulatory region throughout the cell cycle (Figure 6A), suggesting that repression is the default state at *HTA1-HTB1*. We propose that, unlike other well-characterized cell-cycle-sensitive promoters, activation of histone gene expression does not require the action of specific activating transcription factors, although they may play some role. Rather, the key to activating histone gene expression resides with overcoming the repressive chromatin structure established by Rtt106 and its partners. Our functional genomic screen and follow-up experiments suggest that Rtt109 may relieve repression by Asf1/HIR/Rtt106 at *HTA1-HTB1* (Figure 7E). The defect in *HTA1* activation caused by mutation of *RTT109* is partially overcome by deletion of *hir1* $\Delta$  or *rtt106* $\Delta$ . The remaining repressive effect of the *RTT109* deletion when combined with *hir1* $\Delta$  or *rtt106* $\Delta$  could be a consequence of its requirement to remove any remaining nucleosomes in those double mutants. Thus, the activity of Rtt109 may not be required when the *HTA1-HTB1* promoter region is nucleosome-free. This phenomenon mirrors that of the HIR-dependent recruitment of the yeast SWI/SNF complex that also activates transcription at *HTA1-HTB1* (Dimova et al., 1999), since mutation of components of the HIR complex abrogates the requirement for SWI/SNF in transcriptional activation. Vps75, a histone chaperone that associates with Rtt109, was also identified as an activator in our screen (Figure 1B), perhaps because it stabilizes Rtt109 (Fillingham et al., 2008).

Rtt109 acetylates H3K56, a modification that is enriched at the yeast histone gene promoters (Xu et al., 2005) in a cell-cycle-dependent manner. The acetylation of H3K56 is required for the recruitment of the SWI/SNF complex member Snf5 (Xu et al., 2005). Thus SWI/SNF could act directly downstream of Rtt109 in a cell-cycle-dependent manner to overcome HIR/Rtt106-mediated repression and activate transcription of *HTA1*. Rtt109 and H3K56ac have been implicated in the process of nucleosome disassembly leading to transcriptional activation at the *PHO5* locus (Williams et al., 2008). Based on these observations, a plausible model for Rtt109 action at the *HTA1-HTB1* promoter involves the coordinated action of Rtt109 and SWI/SNF to disassemble nucleosomes leading to transcriptional activation (Figure 7F). SWI/SNF functions in nucleosome eviction pathways (for example, at *SUC2*, where it binds to the UAS and mediates nucleosome eviction [Schwabish and Struhl, 2007]). An important



caveat to these models is the unclear relationship of Rtt109 to Spt10, a protein initially suggested to be the H3K56-specific HAT at the histone genes in *Sc* (Xu et al., 2005). Our results indicate that both Rtt109 and Spt10 function to activate *HTA1* (Figure 2). Clearly, more work will be required to discover whether Spt10 directly acetylates H3K56 or if it stabilizes H3K56ac-containing nucleosomes, as well as what (if any) relationship exists between Spt10 and Rtt109 at *HTA1-HTB1*.

Rtt106 interacts physically with CAF-1 (Huang et al., 2005) to function in replication-coupled chromatin assembly (Li et al., 2008). In contrast, both the yeast Asf1/HIR complex and higher-organism versions of HIR function in the replication-independent assembly of chromatin (Green et al., 2005, and reviewed in De Koning et al., 2007). We found that the CAF-1 subunit Cac2 is not necessary for Rtt106 localization to *HTA1-HTB1* (Figure 4), suggesting that Asf1/HIR/Rtt106-mediated nucleosome assembly at *HTA1-HTB1* likely occurs in a replication-independent manner. Although our results suggest that the major function of the three histone chaperones at the *HTA1-HTB1* promoter is the assembly of nucleosomes, the precise nature of Asf1/HIR/Rtt106-mediated repression remains to be determined.

