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Direct Interplay among Histones, Histone Chaperones, and a Chromatin Boundary Protein in the Control of Histone Gene Expression

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In *Saccharomyces cerevisiae*, the histone chaperone Rtt106 binds newly synthesized histone proteins and mediates their delivery into chromatin during transcription, replication, and silencing. Rtt106 is also recruited to histone gene regulatory regions by the HIR histone chaperone complex to ensure S-phase-specific expression. Here we showed that this Rtt106:HIR complex included Asf1 and histone proteins. Mutations in Rtt106 that reduced histone binding reduced Rtt106 enrichment at histone genes, leading to their increased transcription. Deletion of the chromatin boundary element Yta7 led to increased Rtt106:H3 binding, increased Rtt106 enrichment at histone gene regulatory regions, and decreased histone gene transcription at the *HTA1-HTB1* locus. These results suggested a unique regulatory mechanism in which Rtt106 sensed the level of histone proteins to maintain the proper level of histone gene transcription. The role of these histone chaperones and Yta7 differed markedly among the histone gene loci, including the two H3-H4 histone gene pairs. Defects in silencing in *rtt106* mutants could be partially accounted for by Rtt106-mediated changes in histone gene repression. These studies suggested that feedback mediated by histone chaperone complexes plays a pivotal role in regulating histone gene transcription.

Cell cycle-regulated transcription of the canonical histone genes is a hallmark of eukaryotic organisms. During S phase, a coordinated burst of histone gene transcription is required to double the level of histone proteins to package newly replicated DNA into chromatin (13, 22). The tight coupling of both the timing and the level of histone gene expression with DNA synthesis is critical for cell viability. Mutations that perturb DNA replication lead to altered histone gene transcription (3, 23). Conversely, misregulation of the timing and/or level of histone gene expression leads to genomic instability and cell cycle defects (23, 43). Consequently, cells have evolved a complex regulatory mechanism to repress histone gene transcription outside S phase and to promote a precise level of transcription during S phase.

The *Saccharomyces cerevisiae* genome contains two nonallelic copies of each canonical histone gene which are organized in head-to-head pairs of H2A-H2B (*HTA1-HTB1* and *HTA2-HTB2*) and H3-H4 (*HHT1-HHF1* and *HHT2-HHF2*). Nucleosomes, the fundamental units of chromatin structure, contain two H2A/H2B dimers, one H3/H4 tetramer, and ~147 bp of DNA wrapped around the outer surface (37). Repression of histone transcription outside S phase is maintained by the HIR H3/H4 histone-protein chaperone complex, referred to here as the HIR complex (Hir1, Hir2, Hir3, and Hpc2). The HIR complex localizes to negative (NEG) sequence elements within the regulatory regions of *HTA1-HTB1*, *HHT1-HHF1*, and *HHT2-HHF2* (44, 45, 59). Although *HTA2-HTB2* transcription is S-phase specific, its promoter does not contain NEG elements, and the factors involved in its HIR-independent repression are unknown (45). A recent study implicated two additional H3/H4 chaperones, Rtt106 and Asf1, in maintaining histone gene repression at the HIR-regulated loci (16). Rtt106 is recruited to histone gene regulatory regions in a HIR-dependent manner (16), and it contributes to transcriptional repression through the direct recruitment of the RSC chromatin-remodeling complex outside S phase (15, 42). Mutations that disrupt Rtt106:H3 binding lead to reduced Rtt106 enrichment at histone gene promoters (32, 51), suggesting that the Rtt106:HIR interaction might be mediated by histone proteins. Asf1 copurifies with the HIR complex (21), and in *asf1*Δ cells, Rtt106 localization is reduced, leading to inappropriate histone expression outside S phase (51, 53). Although these data suggest that Asf1 plays a direct role in histone gene regulation, whether Asf1 localizes to histone gene promoters as a member of the HIR:Rtt106 regulatory complex was unclear.

During late G_1, histone gene repression is alleviated by the dissociation of the RSC complex and by the Rtt106-dependent recruitment of the SWI/SNF chromatin-remodeling complex (8, 15). SWI/SNF promotes active transcription, perhaps by exposing the upstream activating sequences (UAS) to the cell cycle-regulated transcription factors Spt10 and SBF (9, 12, 58). Additional activating factors include Rtt109, a histone acetyltransferase that acetylates newly synthesized histone H3 proteins at lysine 56 (H3K56ac) (10, 24, 48, 55), and Yta7, an AAA-ATPase protein that maintains certain chromatin boundaries (16, 20, 31, 54). The H3K56 acetylation is S-phase specific (40) and is required to overcome chaperone-mediated histone gene repression through an unknown mechanism (16). Yta7 blocks the apparent spreading of Rtt106 and RSC into the histone gene open reading frames and facilitates efficient promoter escape and elongation by RNA polymerase II (16, 31). In *rtt109*Δ and *yta7*Δ cells, *HTA1* transcription is reduced (16). The transcriptional effects caused by these mutations at the other histone loci were unknown.

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The H3/H4 chaperones that regulate histone gene transcription also facilitate nucleosome turnover throughout the genome (6, 7). During S phase, newly synthesized H3/H4 histones are bound by Asf1 and presented to Rtt109 for acetylation at H3K56 (1). These new histones are then delivered into chromatin by the CAF-1 and Rtt106 histone chaperones through a direct interaction with the replication machinery (5, 33, 50, 61). Rtt106 also facilitates replication-independent nucleosome turnover, along with the HIR complex and Asf1 during both transcription initiation and elongation (21, 27, 47). Additionally, CAF-1 performs an overlapping function with Asf1, Rtt106, and the HIR complex to maintain silent chromatin (25, 26, 30, 49, 56). The involvement of histone chaperones in both the assembly of histone proteins into chromatin and the regulation of histone gene transcription suggests that the two processes might be linked (13, 51). Thus, histone chaperones might facilitate a negative-feedback loop to allow cells to adjust histone gene transcription as a function of free histone protein levels.

In this study, we expanded our understanding of histone gene regulation by establishing the nature of the holocomplex at histone regulatory regions. Our data provided evidence that Asf1 acted directly at histone promoters to mediate repression. Additionally, we observed a relationship between Rtt106's physical interaction with histone proteins, its localization at histone promoters, and histone gene transcription. These results suggested that the histone binding activity of Rtt106 might facilitate negative-feedback regulation of histone gene transcription. Our data revealed an unexpected diversity of responses among the distinct histone gene pairs to changes in the composition of the regulatory complex, suggesting the existence of locus-specific mechanisms controlling histone gene transcription. Moreover, our data reveal a new distinction between the two H3-H4 histone gene pairs and implicate changes in histone gene transcription as a foundation for Rtt106's role in silencing.

MATERIALS AND METHODS

Yeast strains, plasmids, and culture. All yeast strains were derived from *S. cerevisiae* W303 (Table 1). Gene deletions were generated by one-step integration of knockout cassettes (36) followed by PCR analysis of the 5′ and 3′ ends of the targeted gene. A C-terminal 3× FLAG tag (17) was integrated in frame with the targeted genes by one-step integration and verified by PCR. Strains containing mutant *rtt106 S80E*, *R86A*, and *T265E* plasmids or the *hmr-a1*Δ::*URA3* reporter were previously described (60, 61).

Site-directed mutagenesis was used to delete residues 302 to 455 from *RTT106-3×FLAG* (pJR2879) to generate *rtt106*(*1-301*)*-3×FLAG* (pJR3186) as described previously (39). All plasmids were introduced into each strain by transformation (18).

Silencing of the *hmr-a1*Δ::*URA3* reporter and sensitivity to genotoxic chemicals were monitored by spotting 5-fold serial dilutions of mid-log-phase cultures onto selective media, as described previously (61).

Yeast whole-cell extract analysis. Yeast whole-cell extracts were precipitated with 20% trichloroacetic acid. Extracts were solubilized in SDS loading buffer and visualized by SDS-PAGE and immunoblotting using standard procedures. Immunoblots were visualized and quantified with a LI-COR Odyssey imaging system. Anti-Flag M2 antibody (F3165; Sigma) was used to detect FLAG-tagged proteins. Anti-Pgk1 (Invitrogen) was used as a loading control.

Yeast coimmunoprecipitation. Rtt106-FLAG coimmunoprecipitations (co-IP) were performed as described previously (61). Yeast lysate was incubated with 50 μl of anti-FLAG M2 agarose (Sigma). SDS-PAGE, immunoblotting, and LI-COR imaging were performed using standard procedures. Anti-Flag M2 antibody (F3165; Sigma), anti-H3 (Ab1791), and anti-H3K56ac (07-677; Millipore) were used to detect Rtt106-FLAG and copurifying histone proteins, respectively.

RNA preparation and analysis. Reverse transcription-quantitative PCR (RT-qPCR) analysis was performed as described previously (61). Total RNA was extracted using an RNeasy minikit (Qiagen). Genomic DNA was digested on the column using RNase-free DNase (Qiagen). cDNA was synthesized using the SuperScript III first-strand synthesis system for RT-PCR kit (Invitrogen) and oligo(dT) primers. qPCR was performed using a DyNAmo HS SYBR green qPCR kit (New England BioLabs) and an Mx3000P machine (Stratagene). Amplification values for each primer set were normalized to the amplification values from *ACT1* cDNA. Each background was analyzed in triplicate and for three independent RNA preparations. Statistical comparisons were performed using a two-tailed unpaired *t* test. Oligonucleotides for qPCR are listed in Table 2.

Chromatin immunoprecipitation. Chromatin immunoprecipitations (ChIP) were performed as described previously (61). A total of 50 optical-density-at-600-nm (OD_{600}) units of log-phase yeast culture were cross-linked with 1% formaldehyde for 30 min at room temperature. Chromatin was sonicated to ~500 bp. Rtt106-FLAG, Hir1-FLAG, and Asf1-FLAG were immunoprecipitated using anti-FLAG M2 agarose (Sigma). Precipitated DNA fragments were analyzed by qPCR as described above. Amplification values for all primer sets were normalized to a previously described reference locus within an untranscribed region on chromosome V (16). Samples were analyzed in triplicate and for at least three independent chromatin preps. Statistical comparisons were performed using a two-tailed unpaired *t* test. Oligonucleotides for qPCR are listed in Table 2.

