The Replication-independent Histone H3-H4 Chaperones HIR, ASF1, and RTT106 Co-operate to Maintain Promoter Fidelity

Andrea C. Silva, Xiaomeng Xu, Hyun-So Kim, Jeffrey Fillingham, Thomas Kislinger, Thomas A. Mennella, and Michael-Christopher Keogh

From the Department of Cell Biology, Albert Einstein College of Medicine, New York, New York 10461, the Department of Chemistry and Biology, Ryerson University, Toronto, Ontario ONB 2K3, Canada, the Ontario Cancer Institute, Toronto, Ontario M5G 1L7, Canada, and the Department of Biological Sciences, Delaware State University, Dover, Delaware 19901

Background: Transcription is disruptive to chromatin structure and can expose cryptic promoters.

Results: We identify those factors that might regulate cryptic transcription from within inactive and transcribed locations.

Conclusion: Nucleosome shielding prevents cryptic transcription, and replication-independent histone replacement is co-operatively mediated by three H3-H4 chaperones.

Significance: Understanding how cryptic transcription is regulated and lost histones replaced is of fundamental importance.

RNA polymerase II initiates from low complexity sequences so cells must reliably distinguish “real” from “cryptic” promoters and maintain fidelity to the former. Further, this must be performed under a range of conditions, including those found within inactive and highly transcribed regions. Here, we used genome-scale screening to identify those factors that regulate the use of a specific cryptic promoter and how this is influenced by the degree of transcription over the element. We show that promoter fidelity is most reliant on histone gene transactivators (Spt10, Spt21) and H3-H4 chaperones (Asf1, HIR complex) from the replication-independent deposition pathway. Mutations of Rtt106 that abrogate its interactions with H3-H4 or dsDNA permit extensive cryptic transcription comparable with replication-independent deposition factor deletions. We propose that nucleosome shielding is the primary means to maintain promoter fidelity, and histone replacement is most efficiently mediated in yeast cells by a HIR/Asf1/H3-H4/Rtt106 pathway.

The first step in the production of a functional mRNA requires RNA polymerase II to identify and initiate from the appropriate location. To this end, basal promoter elements recruit transcription initiation factors to position and orient the polymerase (for review, see Ref. 1). These elements are composed of short, low complexity sequences (e.g. TATA) that appear frequently by chance, so the cell must distinguish the genuine from the imitation. A major distinction is relative accessibility: DNA is wrapped with histones into nucleosomes, and “real” (i.e. canonical) promoters are typically located in nucleosome-depleted regions. Furthermore, nucleosomes inhibit transcription initiation in vitro, and regulated nucleosome positioning is the primary means of controlling numerous promoters (e.g. PHO5, GAL1–10, CLN2) (for review, see Ref. 2). Nucleosome-depleted regions can be generated by various means, including the intrinsic resistance of certain sequences to nucleosome formation or the targeted removal of histones by various enzymatic activities (2–4).

Chromatin is highly dynamic, particularly in actively transcribed regions (5, 6). This could expose promoter-like elements (i.e. “cryptic” promoters), so eukaryotes have evolved mechanisms to maintain a repressive environment where appropriate. Such regulators have typically been identified by mutant alleles that allow RNA polymerase II to initiate at an increased frequency from within active genes (7–10). In this manner, intergenic cryptic transcription (cryptic-Tx+6) is observed in mutants of various histone chaperones, histone modifiers, and transcription initiation/elongation factors. However, the relative contribution of each factor to the maintenance of promoter fidelity within transcriptionally inactive and active regions is unclear.