A striking feature of the spectrum of regulatory proteins at the *HTA1-HTB1* locus is the physical separation of HIR/Rtt106 from Spt4, Spt5, Spt6, and FACT (Figure S1). Rtt106 was recently found to crosslink to the coding region of *PMA1* (Imbeault et al., 2008), as do Spt4, Spt5, Spt6, and FACT (Kim et al., 2004). Spt6 and Rtt106 are also known to function in parallel to suppress cryptic initiation at an internal promoter within the *FLO8* gene (Imbeault et al., 2008). Since Rtt106 has a defined role in transcriptional elongation, a mechanism may exist to restrict Rtt106 to the promoter region of *HTA1-HTB1*. Yta7 was originally identified as a protein whose absence led to the spreading of the silent state of chromatin at HMR to surrounding genes, consistent with its proposed function as a barrier between regions of heterochromatin and euchromatin at HMR (Tackett et al., 2005). We found that Rtt106, but not Hir1, crosslinks throughout *HTA1-HTB1* in the absence of *YTA7*, including the transcribed regions (Figures 7C and 7D). Thus, the loss of a barrier protein (Yta7) at *HTA1-HTB1* appears to cause the lateral spread of Rtt106 from the promoter through the ORFs. Since Rtt106 is a histone chaperone specifically associated with the formation of repressive chromatin, its lateral spreading across the coding region of *HTA1* could repress transcription by propagating a repressive chromatin structure. The regulation of Rtt106 localization could represent a more general mechanism underlying heterochromatin spreading. For example, Yta7 may influence Rtt106 at heterochromatin boundaries at HMR, a region where both proteins have been functionally implicated (Huang et al., 2007; Jambunathan et al., 2005; Tackett et al., 2005). More generally, Yta7 could function together with Asf1/HIR/Rtt106 to create short domains of repressed chromatin throughout the genome. Our nucleosome occupancy data hint that the promoter regions of other genes are indeed subject to HIR/Rtt106 regulation, and Yta7 may also be involved in their regulation. Global approaches such as ChIP-chip combined with nucleosome occupancy studies will address this question and are currently in progress.

We present here a detailed analysis of histone gene regulation based on the use of a dual-reporter screen to discover regulators. We have elucidated mechanisms of histone gene control both for well-studied regulators like HIR and previously unknown proteins like the histone chaperone Rtt106 and the Yta7 boundary element. We also note that the functional genomics approach presented here can be applied to study virtually any pathway for which an appropriate fluorescent reporter gene can be devised, providing a powerful means to link gene function to transcriptional control.

## EXPERIMENTAL PROCEDURES

### Yeast Strains and Plasmids

Yeast strains are listed in Table S1. Strains were constructed by using standard yeast media and genetic approaches. To generate the promoter-reporter construct, GFP (S65T) followed by the *ADH1* terminator sequence was amplified from the plasmid *pFA6a-GFP S65T-ADH1 HIS3MX6* (Wach et al., 1997) and cloned into the *SacI-PstI* sites of plasmid pRS315 (Sikorski and Hieter, 1989), giving rise to the plasmid BA1926. The entire *HTA1-HTB1* intergenic region was PCR amplified and cloned into plasmid BA1926 adjacent to GFP, which was subsequently transformed into the strain BY4256 (Kainth et al., 2009).

### SGA-Based Functional Genomic Screen for Regulators of *HTA1* Expression

A detailed description of this method is described in Kainth et al. (2009). By using the SGA methodology (Tong et al., 2004), the *HTA1pr-GFP* reporter plasmid (BA1930) along with the control RFP reporter gene was combined with each yeast deletion mutant. Colony size for each arrayed mutant was derived by using Qt ColonyImager software version 1.01 (H. Ding, B.J.A., and C. Boone, unpublished data), and positions on the array with no or slow colony growth were removed from further analysis. Colony fluorescence was assayed by using the Typhoon Trio variable mode imager (GE Healthcare), and quantification was carried out with GenePix Pro 3.0 software. Each screen was carried out in duplicate where deletion mutants are represented twice on the array. GFP and RFP intensities were averaged from replicate deletion mutant colonies on the array and  $\log_2$  GFP:RFP ratios computed. LOESS-normalized (Cleveland, 1979)  $\log_2$  GFP:RFP ratios from duplicate screens were averaged, giving rise to a single gene expression measurement for each deletion strain derived from a total of four independent colonies. These  $\log_2$  ratios were transformed to robust Z scores using median and median absolute deviation, and P values were assigned to these Z scores using the normal distribution. From a diverse data set of 27 promoter-GFP reporter screens, we obtained a list of 260 deletion mutants that usually appear as hits in these screens (P.K., H. Sassi, L.P.-C., T.R.H., and B.J.A., unpublished data), which were removed from further analysis of specific *HTA1* promoter regulators.

### Genome-wide Nucleosome Occupancy

Isolation of nucleosome-bound DNA and hybridization onto the yeast tiling array (Affymetrix) were carried out according to Lee et al. (2007).

### Additional Information

Additional experimental procedures are available in the Supplemental Data. Data from the *HTA1pr-GFP* screen and nucleosome occupancy experiments are available in Tables S3–S5.

### ACCESSION NUMBERS

The GEO accession number for the *hir1Δ*, *rtt106Δ*, and WT genome-wide nucleosome positioning data reported in this paper is GSE16693.

### SUPPLEMENTAL DATA

Supplemental Data include five tables, four figures, Supplemental Experimental Procedures, and Supplemental References and can be found with

this article online at [http://www.cell.com/molecular-cell/supplemental/S1097-2765\(09\)00461-4](http://www.cell.com/molecular-cell/supplemental/S1097-2765(09)00461-4).

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