RESULTS

Rtt106:H3 binding was required for histone gene repression. Throughout the cell cycle, the HIR complex recruits Rtt106 to histone gene regulatory regions, where together they facilitate transcriptional repression outside S phase and transcriptional activation during S phase (13, 22). Although Rtt106 coimmunoprecipitates (co-IP) with the HIR complex, it is unclear whether this interaction is direct or involves intermediate proteins (16). Notably, the deletion of *RTT109* or *ASF1*, which prevents H3K56 acetylation and consequently decreases Rtt106:H3 binding, reduces Rtt106 enrichment at histone promoters (Fig. 1A and B) (32, 33, 51) without reducing HIR localization (Fig. 1C) (16). Therefore, the HIR:Rtt106 interaction might be bridged by additional proteins, with histones H3/H4 being likely candidates. To test whether Rtt106:H3 binding directly recruited Rtt106 to histone gene promoters, we monitored the localization of three Rtt106 point mutants that we previously discovered disrupt the histone binding surface along its double pleckstrin homology (PH) domain (Rtt106 S80E, R86A, and T265E) (61). S80E and R86A disrupt the charge of a basic patch within the N-terminal PH domain, and T265E alters the charge on a loop connecting two β strands within the C-terminal PH domain. Consistent with the histone-bridging hypothesis, each Rtt106 mutant protein had reduced H3 binding and reduced enrichment at the *HTA1-HTB1* promoter (Fig. 1A and B). The lower levels of enrichment for Rtt106 S80E and T265E compared to that for R86A suggested that R86A had subtle changes in histone binding that were detectable by ChIP but not by earlier co-IP experiments. Our previous work found a similar increase in the severity of replication and silencing defects associated with the *S80E* and *T265E* alleles compared to those associated with *R86A*, further suggesting that Rtt106 R86A is partially functional (61). Like most histone chaperones, Rtt106 contains an acidic C-terminal tail. Although the tail of Rtt106 is not

TABLE 1 Strains used in this study[a]

Strain	Genotype	Source or reference	Figure(s)
JRY3009	*MAT*α *ade2-1 his3-11 leu2-3,112 trp1-1 ura3-52 can1-100*	R. Rothstein	Fig. 1C; Fig. 2A, B, and D
JRY9253	*MAT*α *rtt106*Δ::*KanMX* [pJR2877 *HIS3 RTT106*]	61	Fig. 1A and B; Fig. 2D and E; Fig. 3A to F
JRY9255	*MAT*α *rtt106*Δ::*KanMX* [pJR2879 *HIS3 RTT106-3×FLAG*::*KanMX*]	61	Fig. 1A and B; Fig. 2D and E; Fig. 3A to F; Fig. 4A to C
JRY9262	*MAT*α *rtt106*Δ::*KanMX hir1*Δ::*HygMX* [pJR2879 *HIS3 RTT106-3×FLAG*::*KanMX*]	61	Fig. 1A and B; Fig. 3B
JRY9258	*MAT*α *rtt106*Δ::*KanMX* [pJR2899 *HIS3 rtt106*(*S80E*)*-3×FLAG*::*KanMX*]	61	Fig. 1A, B, and D; Fig. 3D; Fig. 4B
JRY9259	*MAT*α *rtt106*Δ::*KanMX* [pJR2972 *HIS3 rtt106*(*R86A*)*-3×FLAG*::*KanMX*]	61	Fig. 1A, B, and D; Fig. 3D; Fig. 4B
JRY9260	*MAT*α *rtt106*Δ::*KanMX* [pJR2978 *HIS3 rtt106*(*T265E*)*-3×FLAG*::*KanMX*]	61	Fig. 1A, B, and D; Fig. 3D; Fig. 4B
JRY9401	*MAT*α *rtt106*Δ::*KanMX asf1*Δ::*HygMX* [pJR2879 *HIS3 RTT106-3×FLAG*::*KanMX*]	This study	Fig. 1A and B
JRY9266	*MAT*α *rtt106*Δ::*KanMX rtt109*Δ::*HygMX* [pJR2879 *HIS3 RTT106-3×FLAG*::*KanMX*]	61	Fig. 1A and B; Fig. 3E; Fig. 4C
JRY9402	*MAT*α *rtt106*Δ::*KanMX* [pJR3186 *HIS3 rtt106*(*1-301*)*-3×FLAG*::*KanMX*]	This study	Fig. 1A, B, and D
JRY9268	*MAT*α *rtt106*Δ::*KanMX* [pRS313]	61	Fig. 1D; Fig. 4A
JRY9403	*MAT*α *HIR1-3×FLAG*::*KanMX*	This study	Fig. 1C; Fig. 2D and E
JRY9404	*MAT*α *HIR1-3×FLAG*::*KanMX rtt106*Δ::*HygMX*	This study	Fig. 1C
JRY9405	*MAT*α *HIR1-3×FLAG*::*KanMX rtt109*Δ::*HygMX*	This study	Fig. 1C
JRY9406	*MAT*α *HIR1-3×FLAG*::*KanMX hst3*Δ::*HygMX hst4*Δ::*NatMX*	This study	Fig. 1C
JRY9407	*MAT*α *HIR1-3×FLAG*::*KanMX yta7*Δ::*HygMX*	This study	Fig. 2D and E
JRY9408	*MAT*α *ASF1-3×FLAG*::*KanMX*	This study	Fig. 2A to E
JRY9411	*MAT*α *ASF1-3×FLAG*::*KanMX rtt106*Δ::*HygMX*	This study	Fig. 2B and C
JRY9412	*MAT*α *ASF1-3×FLAG*::*KanMX hir1*Δ::*HygMX*	This study	Fig. 2B and C
JRY9413	*MAT*α *ASF1-3×FLAG*::*KanMX yta7*Δ::*HygMX*	This study	Fig. 2D and E
JRY9414	*MAT*α *rtt106*Δ::*KanMX yta7*Δ::*HygMX* [pJR2879 *HIS3 RTT106-3×FLAG*::*KanMX*]	This study	Fig. 2D and E; Fig. 3A to E; Fig. 4A to C
JRY9415	*MAT*α *rtt106*Δ::*KanMX hir1*Δ::*HygMX yta7*Δ::*NatMX* [pJR2879 *HIS3 RTT106-3×FLAG*::*KanMX*]	This study	Fig. 3A and B
JRY9416	*MAT*α *rtt106*Δ::*KanMX yta7*Δ::*HygMX* [pJR2899 *HIS3 rtt106*(*S80E*)*-3×FLAG*::*KanMX*]	This study	Fig. 3C and D; Fig. 4B
JRY9417	*MAT*α *rtt106*Δ::*KanMX yta7*Δ::*HygMX* [pJR2972 *HIS3 rtt106*(*R86A*)*-3×FLAG*::*KanMX*]	This study	Fig. 3C and D; Fig. 4B
JRY9418	*MAT*α *rtt106*Δ::*KanMX yta7*Δ::*HygMX* [pJR2978 *HIS3 rtt106*(*T265E*)*-3×FLAG*::*KanMX*]	This study	Fig. 3C and D; Fig. 4B
JRY9419	*MAT*α *rtt106*Δ::*KanMX yta7*Δ::*HygMX rtt109*Δ::*NatMX* [pJR2879 *HIS3 RTT106-3×FLAG*::*KanMX*]	This study	Fig. 3C and E; Fig. 4C
JRY9420	*MAT*α *rtt106*Δ::*KanMX yta7*Δ::*HygMX* [pRS313]	This study	Fig. 4A
JRY9267	*MAT*α *rtt106*Δ::*KanMX hst3*Δ::*HygMX hst4*Δ::*NatMX* [pJR2879 *HIS3 RTT106-3×FLAG*::*KanMX*]	61	Fig. 3F
JRY8883	*MAT***a** *hmr-a1*Δ::*KlURA3*	60	Fig. 5A to C
JRY9421	*MAT***a** *hmr-a1*Δ::*KlURA3 sir3*Δ::*KanMX*	This study	Fig. 5A to C
JRY9422	*MAT***a** *hmr-a1*Δ::*KlURA3 hht1-hhf1*Δ::*KanMX*	This study	Fig. 5A and B
JRY9423	*MAT***a** *hmr-a1*Δ::*KlURA3 hht2-hhf2*Δ::*KanMX*	This study	Fig. 5A and B
JRY9424	*MAT***a** *hmr-a1*Δ::*KlURA3 rtt106*Δ::*KanMX*	This study	Fig. 5A and B
JRY9425	*MAT***a** *hmr-a1*Δ::*KlURA3 cac1*Δ::*KanMX*	This study	Fig. 5A and B
JRY9426	*MAT***a** *hmr-a1*Δ::*KlURA3 hir1*Δ::*KanMX*	This study	Fig. 5A and B
JRY9427	*MAT***a** *hmr-a1*Δ::*KlURA3 hht1-hhf1*Δ::*HygMX rtt106*Δ::*KanMX*	This study	Fig. 5A and B
JRY9428	*MAT***a** *hmr-a1*Δ::*KlURA3 hht2-hhf2*Δ::*HygMX rtt106*Δ::*KanMX*	This study	Fig. 5A and B
JRY9429	*MAT***a** *hmr-a1*Δ::*KlURA3 hht1-hhf1*Δ::*KanMX cac1*Δ::*HygMX*	This study	Fig. 5A and B
JRY9430	*MAT***a** *hmr-a1*Δ::*KlURA3 hht2-hhf2*Δ::*KanMX cac1*Δ::*HygMX*	This study	Fig. 5A and B
JRY9431	*MAT***a** *hmr-a1*Δ::*KlURA3 hht1-hhf1*Δ::*KanMX hir1*Δ::*HygMX*	This study	Fig. 5A and B
JRY9432	*MAT***a** *hmr-a1*Δ::*KlURA3 hht2-hhf2*Δ::*KanMX hir1*Δ::*HygMX*	This study	Fig. 5A and B
JRY9243	*MAT***a** *hmr-a1*Δ::*KlURA3 rtt106*Δ::*KanMX cac1*Δ::*HygMX*	61	Fig. 5A to C
JRY9433	*MAT***a** *hmr-a1*Δ::*KlURA3 rtt106*Δ::*KanMX cac1*Δ::*HygMX hht1-hhf1*Δ::*NatMX*	This study	Fig. 5A to C
JRY9434	*MAT***a** *hmr-a1*Δ::*KlURA3 rtt106*Δ::*KanMX cac1*Δ::*HygMX hht2-hhf2*Δ::*NatMX*	This study	Fig. 5A to C

[a] *KlURA3*, *Kluyveromyces lactis URA3*.

necessary for histone binding *in vitro* (33, 34, 52), the tail was necessary for histone binding *in vivo* and for *HTA1-HTB1* promoter recruitment (Fig. 1A and B). Together these results suggested that HIR recruited Rtt106 to histone gene promoters through a histone protein bridge.