Here, we asked whether the degree of co-transcriptional disruption influenced the use of a specific cryptic promoter (FLO8TATA+1626) in mutant backgrounds covering ~95% of budding yeast (Saccharomyces cerevisiae) genes. We show that most mutants that support cryptic transcription from FLO8TATA+1626 only do so in the context of extensive co-transcriptional disruption. The strongest/most penetrant phenotype is observed on deletion of histone gene transactivators (SPT10, SPT21) and H3-H4 chaperones (HIR complex and ASF1) from the replication-independent deposition (RID) pathway. Mutations of the H3-H4 chaperone Rtt106 that abro-
RID Histone Chaperones Maintain Promoter Fidelity

**EXPERIMENTAL PROCEDURES**

**Materials**—Antibodies, strains, plasmids, and ChIP oligonucleotides are listed in supplemental Tables 1–4.

**ChIP/Quantitative PCR**—ChIP samples were prepared as described previously (11, 12). Quantitative PCR data were obtained with an iCycler (Bio-Rad), SYBR Green (Molecular Probes), platinum Taq (Invitrogen), and the primer sets in supplemental Table 4. Quantitations were as described previously (13), with all enrichments expressed relative to a subtelomeric gene-free region (9716–9823) from chromosome V (11, 12).

**Cryptic Transcription Screening by Synthetic Genetic Array Technology**—Each cryptic-transcription reporter (e.g. Fig. 1A) and mutant alleles of asf1 or rtt106 (supplemental Table 3) were PCR megaprimering (see below) and homologous recombination in a Synthetic Genetic Array Technology-compatible strain (KFY1309; see supplemental Table 2). HIS3 was from yeast genomic DNA; each promoter was from plasmids (14).

Each reporter strain was crossed to two mutant libraries: /H11011 4800 nonessential genes deleted by KanMX (15) or 842 essential gene hypomorphs with KanMX disrupting their polyadenylation site (16). Libraries were arrayed in duplicate at 1536 colonies per Singer RoToR 12.5/8.5-cm plate and replica-plated in duplicate, giving four independent colonies for each measurement. At the final screen step each NatR/KanR haploid was pinned onto media lacking histidine to identify those expressing HIS3 (HIS+/H11001; cryptic-Tx+/H11001), with each plate scored daily for up to 7 days to determine the strength of the phenotype. Factors were considered potential regulators of promoter fidelity if we observed ≥3 of 4 HIS+ colonies of similar size from at least two reporters in the mutant background.

**PCR Megaprimering**—Cryptic transcription reporters (e.g. Fig. 1A) and mutant alleles of asf1 or rtt106 (supplemental Table 3) gate its interaction with either histones or dsDNA permit extensive cryptic transcription comparable with RID gene deletions. We propose that nucleosome shielding is the primary means to maintain promoter fidelity, and histone replacement is most efficiently mediated in yeast cells by a (HIR/Asf1/H3-H4/Rtt106)-containing pathway.

**FIGURE 1. Screening to identify cryptic-Tx\(^+\) mutants and relate the phenotype to the degree of co-transcriptional disruption.** A, schematic shows each cryptic-Tx\(^+\) reporter (YFG\(_{pr}\).HA3-FLO8::HIS3). HIS3 is out of frame with the FLO8\(_{pr}\), ATG such that transcription must initiate from an internal TATA (FLO8\(_{1626}\)) to allow growth in the absence of histidine (8). B, relative output of ~80% of the yeast RNA polymerase II transcriptome (5065 of ~6000 genes) is shown (19). For this study the promoter upstream of FLO8::HIS3 was retained (FLO8\(_{pr}\)) or replaced by one of seven alternates. C, degree of cryptic transcription is strongly influenced by the strength of the upstream promoter. Reporter arrays were pinned onto synthetic complete SC medium lacking histidine (GAL1-S\(_{pr}\), with 2%/1% galactose/raffinose; TDH3\(_{pr}\)/TEF1\(_{pr}\), with 2% glucose), incubated at 30 °C, and photographed on days indicated. Panels of the indicated mutants (pinned in duplicate) are size-standardized to facilitate cross-comparison. D, mutants in most transcription-related factors that support cryptic-Tx\(^+\) primarily do so when the cryptic promoter is located in a highly transcribed region. Factors are grouped by complex or pathway. The transcriptional strength of each promoter (e.g. TDH3\(_{pr}\)) upstream of the cryptic promoter is arranged by decreasing output (left to right). The color within each box indicates when HIS+ colonies were visible above the relevant plate background (days 1–7 after pinning on media lacking histidine). This measures the strength of cryptic-Tx\(^+\) in each case. A indicates a complete gene deletion; D, a DAmP allele; *, as for set2\(_\Delta\) and yjl169w\(_\Delta\), the deletion of adjacent overlapping genes. BTFs, basal transcription factors; CompAsS, complex associated with Set1; CTDK, C-terminal domain kinase.
were created by two-step megaprimer (11). First-round reactions used 5'-flanking and 3'-mutagenic (or overlapping homology) primers, template (plasmid or S. cerevisiae genomic DNA), and Phusion polymerase (Finzymes). Purified first-round products were used as megaprimer in the second-round reaction with a suitable 3'-primer, template, and Phusion polymerase. Second-round products were transformed into yeast for integration by homologous recombination or cloned to plasmid vectors as required.