Similar trends in defective recruitment of Rtt106 were observed at the *HHT1-HHF1* and *HHT2-HHF2* promoters (data not shown). However, the overall enrichment signal at the two H3-H4 gene pairs was reduced compared to that at *HTA1-HTB1*. Currently, it is unclear whether the smaller dynamic range of wild-type Rtt106 enrichment at the H3-H4 promoters, compared to that at H2A-H2B, reflected a biological distinction or an experimental limitation of ChIP at the H3-H4 promoter sequences. The *HTA2-HTB2* locus, which is not subject to regulation by the HIR complex (45), exhibited minimal Rtt106 enrichment above background (Fig. 1B).

TABLE 2 Oligonucleotides used in this study

Method and oligonucleotide	Forward	Reverse	Annealing temp (°C)	Source or reference
ChIP				
Chromosome V UTR[a]	GCA ATC AAC ATC TGA AGA AAA GAA AGT AGT	CAT AAT CTG CGT AAA AAT GGC GTA AAT	55	16
HTA1-HTB1 promoter	ATA GTT AAC GAC CCA ACC GCG T	ACG GGC GTT TCT TCA ACA ACG A	55	This study
HTA1 5′ ORF	ACG GAT TTG GTT ATT TCT CAG TGA A	ATT CCA AGA CAG CAG TCA AGT AGA C	55	16
HTA1 3′ ORF	CAA AGA AGT CTG CCA AGG CT	AGC AGT TTA GTT CCT TCC GC	55	This study
HTA1 3′ UTR	AGG TTC ATT GGG CAC TGT TG	ACA GTT CTC CGT GAC AGG AT	55	This study
HTB1 5′ ORF	CCA CAA ATA AAC CAT ACA CAC	GGA AAT ACC AGT GTC AGG GT	55	This study
HTB1 3′ ORF	CCA GGT GAA TTG GCT AAG CA	GCA TTC CCT CTA TGA GAC CA	55	This study
HTB1 3′ UTR	CGA AAC TTC AGA GCA TTG GC	GGG TTC AAT CTC CAA GGC AT	60	This study
HHT1-HHF1 promoter	ATT TAC CAC CGT ATT CGC GG	AGG TGC AGA GCA AGG AAA TG	55	This study
HHT1 ORF	GCA ATT AGC TTC TAA GGC TGC CAG	GCA GCC AAG TTG GTA TCT TCA A	60	This study
HHT1 3′ UTR	GCC TTG TAG GAG GCA AGA TT	CGT ATG CGG CTT CAA GTT GT	55	This study
HHF1 5′ ORF	CCG CGA ATA CGG TGG TAA AT	TGG CAC CAC CTT TAC CTA GA	55	This study
HHF1 3′ ORF	TCA TCA GAG ACT CTG TTA CC	GTT ACC GTT TTC TTA GAA TTA G	55	31
HHF1 3′ UTR	ATC TGA GAG CAG GAA GAG CA	GTG TGT CAG CAT CAG AGG TT	55	This study
HHT2-HHF2 promoter	AAA TGA CCA ACT CCC ATC CG	TTT GTT CTG GTC TGG TCT GC	55	This study
HTA2-HTB2 promoter	AAT GGT AGC ACG TCG CGT TT	TGA CGG CAA GTG TCT CAC TGT T	55	This study
RT-qPCR				
ACT1	GTC GGT AGA CCA AGA CAC C	GGG TGT TCT TCT GGG GC	55	31
HTA1	ACG TTA CCA TTG CCC AAG G	GTT TAG TTC CTT CCG CCT TC	55	31
HHT1	AAT CTT CTG CCA TCG GTG CC	CTA AAA CTG ATG ACA ATC AAC	55	31
HHF1	TCA TCA GAG ACT CTG TTA CC	GTT ACC GTT TTC TTA GAA TTA G	55	31
HTA2	TAT TGG GTA ATG TTA CCA TC	GCT TTG TTT CTT TTC AAC TCA G	55	31
hmr-a1::*URA3*	CTT CCA AGG GTT CTC TAG CAC ACG	CTG TAC TGC TGA CCC AAT GCA TCG	55	61
HML-α2	TCC ACA AAT CAC AGA TGA GT	GTT GGC CCT AGA TAA GAA TCC	55	This study
*MAT***a**	TGG ATG ATA TTT GTA GTA TGG CGG A	TCC CTT TGG GCT CTT CTC TT	55	61

[a] UTR, untranscribed region; ORF, open reading frame.

To promote S-phase-specific transcription, Rtt106 directly recruits the chromatin remodeling enzymes RSC and SWI/SNF to generate repressive and active chromatin architectures, respectively (8, 15, 42). In *rtt106*Δ strains, histone genes are transcribed outside S phase, leading to an increase in total histone mRNA in asynchronously growing cultures (16). Each *rtt106* mutant strain, in which Rtt106 failed to localize to the promoter, had increased histone mRNA levels (Fig. 1D). *HTA1* and *HHT1* transcription in *rtt106 R86A* and *rtt106*(*1-301*) strains was similar to that in the wild type, suggesting that a threshold amount of Rtt106 was able to maintain repression. In the absence of Rtt106, the extent of transcriptional derepression was greater at *HHF1* than at *HTA1* and *HHT1*, suggesting nonidentical regulatory mechanisms at each histone gene pair. The direct inverse relationship between the extent of Rtt106:H3 binding and the level of histone gene expression suggested a potential Rtt106-mediated negative-feedback loop, as discussed below.

Asf1 localized to histone gene regulatory regions with Rtt106 and the HIR complex. Because Rtt106 enrichment is reduced and histone transcription is increased in *asf1*Δ cells, it has been suggested that Asf1 itself localizes to histone promoters and recruits Rtt106 (51). However, since Rtt106:H3 binding was necessary for Rtt106 recruitment to the promoter (Fig. 1A) and the affinity of Rtt106 for H3 is enhanced by the Asf1-dependent acetylation of H3K56 (33), Rtt106 mislocalization in *asf1*Δ cells could result from reduced Rtt106:H3 binding due to the absence of H3K56ac. To test whether Asf1 directly regulated histone gene transcription, we evaluated Asf1's enrichment in the regulatory region of the *HTA1-HTB1* gene pair and at the other histone gene loci. Intriguingly, like Rtt106 and the HIR complex (16, 21), Asf1 localized to the center of the *HTA1-HTB1* promoter (Fig. 2A). This localization profile was consistent with Asf1's known direct physical interaction with HIR and its indirect interaction with Rtt106 , which is thought to be mediated by H3/H4 binding (21, 32). Together, these findings supported a previous suggestion that the HIR complex, localized to the center of the *HTA1-HTB1* regulatory region, recruits Asf1, which in turn recruits Rtt106 through an H3/H4 bridge (51).

Consistent with this proposed order of recruitment, Asf1 enrichment was Rtt106 independent and was reduced in the absence of Hir1 (Fig. 2B). The cellular levels of Asf1 in *rtt106*Δ, *hir1*Δ, and wild-type cells were similar (Fig. 2C). Therefore, changes in Asf1 enrichment were not due to altered protein levels. Both Asf1 and Hir1 exhibited increased promoter enrichment in *rtt106*Δ cells (Fig. 2B and Fig. 1C). Therefore, the absence of Rtt106 may have caused greater exposure of the Hir1 and Asf1 epitopes, leading to the appearance of increased enrichment. Alternatively, since histone gene derepression is greater in *hir1*Δ cells than in *rtt106*Δ cells (16), the increased enrichment of HIR and Asf1 in *rtt106*Δ cells may partially compensate for the absence of Rtt106. Nevertheless, this similar trend in Hir1 and Asf1 enrichment strongly suggested that Asf1 played a direct role at histone gene promoters and was recruited prior to Rtt106.

Similarly to Rtt106 and the HIR complex (Fig. 1B and below), Asf1 exhibited a low level of enrichment at the *HTA2-HTB2* promoter (Fig. 2B). Since *HTA2-HTB2* does not contain the negative

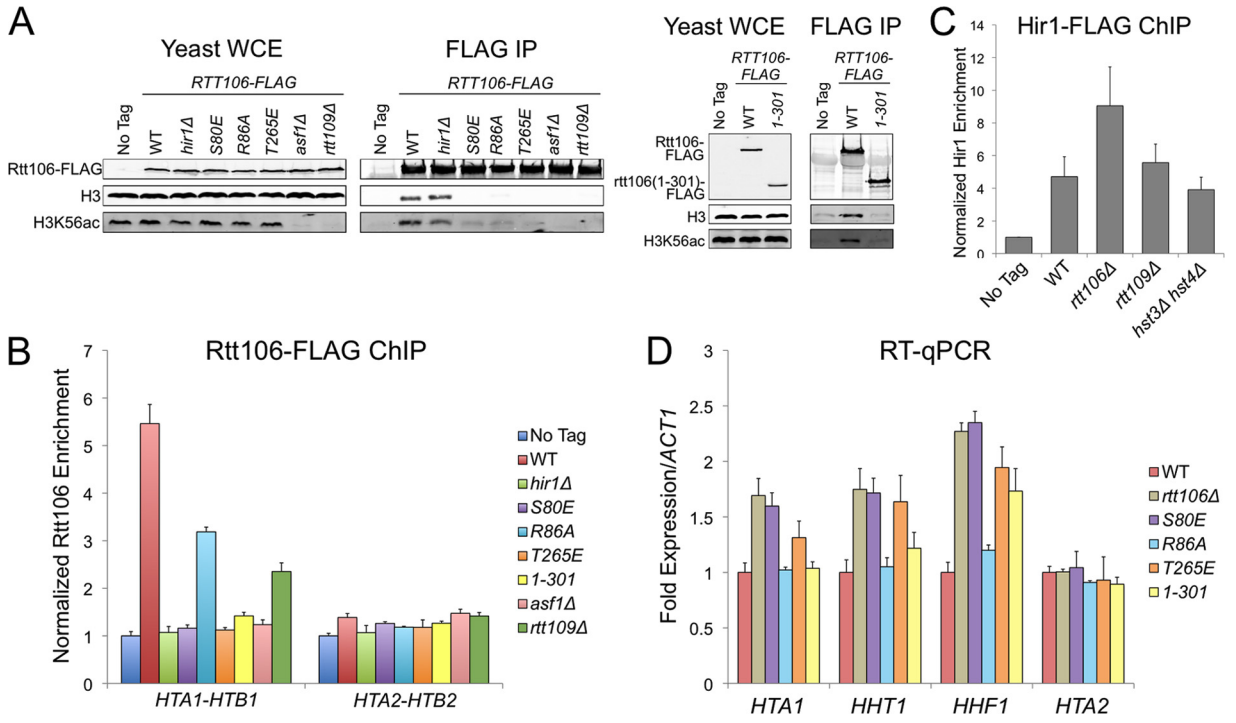

FIG 1 Rtt106:H3 binding directly regulated histone gene transcription. (A) Mutants that disrupted Rtt106:H3 binding. Rtt106-FLAG was immunoprecipitated (IP) from yeast whole-cell extract (WCE) of the indicated mutant strains with anti-FLAG resin. Copurifying proteins were detected by immunoblotting with antibodies against FLAG, H3, and H3K56ac. WT, wild type. (B) ChIP analysis of Rtt106-FLAG at the *HTA1-HTB1* and *HTA2-HTB2* promoters. Values were obtained with qPCR and were normalized to a previously described negative control within an untranscribed region of chromosome V. No-tag ratios were normalized to 1. Error bars here and elsewhere represent standard deviations from the mean ($n \geq 3$). (C) ChIP analysis of Hir1-FLAG at the *HTA1-HTB1* promoter. Data were analyzed as described for panel B. (D) RT-qPCR analysis of histone transcripts in *rtt106* mutant strains. Transcript values were normalized to *ACT1* mRNA and adjusted to the WT value, which was normalized to 1. *rtt106*Δ, *S80E*, and *T265E* each had significantly increased *HTA1*, *HHT1*, and *HHF1* transcription compared to the WT ($P < 0.01$). *R86A* and *rtt106*(*1-301*) each had significantly increased *HHF1* transcription compared to the WT ($P < 0.01$).

regulatory sequence elements that recruit the HIR complex at the other three histone gene loci, this low-level enrichment of histone chaperones was enigmatic. The enrichment of Hir1, Rtt106, and Asf1 at *HTA2-HTB2* was unchanged in mutant backgrounds that altered chaperone recruitment at the three HIR-dependent histone gene loci (Fig. 2B and D). Additionally, *HTA2* transcription was similarly unaffected in such mutants compared to that of loci containing negative regulatory sequence elements (Fig. 1D) (45). Therefore, if these low-level enrichments reflected a true histone chaperone complex at *HTA2-HTB2*, the complex was clearly subject to distinct regulation.

Next we tested whether Asf1 localization was restricted to the center of histone regulatory sequences by the AAA-ATPase chromatin boundary protein Yta7, which regulates the localization of Rtt106. In *yta7*Δ cells, Rtt106 has increased enrichment at the promoter and in the 5′ end of the histone open reading frames (ORFs) (Fig. 2D and E) (16). Subsequent increased recruitment of RSC throughout the region is thought to create a transcriptionally refractive chromatin environment (31). Unlike Rtt106, the HIR complex exhibits similar levels of enrichment between *yta7*Δ and wild-type cells (Fig. 2D and E) (16). Like the HIR complex and unlike Rtt106, in the absence of Yta7, Asf1 enrichment was similar to its enrichment in wild-type cells (Fig. 2D and E). Therefore, the HIR complex and Asf1 were nucleating factors that recruited Rtt106 to the regulatory region of histone genes. In the absence of the Yta7-mediated boundary, only Rtt106 and RSC showed increased enrichment in both histone gene promoters and ORFs to repress transcription (31). In the absence of Yta7, Rtt106 enrichment was more extensive at *HHT1-HHF1* than at *HTA1-HTB1*, suggesting a locus-specific variation of this regulation (Fig. 2E).

Yta7 in Rtt106:H3 binding and histone gene localization. The relationship between Rtt106's histone binding activity and its recruitment to histone promoters suggested that Rtt106 plays a pivotal role in a negative-feedback loop for histone gene regulation. Reduced Rtt106:H3 binding led to reduced Rtt106 enrichment at the promoter and increased histone gene transcription (Fig. 1). Therefore, Rtt106 may act as a sensor to fine-tune histone transcription as a function of histone protein levels. A recent study reported an increased level of chromatin-bound H3 in *yta7*Δ cells (35). The negative-feedback model predicted that the increased promoter enrichment of Rtt106 and the reduced histone transcription observed in *yta7*Δ cells might depend on Rtt106 "sensing" these elevated histone protein levels. Indeed, co-IP experiments revealed an increased amount of H3 bound to Rtt106 in *yta7*Δ cells compared to wild-type cells (Fig. 3A). This increase was maintained in *hir1*Δ *yta7*Δ double mutants, indicating that the elevated Rtt106:H3 binding in *yta7*Δ single mutants occurred prior to Rtt106 recruitment to histone gene promoters (Fig. 3A and B). Eliminating Rtt106's ability to "sense" histone proteins, either by mutating its histone binding surface (*S80E*, *R86A*, or *T265E*) or by depleting H3K56ac (*rtt109*Δ), suppressed both the elevated Rtt106:H3 binding and the increased Rtt106 enrichment

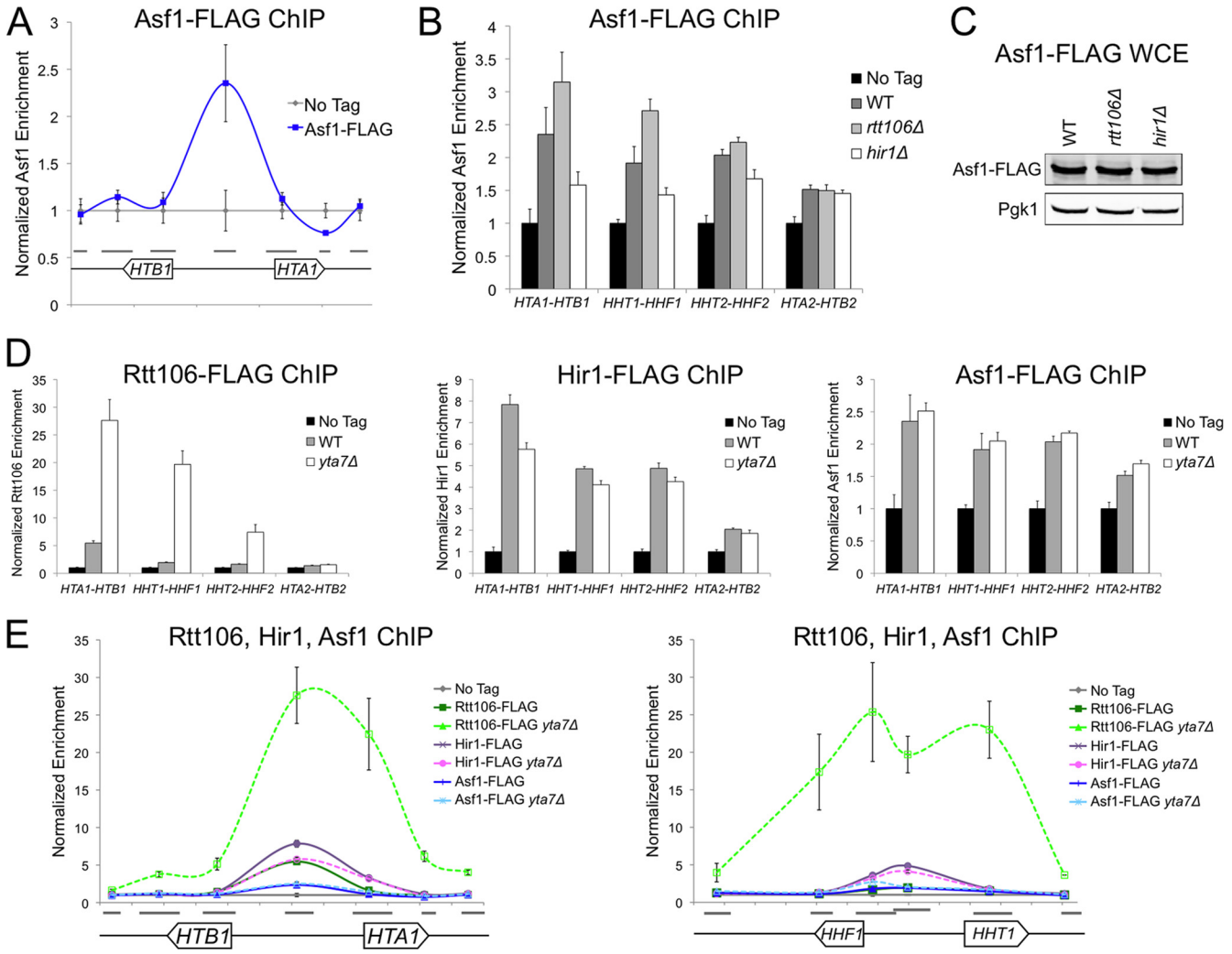

FIG 2 Asf1 localized with Rtt106 and the HIR complex at histone gene regulatory regions. (A) ChIP analysis of Asf1-FLAG at the *HTA1-HTB1* locus (chromosomal coordinates from 913.9 to 916.3 kb). Gray bars indicate the sequences amplified by each qPCR primer set. qPCR values were normalized as described for Fig. 1B. (B) ChIP analysis of Asf1-FLAG at the center of the *HTA1-HTB1*, *HHT1-HHF1*, *HHT2-HHF2*, and *HTA2-HTB2* promoters in the indicated mutant strains. WT, wild type. (C) Yeast whole-cell extract (WCE) immunoblotted with anti-FLAG and anti-Pgk1 (loading control) antibodies. (D) ChIP analysis of Rtt106-FLAG (left), Hir1-FLAG (center), and Asf1-FLAG (right) as described for panel B. (E) ChIP analysis of Rtt106-FLAG, Hir1-FLAG, and Asf1-FLAG at the *HTA1-HTB1* (left) and *HHT1-HHF1* (right, chromosomal coordinates 254.6 to 257.1 kb) loci, as described for panel A.

at histone promoters in *yta7*Δ cells (Fig. 3C to E). This suppression indicated that elevated enrichment of Rtt106 in the *yta7*Δ mutant relied upon the previously characterized Rtt106:H3 binding surface, implying that the additional Rtt106 was bound to H3/H4. Moreover, in the presence of excess histone protein, increased Rtt106:H3 binding was necessary for Rtt106 recruitment and subsequent repression of histone gene transcription.