**TCA Cell Extracts for Western Blotting**—Whole cell extracts were isolated by the trichloroacetic acid (TCA) method to extract chromatin and preserve labile modifications efficiently (17, 18), with supernatants analyzed by immunoblotting.

**RESULTS**

**Most Cryptic-Tx⁺ Mutants Compromise Promoter Fidelity Only in Highly Transcribed Regions**—The disruption of chromatin structure that accompanies transcription might be expected to influence cryptic promoter usage. To examine this, we placed HIS3 under the control of a cryptic promoter within FLO8_TATA-T-1626 which is unused in normal conditions (7, 8). To vary transcription through this region the endogenous upstream promoter (FLO8₃₅) was then deleted (Δ₃₅) or replaced with that from a selection of constitutively expressed or inducible genes (Fig. 1A). Together, this series of nine reporters (supplemental Table 2A) is expected to cover a >150-fold range in constitutive and >10-fold range in galactose-dependent transcriptional output (19) (Fig. 1B). By studying the same cryptic promoter in the context of varying transcriptional outputs we could directly determine the impact of co-transcriptional disruption without any confounding variables: the alternate approach of a range of cryptic promoters in various locations would add the potential complication of a different sequence context/co-activator dependence for each (20).

Synthetic Genetic Array Technology (21) was used to place all nine reporters in the context of ~5600 S. cerevisiae mutant backgrounds (>95% genome coverage by complete deletions or DAmP hypomorphs; see "Experimental Procedures"). In total, 12 transcription outputs were tested: one promoterless, five constitutive, and three conditional on activating (galactose) or repressive (glucose) conditions. In each case, haploid colonies were plated in quadruplicate onto media lacking histidine to identify those expressing HIS3, with growth scored daily for up to 7 days to determine the strength of the cryptic-Tx⁺ phenotype (see "Experimental Procedures" and Fig. 1C). Factors were considered positive if we observed ≥3 of 4 HIS⁺ colonies of similar size from at least two reporters in the mutant background.

Screening by the above criteria identified >100 candidate regulators of promoter fidelity, including the vast majority of factors previously annotated to the cryptic-Tx⁺ phenotype (8, 10). We then used genomic/proteomic resources to assign each factor to specific complexes or pathways. The largest group of cryptic-Tx⁺ mutants included factors related to transcriptional regulation (Fig. 1D). This includes deletions of each member of the Ctk1/2/3 kinase → Set2 methyltransferase → Eaf3/Rco1/Rpd3 deacetylase pathway. The trimethylation of H3-Lys36 by Set2 targets the Rpd3S complex to gene coding regions to deacetylate histones H3 and H4 (9, 22, 23). This is presumed to compact chromatin and thus suppress cryptic promoter usage. The observation of cryptic-Tx⁺ in multiple members of the same complex or pathway increases confidence, even when the phenotype is consistently weak. This is the case with the PAF, Rad6, and Set1 complexes (24, 25) (Fig. 1D). PAF and the ubiquitylation of H2B-K123 by Rad6-Bre1-Lge1 are required for the efficient trimethylation of H3-K4 by Set1 (26, 27). Thus, an appropriate level of H3-K4me3 may be required to maintain promoter fidelity (28, 29). Alternatively, the cryptic-Tx⁺ in PAF mutants may be linked to its role with the Bur1-Bur2 cyclin-dependent kinase complex in regulating H3-K36me3 or H3-H4 acetylation levels (24). We also observed cryptic-Tx⁺ in numerous mutants of the proteasome (20 S core and 19 S regulatory subunits; supplemental Fig. 1A), which may be related to various nonproteolytic roles of this complex in transcription initiation and elongation (30). The cryptic-Tx⁺ in mutants of the mismatch repair machinery (MutS and MutL, supplemental Fig. 1B) suggests a novel role for mismatch repair factors in promoting promoter fidelity, with the mechanism currently unknown.