Monitoring Rtt106 localization in *yta7*Δ cells revealed a previously unappreciated distinction between the severity of the S80E and T265E mutations, which had similar phenotypes in wild-type cells (Fig. 1) (61). A small but significant amount of both Rtt106 S80E and R86A spread into histone gene ORFs in *yta7*Δ cells compared to each mutant's localization in wild-type cells ($P < 0.05$ at *HTA1*, $P < 0.01$ at *HHT1*), whereas Rtt106 T265E localization in *yta7*Δ was similar to that of the wild type (Fig. 3D). These results suggested that mutations within Rtt106's C-terminal loop (T265E) led to stronger H3 binding defects than mutations within the N-terminal basic patch (S80E and R86A).

Surprisingly, unlike in *rtt109*Δ single mutants (Fig. 1A), a low level of Rtt106:H3 binding was detectable by co-IP in *rtt109*Δ *yta7*Δ cells (Fig. 3C). Additionally, compared to *rtt109*Δ single mutants, a small amount of Rtt106 spread into histone gene ORFs in *rtt109*Δ *yta7*Δ double mutants ($P < 0.01$)(Fig. 3E). Therefore, in the absence of Yta7, the acetylation of H3K56 by Rtt109 was no longer required for detectable Rtt106:H3 binding. These results were consistent with *in vitro* binding experiments, which show that although the acetylation of H3 at K56 increases the affinity of Rtt106 for H3 by ~15- to 20-fold, Rtt106:H3 binding is still detectable with recombinant, unacetylated H3/H4 (K_d [dissociation constant], ~1 μM) (52). Therefore, it is possible that in *rtt109*Δ cells a low level of Rtt106:H3 binding that was below the level of detection by co-IP occurred, whereas in *yta7*Δ cells the increased cellular concentration of histones allowed the visualization of Rtt106's weak *in vivo* interaction with H3 proteins lacking acetylation at K56. This idea was supported by the Rtt106 enrichment at the *HTA1-HTB1* promoter in *rtt109*Δ which, though substantially

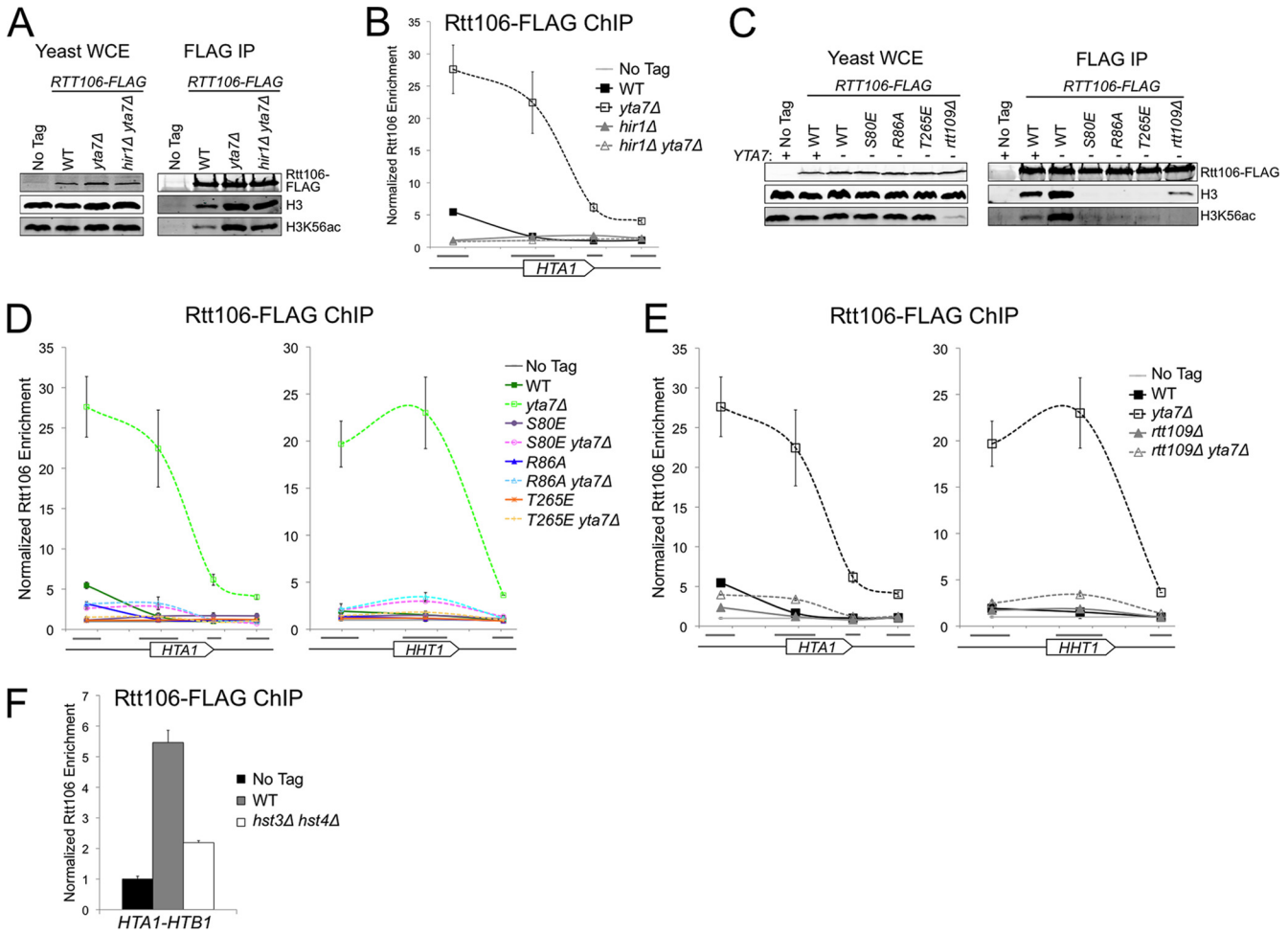

FIG 3 Elevated Rtt106:H3 binding in *yta7*Δ cells was necessary for increased Rtt106 enrichment at histone genes. (A) Rtt106-FLAG immunoprecipitated from the indicated mutant strains as in Fig. 1A. WT, wild type. (B) ChIP analysis of Rtt106-FLAG at the *HTA1* locus, as described in Fig. 2E (chromosomal coordinates from 915.0 to 916.3 kb). (C) Rtt106-FLAG immunoprecipitated from yeast whole-cell extract (WCE) as in panel A. (D and E) ChIP analysis of Rtt106-FLAG at the *HTA1* (left) and *HHT1* (right, chromosomal coordinates 255.8 to 257.1 kb) loci, as in panel B. (F) ChIP analysis of Rtt106-FLAG at the *HTA1-HTB1* promoter, as in Fig. 1B.

reduced with respect to the wild type, was nevertheless higher than the enrichment of Rtt106 carrying the S80E and T265E point mutations along the Rtt106:H3 interaction surface (Fig. 1B).

The correlation between increased Rtt106:H3 binding and Rtt106 enrichment at histone gene promoters in *yta7*Δ cells predicted that additional mutant backgrounds with elevated Rtt106:H3 would lead to similarly increased Rtt106 enrichment within the regulatory region of histone genes. Cells lacking the H3K56ac-specific histone deacetylases Hst3 and Hst4 have increased Rtt106:H3 binding, presumably due to increased levels of H3K56ac (4, 33). However, unlike in *yta7*Δ cells, Rtt106 enrichment was reduced at the regulatory region of *HTA1-HTB1* in *hst3*Δ *hst4*Δ cells (Fig. 3F). Therefore, elevated Rtt106:H3 binding does not always lead to increased Rtt106 localization at the histone gene loci, as discussed further below.

Feedback regulation of histone gene transcription by Yta7 and Rtt106. According to the negative-feedback model, changes in Rtt106 enrichment at histone loci in *yta7*Δ cells should lead to changes in transcription. However, the effect of *yta7*Δ on the level of histone transcripts is somewhat controversial. Gradolatto et al. reported precocious increases in histone mRNA levels prior to S phase in *yta7*Δ cells, though the trend was dependent upon the type of synchronizing agent and was observed only at a subset of histone gene loci (19). In contrast, Fillingham et al. reported reduced total *HTA1* mRNA in *yta7*Δ cells, whereas Lombardi et al. reported that the *HHT1* mRNA level in *yta7*Δ cells is similar to the level in wild-type cells (16, 35). Here, we recapitulated that in *yta7*Δ cells total *HTA1* transcripts were significantly reduced, whereas *HHT1* and *HHF1* mRNA levels were indistinguishable from those in the wild type (Fig. 4A). The repression of *HTA1* expression in *yta7*Δ cells was suppressed in an isogenic *yta7*Δ *rtt106*Δ double mutant, suggesting that Rtt106 spreading caused the *HTA1* repression (Fig. 4A). In summary, enhanced Rtt106 enrichment at *HTA1-HTB1* correlated with reduced transcription at that locus whereas enhanced enrichment at *HHT1-HHF1* did not, implying the existence of additional feedback mechanisms operating at *HHT1-HHF1* to ensure proper transcriptional regulation.

Changes in *HTA1*, *HHT1*, and *HHF1* transcript levels mirrored the changes in Rtt106 S80E, R86A, and T265E localization be-

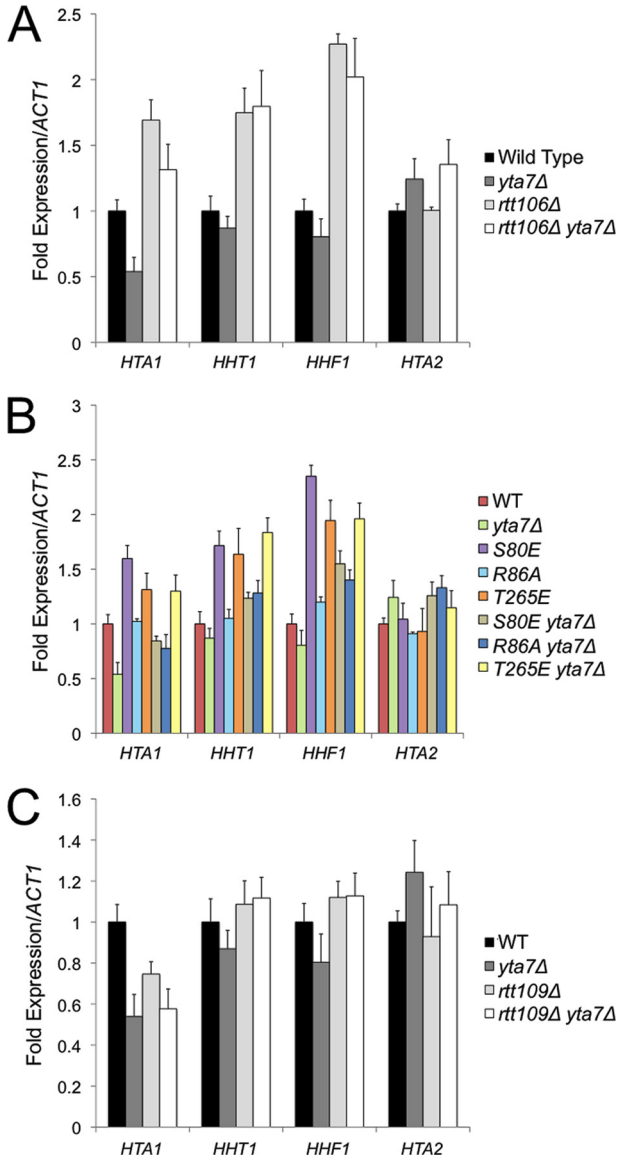

FIG 4 Increased Rtt106 enrichment at histone gene loci repressed *HTA1* transcription. RT-qPCR of histone gene mRNA levels in the indicated strains as described for Fig. 1D. *HTA1* transcription was significantly reduced in *yta7*Δ and *rtt109*Δ strains compared to that in the wild type (WT) ($P < 0.01$).

tween wild-type and *yta7*Δ cells. Rtt106 T265E localization was unchanged in *yta7*Δ cells (Fig. 3D), and histone transcript levels were similar in *T265E* and *T265E yta7*Δ mutants (Fig. 4B). Although Rtt106 S80E and R86A were strongly reduced in their affinity for H3 compared to wild-type Rtt106, these mutants displayed some spreading in the absence of Yta7 compared to the promoter-specific localization of Rtt106 in wild-type cells (Fig. 3D). This partial formation of a repressive complex at the promoter led to reduced *HTA1*, *HHT1*, and *HHF1* transcripts in S80E *yta7*Δ and R86A *yta7*Δ compared to T265E *yta7*Δ and *rtt106*Δ *yta7*Δ, where no spreading occurred (Fig. 4B). Therefore, the enhanced dynamic range of Rtt106 enrichment at histone gene regulatory regions in *yta7*Δ cells allowed further recognition of functionally significant differences in the localization of Rtt106 mutants.

Previous studies classified Rtt109 along with Yta7 as an activator of *HTA1* transcription (16). However, as in *yta7*Δ, we observed transcriptional repression in *rtt109*Δ cells only at *HTA1*; *HHT1* and *HHF1* mRNA levels were similar to those of the wild type (Fig. 4C). Rtt106 enrichment was partially reduced at the *HTA1-HTB1* promoter in *rtt109*Δ cells (Fig. 1B) presumably due to the reduced level of H3K56ac, the preferred histone ligand of Rtt106. Nevertheless, *HTA1* transcript levels are reduced due to the lack of Rtt109's activator function, which is required to overcome histone chaperone-mediated repression (see below) (Fig. 4C) (16). As expected, *HTA1* transcription was reduced in *rtt109*Δ *yta7*Δ cells due to both the absence of the Rtt109 activator and the small but significant amount of Rtt106 enrichment within the *HTA1* ORF (Fig. 4C and Fig. 3E).

Relationship between Rtt106's roles in replication, silencing, and histone gene transcription. In addition to the cell cycle regulation of histone gene transcription, Rtt106 mutations also exhibit genetic interactions with mutations in the CAF-1 histone chaperone complex during replication and silencing (25, 26, 33). The direct physical interaction of Rtt106 with CAF-1 suggests that Rtt106's role in silencing and replication is direct (25). Nevertheless, it was also possible that *rtt106*Δ *cac1*Δ phenotypes regarding replication and silencing were at least partially due to inappropriate histone gene transcription throughout the cell cycle. By this hypothesis, a reduction in histone gene dosage might suppress the silencing defects and genotoxic chemical sensitivities observed in *rtt106*Δ *cac1*Δ cells. To test this hypothesis, we monitored growth on media containing chemicals that induce DNA damage during S phase and on media that measure silencing of an *HMRa1* reporter (*hmr-a1*Δ::*URA3*) in *rtt106*Δ *cac1*Δ cells lacking one of the two H3-H4 histone gene pairs (*hht1-hhf1*Δ or *hht2-hhf2*Δ).

In the presence of genotoxic agents, a reduction in H3-H4 dosage did not suppress the growth defects of *rtt106*Δ *cac1*Δ cells, suggesting that increased histone gene transcription was not the primary cause of these chemical sensitivities (Fig. 5A). During these growth assays, we were surprised to discover a synthetic growth defect phenotype between *rtt106*Δ and *hht1-hhf1*Δ but not *hht2-hhf2*Δ (Fig. 5A; most evident in "day 2" panel). This distinction between H3-H4 gene copies was unexpected; the two loci encode identical H3 and H4 proteins and are thought to be functionally equivalent (9). To tease apart whether the synthetic sickness was due to Rtt106's role in histone gene transcription or replication-coupled nucleosome turnover, we tested whether mutants of the HIR or CAF-1 complex had similar synthetic interactions with *hht1-hhf1*Δ. The *cac1*Δ *hht1-hhf1*Δ and *cac1*Δ *hht2-hhf2*Δ double mutants both phenocopied the *cac1*Δ single mutant, indicating that the two H3-H4 gene pairs were equivalent with respect to replication-coupled chromatin assembly. In contrast, like *rtt106*Δ, *hir1*Δ *hht1-hhf1*Δ had a strong synergistic growth defect compared to that of *hir1*Δ *hht2-hhf2*Δ (Fig. 5A). The increased severity of the *hir1*Δ *hht1-hhf1*Δ growth defects compared to that of *rtt106*Δ *hht1-hhf1*Δ was consistent with the larger fold increase in histone gene transcription in *hir1*Δ cells compared to that in *rtt106*Δ cells (16). These results unveiled a previously unappreciated distinction between histone chaperone-mediated repression at *HHT1-HHF1* and that at *HHT2-HHF2*.

Intriguingly, unlike the sensitivities to DNA-damaging agents, knocking out *hht2-hhf2*Δ partially suppressed the silencing defects in *rtt106*Δ *cac1*Δ cells (Fig. 5B, bottom panels). The absence of suppression in *rtt106*Δ *cac1*Δ *hht1-hhf1*Δ cells further high-