In most of the above mutants cryptic-Tx⁺ was primarily observed when the FLO8::HIS3 reporter is located within a highly transcribed location (i.e. manifesting the phenotype requires extensive co-transcriptional disruption; Fig. 1D). This “transcription threshold” is particularly dramatic in cells deleted for SPT2, encoding a DNA-binding protein that helps to refold nucleosomes with “old” histones in the wake of RNA polymerase II (31, 32) (Fig. 1D and supplemental Fig. 2). Our results indicate that any role for the factor in preventing intergenic cryptic-transcription is restricted to highly transcribed regions. This could be because Spt2 only functions in these locations or because it is dispensable outside of them.

**spt10Δ Cells Support Cryptic-Tx⁺ Independent of Co-transcriptional Disruption**—Of the many cryptic-Tx⁺ mutants in Fig. 1D, three stood out by the vigor of their phenotype: spt21Δ, asf1Δ, and rtt106Δ. Spt21 promotes the transcription of numerous histone loci (33), whereas Asf1 and Rtt106 are H3-H4 chaperones (34–36). Because perturbing histone metabolism would be expected to have detrimental effects on nucleosome maintenance and promoter fidelity, we focused our investigations on factors that regulate this process.

The major core histones of budding yeast are encoded by duplicated genes, with each H2A-H2B and H3-H4 pair arranged in a head-to-head orientation (Fig. 2A). On individual deletion of the three nonessential loci (37), only hht1Δ-hhf1Δ supported cryptic transcription (Fig. 2B). Many histone genes increase output to complement a deletion of their sisters (38), compromising this approach to investigate any relationship between histone dosage and promoter fidelity. However, deletion of the Spt21 or Spt10 transactivators reduces the expression of each core histone (33, 39), which likely explains the strong and pervasive cryptic-Tx⁺ in spt21Δ cells (Fig. 1D). Because the severe slow growth of spt10Δ compromised high throughput screening we created these strains to assay any cryptic transcription directly.

Immunoblotting confirmed that the abundance of each histone (or modified species) was reduced in spt10Δ cells (Fig. 2, C
and D). On assaying cryptic transcription from a range of reporters we found that the phenotype was independent of the strength of the upstream promoter (Fig. 2E: compare TDH3pr and HHT1pr). This suggested that spt10/H9004 cells may not produce enough histones to package even nontranscribed (i.e. relatively stable) regions into nucleosomes. To address this we transformed each spt10/H9004 reporter strain with a high copy plasmid encoding all four core histones and observed that this complements their slow growth (39) and cryptic-Tx+ (Fig. 2F). This rescue required the replenishment of all four histones because it was not achieved with high copy plasmids containing only H2A-H2B or H3-H4 (Fig. 2F).

Rtt106 Histone Chaperone Is a Primary Regulator of Promoter Fidelity—Histone chaperones are central players in histone metabolism. These escort specific histone pairs to prevent nonspecific interactions, control supply, and in some cases directly mediate incorporation into chromatin (for review, see Refs. 40, 41). Recent studies identify Rtt106 as an H3-H4 chaperone that regulates chromatin structure in heterochromatic and transcribed regions (34, 35, 42, 43). Of particular interest, the chaperone is required for efficient histone replacement during active transcription (35), likely explaining the cryptic-Tx+ in rtt106Δ cells. To investigate further how this might be mediated we examined the domain structure and interaction partners of the chaperone.
Rtt106 contains two N-terminal Plekstrin Homology (PH) domains and an acidic C terminus (Fig. 3A). Each region could regulate an association with histones; yeast Pob3 and mammalian SSRP1 (components of the FACT chaperone complex) also regulate an association with histones; yeast Pob3 and mammalian SSRP1 (components of the FACT chaperone complex) also contain a PH domain, and many histone chaperones contain acidic regions that may interact with the highly basic histones (40, 41). We thus created a series of Rtt106 regional deletions, including alleles lacking the first or second PH domains (PH1Δ or PH2Δ), acidic patch (APΔ), or acidic C terminus (CΔ) (Fig. 3, A and B). Strikingly, both PH domains of Rtt106 are required to suppress cryptic transcription. Spots are 10-fold dilutions onto SC media containing 2% galactose and 1% raffinose (Fig. 3C). Rtt106 function depends on its ability to bind dsDNA and H3-H4. WT is a C-terminal HA3 tag of genomic Rtt106. Indicated alleles were C-terminally tagged with HA3 in a low copy plasmid vector (i.e. pRS315). AP, acidic patch; C-term, C terminus; FL, full-length; MUT, point-mutated; PH, Plekstrin homology. Single domain deletions have no impact on Rtt106 protein stability. In contrast, rtt106-APΔ/CΔ (deleted of the acidic patch and acidic C terminus) is hypomorphic. Each rtt106-HA3 was determined by immunoblotting with anti-HA. Rpn10 is a loading control. CΔ, both PH domains of Rtt106 are required to suppress cryptic transcription. Spots are 10-fold dilutions onto SC media containing 2% galactose and 1% raffinose (Fig. 3E). This suggested that Rtt106 must interact with DNA to deliver H3-H4. This is most likely to be necessary within transcribed regions (35), although we have been unable to demonstrate Rtt106 enrichment by ChIP at a number of active genes (data not shown). We thus tested the ability of each rtt106 allele from above to bind the HTA1-HTB1 locus, where the chaperone is required for efficient chromatin assembly (44). WT Rtt106 and alleles deleted of the acidic patch (APΔ or CΔ) were highly enriched at the appropriate region (primer C, Fig. 3F). In contrast, each allele that supports cryptic transcription, including those point mutants that are selectively unable to bind either DNA or H3-H4, failed ChIP at this location.

Rtt106 Maintains Promoter Fidelity in Co-operation with HIR and Asf1—Many H3-H4 chaperones can be categorized by whether they act to replace histones during or outside DNA replication. In this manner replication-dependent deposition (RDD) factors (e.g. CAF complex) are recruited to the replication fork to fill in the gaps as nucleosomes divide between mother and daughter strands. RID factors (e.g. HIR complex) replace histones lost at other times, such as during transcription.
The same N-terminal region of Asf1 contacts both HIR and CAF (50, 51), and mutation of the putative interaction surface (asf1-HD36-37AA or asf1-D37R,E39R) impairs co-operative histone deposition by Asf1 and HIR in vitro (52). However, these alleles (Fig. 5A) likely retain the HIR-Asf1 interaction in vivo because they (in contrast to hir1Δ or asf1Δ) supported the efficient recruitment of Rtt106 to HTA1-HTB1 (Fig. 5B) and had no impact on promoter fidelity (Fig. 5C).

The opposite face of Asf1 binds the C-terminal helix of histone H3 from a H3-H4 dimer, and mutation of this region (e.g. asf1-V94R) largely phenocopies asf1Δ (53, 54). The asf1-V94R allele also abrogates the co-purification of Asf1 and Rtt106, suggesting that H3-H4 bridges the association between these chaperones (47). In this manner the recruitment of Rtt106 to HTA1-HTB1 was abolished in asf1-V94R and asf1Δ cells (Fig. 5B), both of which also supported cryptic transcription (Fig. 5C).

Previous studies have focused on HIR/Asf1 and Rtt106 as distinct pathways to mediate histone replacement within actively transcribed regions (35, 41). However, our findings suggest that a single grouping of HIR/Asf1/H3-H4/Rtt106 (as in Fig. 5D) may operate to deliver replacement histones and maintain promoter fidelity. We thus measured the cryptic transcription in hir1Δ/rtt106Δ relative to hir1Δ or rtt106Δ cells, predicting that if these chaperones operate in parallel pathways the phenotype would be enhanced. However, this was not observed (Fig. 5E), with such epistasis supporting a model where the chaperones operate in a single pathway.