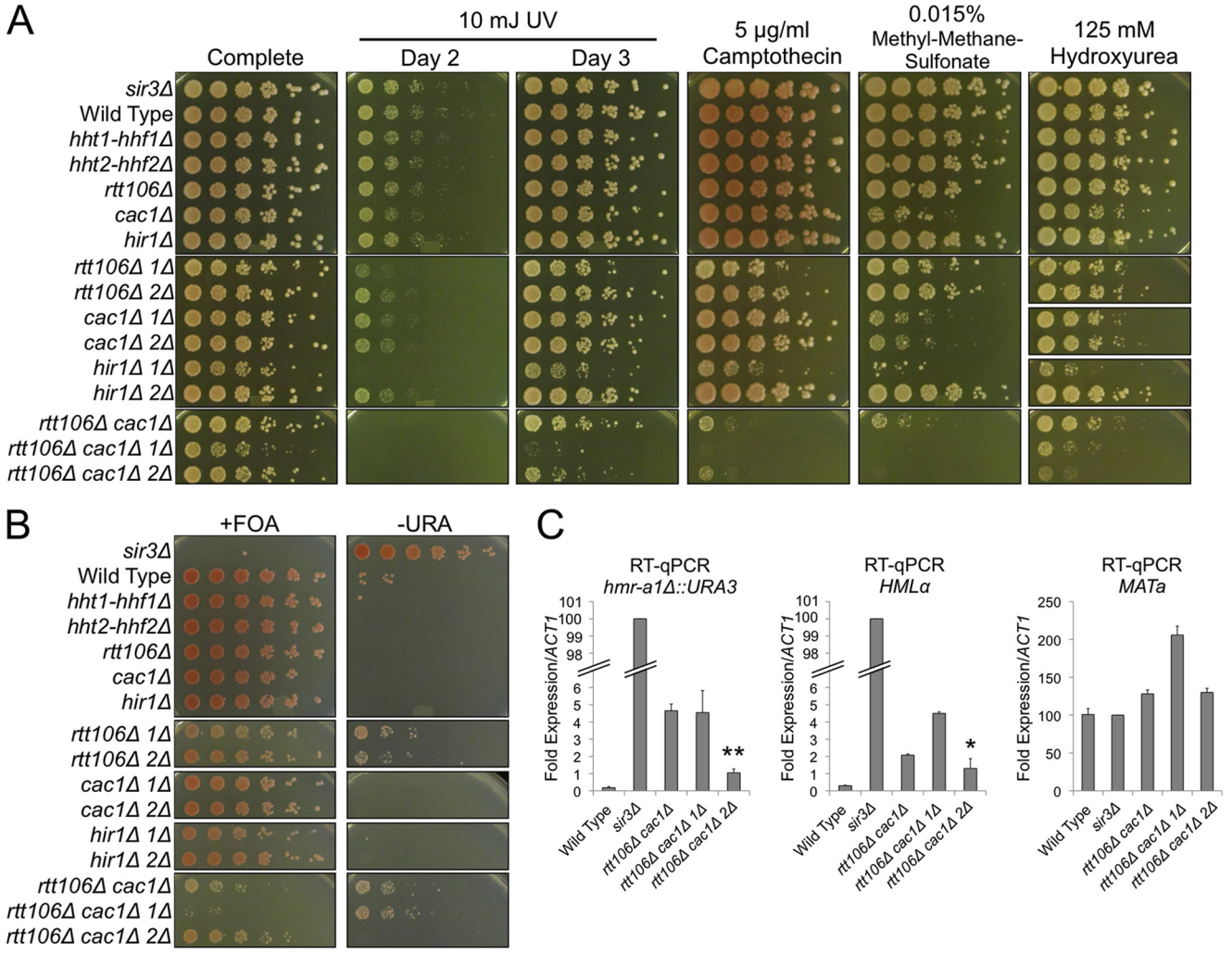

FIG 5 Reduced H3-H4 dosage partially suppressed *rtt106*Δ *cac1*Δ silencing phenotypes. (A) Five-fold serial dilutions of yeast culture were spotted onto media containing the indicated genotoxic agents (*1*Δ, *hht1-hhf1*Δ; *2*Δ, *hht2-hhf2*Δ). (B) Silencing of the *hmr-a1*Δ::*URA3* reporter was monitored by spotting 5-fold serial dilutions of yeast culture onto medium containing 5-fluoroorotic acid (+FOA), a counterselection for *URA3* expression, and onto medium lacking uracil (−URA), a selection for *URA3* expression. (C) RT-qPCR analysis of *hmr-a1*Δ::*URA3*, *HML*α, and *MAT***a**. Transcript values were normalized to *ACT1* mRNA and adjusted to the *sir3*Δ value, which was normalized to 100. Transcription was significantly reduced in *rtt106*Δ *cac1*Δ *hht2-hhf2*Δ cells compared to that in *rtt106*Δ *cac1*Δ cells (*, $P < 0.05$; **, $P < 0.01$).

lighted differences between the two copies of H3-H4. Direct mRNA measurements revealed that *rtt106*Δ *cac1*Δ *hht2-hhf2*Δ cells partially suppressed the *rtt106*Δ *cac1*Δ silencing defects at both the *hmr-a1*Δ::*URA3* reporter and endogenous *HML*α (Fig. 5C). In contrast, transcription at the *MAT***a** locus in *rtt106*Δ *cac1*Δ *hht2-hhf2*Δ cells was similar to that in the wild type (Fig. 5C). Therefore, the enhanced silencing at *hmr-a1*Δ::*URA3* and *HML*α in *rtt106*Δ *cac1*Δ *hht2-hhf2*Δ cells compared to that in *rtt106*Δ *cac1*Δ cells was not due to a general transcription defect. Together, these results suggested a partial overlap of Rtt106's roles in regulating histone gene transcription and maintaining repression at the silent mating-type loci.

DISCUSSION

In this study, we evaluated the composition and function of protein complexes at the negative regulatory regions of histone gene pairs. Our data suggested that in wild-type cells, the HIR complex at the center of the bidirectional promoters recruited Asf1, which in turn recruited Rtt106 via a histone protein bridge (Fig. 6A, left). Rtt106 directly recruits either the RSC or SWI/SNF chromatin remodeling complex to facilitate transcriptional repression or activation, respectively (8, 15, 42). We observed a direct relationship between the level of Rtt106:H3 binding and Rtt106 enrichment at histone promoters and an inverse relationship between Rtt106 enrichment and transcriptional output. *rtt106* mutants that reduced Rtt106:H3 binding had reduced Rtt106 enrichment at histone promoters and increased transcription (Fig. 6A, center). Conversely, *yta7*Δ mutants exhibited increased Rtt106:H3 binding, increased Rtt106 enrichment at histone promoters, and reduced transcription (Fig. 6A, right). The relationship between Rtt106's physical interaction with histone proteins and the level of histone gene transcription suggested that Rtt106 acted as a sensor to facilitate a negative-feedback loop to regulate histone levels.

The regulatory complex of histone gene pairs: a sandwich of histone proteins and chaperones. Previous work established that

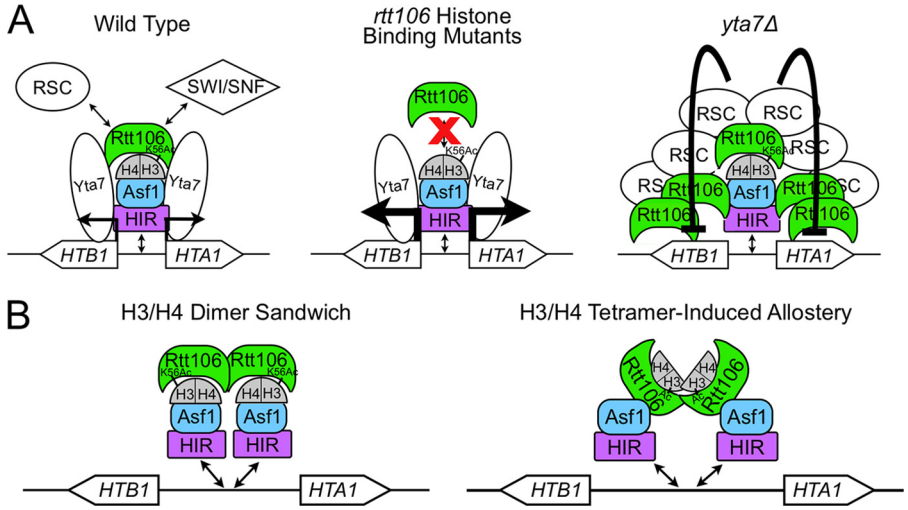

FIG 6 Model for negative-feedback regulation of histone gene transcription. (A) In wild-type cells (left), HIR, Asf1, and Rtt106 histone chaperones along with Yta7 localize to histone gene regulatory regions. Rtt106 directly recruits RSC or SWI/SNF chromatin remodeling enzymes to facilitate S-phase-specific histone gene transcription. *rtt106* mutants that can no longer bind histone H3 (center) have reduced Rtt106 enrichment at histone regulatory regions and increased histone transcription. In *yta7*Δ cells (right), Rtt106:H3 binding was increased, leading to elevated Rtt106 enrichment throughout histone gene loci and reduced transcription. (B) Two models for the histone-mediated interaction between Rtt106 and Asf1.

the HIR complex localizes and recruits Rtt106 to a negative regulatory element in the promoters of three of the four histone gene pairs (16, 44, 45, 59). We found that Asf1, another H3/H4 chaperone previously shown to interact with both the HIR complex and Rtt106 (21, 32), also localized to the promoter regions of all HIR-regulated histone gene pairs (Fig. 2A and B). Recruitment of Asf1 was HIR dependent and Rtt106 independent (Fig. 2B). In contrast, the recruitment of Rtt106 was both Asf1 dependent and HIR dependent (Fig. 1B) (16, 51). These results suggested that a HIR:Asf1:Rtt106 regulatory complex formed at histone promoters to maintain S-phase-specific expression.

The involvement of three different H3/H4 chaperones at histone gene regulatory regions implied that histone proteins themselves might be critical components of the complexes. Indeed, we found that mutations in *RTT106* that reduced the affinity of Rtt106 for histone H3 reduced the enrichment of Rtt106 at histone promoters (Fig. 1A and B) and led to increased histone expression (Fig. 1D). Because HIR and Asf1 enrichment at these control regions was Rtt106 independent (Fig. 1C and Fig. 2B) (16), these data implied that a histone protein bridge mediated the interaction between Asf1 and Rtt106. The histone dependence of this chaperone-chaperone interaction was unique; Rtt106:CAF-1, Asf1:CAF-1, and Asf1:HIR interactions are direct and independent of chaperone-histone binding (21, 25, 57). This additional layer of complexity suggested a new mechanism for chaperone-mediated interactions. Previous studies established that Asf1 fails to co-IP with Rtt106 in *hir1*Δ cells (32). Therefore, the Asf1:H3/H4:Rtt106 interaction likely occurs at histone gene promoters. At this point, we have not resolved whether the HIR complex dependence of this interaction is due to sequential recruitment of Asf1 followed by Rtt106 or whether the HIR complex stabilizes the Asf1:H3/H4:Rtt106 interaction, possibly by making direct contacts with Rtt106.

Since Asf1:H3 binding occludes the tetramerization surface of H3/H4 (2, 11, 41), the proposed Rtt106:H3/H4:Asf1 interaction must utilize H3/H4 dimers. Although Rtt106 was recently shown to bind H3/H4 tetramers *in vivo*, the possibility of Rtt106 binding H3/H4 dimers has not been excluded (14). Additionally, Rtt106 itself was recently shown to function as a dimer (52). Together, these results suggest that the regulatory complex at histone gene promoters contains one Rtt106 dimer bound to two individual H3/H4 dimers each bound by Asf1 (Fig. 6B, left). Asf1's direct interaction with each H3/H4 dimer and the HIR complex would block H3/H4 tetramerization and promote Rtt106 recruitment to histone promoters, respectively. Alternatively, Rtt106 binding to H3/H4 tetramers might induce a conformational change in Rtt106 required for a direct Rtt106:Asf1 interaction (Fig. 6B, right).