For our final analysis we noted that cells individually deleted of HIR, ASF1, or RTT106 maintain a level of promoter fidelity greater than in spt10Δ (compare Figs. 1C and 2E). Thus, replacement histones are delivered/deposited in cells containing mutants of each RID chaperone, just not as efficiently as required. On further testing, histone overexpression partially complemented the cryptic-Tx+ in hir1Δ in rtt106Δ cells, suggesting that if these chaperones operate in parallel pathways the phenotype would be enhanced. However, this was not observed (Fig. 5E), with such epistasis supporting a model where the chaperones operate in a single pathway.

**DISCUSSION**

**Nucleosome Shielding Is Essential to the Maintenance of High Promoter Fidelity**—It is widely assumed that cryptic promoters are repressed by nucleosome shielding, but how this might be maintained in a transcriptionally active relative to an inactive region has never been fully investigated. Here, we show that the ability to repress a cryptic promoter is dramatically compromised if various aspects of histone metabolism are perturbed. This includes deletions of transactivating factors that regulate histone gene expression (SPT21 or SPT10), or chaperones that supply H3-H4 to the RID machinery (HIR, ASF1, and RTT106). In the most extreme case (spt10Δ), we observe equivalent cryptic-Tx+ from within non- and highly transcribed regions (Fig. 2B), likely because of genome-wide defects in nucleosome packaging (39). However, in individual mutants of each RID chaperone the degree of cryptic transcription is proportional to the strength of the upstream promoter (e.g. Fig. 1C). This suggests that the rate of histone replacement always lags turnover in RID mutant backgrounds.

**FIGURE 4.** Mutations in replication-independent histone chaperones show extensive cryptic-Tx+ . Schematic depicts each factor relative to Asf1, a H3-H4 donor to CAF (RDD pathway), HIR (RID pathway), and various acetyltransferases (e.g. SAG, Rtt109, Hat1). The cryptic-Tx+ in each mutant from this network is depicted (scored as in Fig. 1D). The similarity of the phenotype in deletions of *ASF1*, *HIR*, and *RTT106* strongly suggests a common functional relationship. Δ, complete gene deletion; D, DAmP allele; *, as for *mrc1*.

**FIGURE 5.** Schematic depicts each factor relative to Asf1, a H3-H4 donor to CAF (RDD pathway), HIR (RID pathway), and various acetyltransferases (e.g. SAG, Rtt109, Hat1). The cryptic-Tx+ in each mutant from this network is depicted (scored as in Fig. 1D). The similarity of the phenotype in deletions of *ASF1*, *HIR*, and *RTT106* strongly suggests a common functional relationship. Δ, complete gene deletion; D, DAmP allele; *, as for *mrc1*.
Given that nucleosome shielding appears so important to promoter fidelity, the extensive chromatin remodeling that accompanies active transcription (5, 6) would be expected to allow the transient exposure of cryptic promoters, even in wild-type cells. This likely explains the cryptic transcription “background” in cells carrying the highly transcribed TDH3Pr::FLO8::URA3 reporter (supplemental Fig. 2; note the poor growth on 5-fluoroorotic acid). This also suggests an interesting possibility: a cryptic promoter located within a regulatable gene (e.g., inducible or developmental) could derive a noncoding RNA under the same conditions that drive the coding RNA. This would represent an additional, and underappreciated, complexity in the cellular response.

**Many Predeposition Histone Modifications Appear Dispensable for Maintenance of High Promoter Fidelity**—The extensive cryptic-Tx+ in RID mutants indicates that replacement histones are important, but how about the modifications thereon? Asf1 delivers newly synthesized H3-H4 to various acetyltransferases for modification (as in Fig. 4) and then passes these histones to CAF or HIR for deposition. The extensive cryptic-Tx+ of asf1/H9004 is not shared with mutants of any associated acetyltransferases (Fig. 4), suggesting that whereas high promoter fidelity requires replacement histones, their predeposition modification status is either redundant or largely unimportant.