On the spreading of, and repression by, Rtt106. One of the most surprising findings in this study was the lack of a consistent mechanistic link between Rtt106 spreading from the regulatory regions into the coding regions of histone gene pairs and the repression of those genes. In wild-type cells, Yta7 restricts Rtt106 localization to the center of the regulatory region (16, 31), perhaps analogously to the way in which Yta7 acts as a boundary to the spreading of silenced chromatin (20, 28, 54). Our data at the *HTA1-HTB1* gene pair confirmed earlier results: in the absence of Yta7, increased Rtt106 enrichment within the *HTA1-HTB1* locus led to reduced *HTA1* transcription (Fig. 2E and 4A) (16). However, this paradigm was not shared across all histone gene loci. Rtt106 enrichment in *yta7*Δ cells was less extensive within the *HTB1* ORF than within the *HTA1* ORF (Fig. 2E). This asymmetric localization was consistent with previous findings that Rtt106 has no effect on *HTB1* transcription in otherwise wild-type cells (16). Additionally, we found even more extensive spreading of Rtt106 from the regulatory region of *HHT1-HHF1* into the coding sequences in *yta7*Δ cells, yet *HHT1* and *HHF1* expression in *yta7*Δ was similar to that in wild-type cells (Fig. 2E and 4A). Therefore, repression was not an obligate consequence of increased Rtt106 localization. Additional factors, such as SWI/SNF versus RSC recruitment, might be the source of these transcriptional differences. These results highlight the importance of investigating

transcriptional effects at all histone gene pairs, unlike many studies that focused only on the *HTA1* locus.

The potential for histone chaperones spreading along chromatin represents a new mechanism for transcriptional regulation. In the case of Rtt106, its binding preference for H3K56ac over unmodified H3 suggests that spreading may be dependent on the presence of H3K56ac, which is an S-phase-specific modification (40). Indeed, the spreading of Rtt106 in *yta7*Δ mutants was largely suppressed in *yta7*Δ *rtt109*Δ double mutants, which lack the H3K56 acetyltransferase (Fig. 3D). Curiously, in wild-type cells Rtt106 enrichment at histone gene promoters is consistent throughout the cell cycle, whereas Yta7 enrichment is S-phase specific (16, 31). Therefore, either Rtt106 spreading is also S-phase specific or Rtt106 spreading outside S phase is restricted by Yta7-independent factors. These possibilities will be distinguished by monitoring Rtt106 enrichment in synchronized *yta7*Δ cells. Recent studies suggest that Rtt106 might form higher-order oligomeric structures *in vitro* (14, 52), which could also facilitate the increased enrichment in the absence of Yta7. Future studies will address whether, as in *yta7*Δ cells, artificial overexpression of histone proteins leads to increased Rtt106:H3 binding, increased Rtt106 localization at endogenous histone gene regulatory regions, and reduced histone gene transcription.

The altered Rtt106 localization that we observed in *yta7*Δ cells was consistent with results of previous studies that proposed that Rtt106 spreads into histone ORFs (16). However, the increased Rtt106 ChIP signal in *yta7*Δ cells might reflect a change in the dynamics of Rtt106's association with the promoter rather than an increase in the total amount of Rtt106 protein bound to each locus. Additionally, since the histone genes are small (~400 bp), the limitations of conventional ChIP make rigorous assessment of changes in Rtt106 localization below ~500 bp difficult. Future studies will address whether the altered Rtt106 localization represents true spreading or a distinct mechanism of recruitment that is antagonized by Yta7.

In *yta7*Δ cells, the enhanced Rtt106:H3 binding may have resulted from the increased levels of chromatin-bound H3 (35). Although Rtt106:H3 binding is H3K56ac dependent (33) and the ratio of H3K56ac to H3 was unchanged in *yta7*Δ cells (Fig. 3A), a contribution by other posttranslational modifications to Rtt106:H3 binding remains possible. Intriguingly, a cocrystal structure of Rtt106 and acetylhistamine revealed two acetylhistamine-binding sites, one on each PH domain of Rtt106 (52) (Protein Data Bank [PDB] identification [ID] code 3TW1). If these two acetylhistamine binding sites reveal binding sites also capable of binding acetyllysine, then Rtt106:H3 binding might be influenced by a second acetyl modification elevated in *yta7*Δ cells.

Role of H3K56ac in histone gene transcription. Currently, the role of H3K56ac in the regulation of histone gene transcription is paradoxical. In wild-type cells, H3K56ac is highly enriched at histone gene promoters and is thought to induce histone turnover and active transcription (29). Cells lacking the histone gene activators Spt10 or Rtt109 have reduced H3K56ac within the promoter and are transcriptionally repressed (16, 29, 58). Therefore, H3K56ac appears to be necessary for histone gene activation. However, *asf1*Δ cells, which lack H3K56ac (Fig. 1A) (1), have elevated histone gene transcription compared to that of wild-type cells (16, 53), suggesting that H3K56 acetylation is not required for high-level expression. Unlike *rtt109*Δ cells in which Rtt106 enrichment at the *HTA1-HTB1* promoter is partially reduced, Rtt106 is reduced to background levels in *asf1*Δ cells due to Asf1's direct role in Rtt106 recruitment (Fig. 1B). Therefore, H3K56ac may only be necessary for transcription in the presence of histone chaperones. Additionally, in *hir1*Δ and *rtt106*Δ cells, histone promoters are depleted of histone protein and transcription is elevated compared to that of the wild type (16, 45), further suggesting that H3K56ac is not necessary for active transcription. These results suggest that H3K56ac is necessary to overcome the histone chaperone-mediated repression at histone promoters, perhaps by promoting nucleosome turnover to expose the DNA to activators. Monitoring chaperone-dependent histone incorporation within the histone gene regulatory regions throughout the cell cycle will further define the relationship between chaperone-mediated nucleosome assembly and histone gene transcription.

In addition to acting locally at the histone gene regulatory regions, H3K56ac might antagonize chaperone-mediated repression at a distance by titrating chaperones away from histone promoters and into other areas of the genome. Consistent with this hypothesis, although Rtt106:H3 binding was increased in *hst3*Δ *hst4*Δ cells (33), which have elevated levels of H3K56ac, Rtt106 enrichment was partially reduced at histone gene promoters (Fig. 3F). According to a simple negative-feedback model, increased Rtt106:H3 binding should lead to increased Rtt106 localization and reduced histone gene transcription. However, nearly 100% of H3 proteins are acetylated at H3K56 in *hst3*Δ *hst4*Δ cells (4), which could titrate Rtt106 away from the histone gene loci and into other areas of the genome. Additionally, excess H3K56ac in this double mutant leads to elevated DNA damage, which might titrate chaperones to sites of repair (4, 38, 46). Thus, the mechanism of regulation appeared to involve a fine-tuning of affinities and concentrations among histone proteins and their chaperones.

Relationship between histone gene expression, replication, and silencing. In addition to mediating H3K56ac delivery into chromatin (33, 61), the role of Rtt106 in histone gene regulation may promote DNA replication and silencing, most evident in cells lacking CAF-1. A reduction in H3 and H4 gene dosage partially suppressed the silencing defects but not the genotoxic chemical sensitivities associated with *rtt106*Δ *cac1*Δ cells (Fig. 5). These results were consistent with findings that overexpression of the histone genes enhanced the telomeric silencing defects observed in *cac* mutants, further suggesting that in the absence of CAF-1, silencing was sensitive to changes in histone levels (30). Additionally, *rtt106*Δ *rtt109*Δ mutants partially suppress the histone transcription defects associated with each single mutant (16), and *rtt109*Δ *rtt106*Δ *cac1*Δ mutants partially suppress *rtt106*Δ *cac1*Δ silencing phenotypes (61). Together, these results suggest that Rtt106's role in histone gene regulation may at least partially account for the silencing defects associated with *rtt106*Δ.

Analyzing changes in histone gene dosage revealed a genetic interaction between the replication-independent histone chaperones *RTT106* and *HIR1* and one of the two loci encoding H3 and H4 (*HHT1-HHF1*) (Fig. 5A). This distinction between the two loci encoding H3 and H4 suggested that Rtt106 and Hir1 might perform unique functions at *HHT1-HHF1* compared to *HHT2-HHF2*. Although, we did not detect differences in the level of chaperone enrichment at these two loci in wild-type cells (Fig. 2D), differences in Rtt106:H3 binding and Rtt106 enrichment at histone gene regulatory regions in *hht1-hhf1*Δ and *hht2-hhf2*Δ cells may have contributed to the observed phenotypes. Another possibility was that in *hht1-hhf1*Δ cells, the increased transcription

of *HHT2-HHF2* was detrimental in the absence of Rtt106 or Hir1. In *rtt106*Δ and *hir1*Δ cells, the fold increase of *HHT* and *HHF* transcription is larger at the *HHT1-HHF1* locus than at *HHT2-HHF2* (16). Additionally, each H3-H4 locus produces a slightly different ratio of *HHT* to *HHF* transcripts. Because all H3 and H4 proteins are thought to exist as either an H3/H4 dimer or tetramer, altering the amount of each histone type can be detrimental to the cell. Therefore, in the absence of replication-independent histone chaperones, an altered *HHT*/*HHF* ratio might lead to increased sensitivity to DNA-damaging agents. Importantly, strains used to analyze histone point mutants historically have both endogenous copies of H3 and H4 knocked out and *HHT2-HHF2* expressed from a plasmid. These strains are effectively *hht1-hhf1*Δ, and therefore results should be interpreted with caution when analyzing replication-independent histone chaperone phenotypes.

Concluding remarks on histone chaperone-mediated negative feedback. Because all newly synthesized H3 molecules are acetylated on K56 (4), and Rtt106:H3 binding is K56ac dependent (33), our results suggest that Rtt106 may act as a sensor to regulate histone gene transcription as a function of new histone protein levels. This feedback structure positions the HIR:Asf1:H3/H4:Rtt106 interaction as a critical node in regulating histone transcription. Future analysis of the relationship between RNA polymerase II loading and histone chaperone occupancy at the histone gene loci throughout the cell cycle will deepen our understanding of this complex regulatory mechanism. Since histone gene promoters are sites of rapid histone turnover (29), monitoring the colocalization of Rtt106, Asf1, and the HIR complex at other sites in the genome with similar histone dynamics will likely identify genes encoding proteins that are critical for chromatin assembly and thus are regulated by similar feedback loops.

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