Of particular note in this regard Asf1 is required for the efficient acetylation of H3-K56 by the Rtt109 acetyltransferase (53).

**FIGURE 5. Mutants in replication-independent histone chaperones show extensive cryptic-Tx+**. A, indicated point mutants have no impact on asf1 protein stability. The tested alleles selectively abrogate binding to either HIR (HD36 –37AA or D37R, E39R) or H3-H4 (D34A or V94R) (50, 51, 53). Alleles were C-terminally tagged with HA3 in a low copy plasmid vector (i.e., pRS315-asf1.HA3) and expression determined by immunoblotting with anti-HA. H2B is a loading control. B, ability of Asf1 to bind H3-H4 is required to ChiR Rtt106 at HTA1-HTB1. The enrichment of Rtt106.HA3 at region C in the indicated asf1 backgrounds is expressed relative to a gene-free region (GFR) from chromosome V (as in Fig. 3). Error bars represent S.D. from at least three replicate quantitative PCRs. U, untagged; HD, HD36 –37AA; DE, D37R, E39R. C, ability of Asf1 to bind H3-H4 is required to maintain promoter fidelity. Spots are 10-fold dilutions onto SC media (2% glucose ± uracil) followed by incubation at 30 °C for days indicated. D, model depicts specific interactions required to recruit HIR, Asf1, and Rtt106 to HTA1-HTB1. HIR complex binds a specific DNA element (the NEG box) and directly interacts with Asf1, with both factors required to recruit Rtt106 (44). Rtt106 associates with Asf1 via H3-H4 and also binds dsDNA, possibly to stabilize its recruitment. E, hir1Δ/rtt106Δ cells do not support increased cryptic transcription relative to hir1Δ or rtt106Δ alone. The transcriptional activity of TDH3Pr and ADH1Pr upstream of FLO8::HIS3 is as in Fig. 1B. Spots are 10-fold dilutions onto SC media (2% glucose ± histidine) followed by incubation at 30 °C for days indicated. F, cryptic-Tx+ of hir1Δ, rtt106Δ, or asf1/H9004 cells is rescued by histone overexpression. Strains were transformed with an empty high copy plasmid (2 μm) or same encoding all four core histones (as in Fig. 2F). Spots are 10-fold dilutions onto SC media (2% glucose ± histidine) followed by incubation at 30 °C for days indicated.
RID Histone Chaperones Maintain Promoter Fidelity

55, 56). Furthermore, H3-K56ac increases the binding affinity of CAF-1 and Rtt106 for histone H3, enhancing nucleosome assembly by these chaperones in the RDD pathway (46). However, rtt109Δ cells do not support cryptic transcription (8) (Fig. 4), indicating that H3-K56ac has no significant role in the Rtt106-mediated RID pathway.

One Major or Two Overlapping but Independent RID Pathways?—The HIR, Asf1, and Rtt106 chaperones have the independent ability to assemble nucleosomes in vitro (34, 42, 52). Thus, why cells need all three factors is unclear, although co-operative synergy is an obvious possibility: H3-H4 replacement has been proposed to be most efficiently mediated by yeast HIR/Asf1/H3-H4 or mammalian HIRA/ASF1a/H3.3-H4, with the Asf1 homolog possibly delivering histone dimers to the relevant HIR/HIRA (52, 57). The recent identification of DAXX, a mammalian Rtt106-like chaperone, added another component to the mix. Most studies suggest that HIRA/ASF1a and DAXX largely operate in separate RID pathways that deliver replacement histones to distinct genomic locations (58–60). However, there are also indications that DAXX and HIRA might co-operate at certain locations (59).

One interpretation of our data is that budding yeast also contain distinct HIR/Asf1/H3-H4 and Asf1/H3-H4/Rtt106 groups. If this is indeed the case, these chaperone complexes show limited functional specialization because the individual components of each group are equally important for the maintenance of promoter fidelity within FLO8 (e.g. Fig. 4). Furthermore hir1Δ/rtt106Δ shows a level of cryptic transcription comparable with hir1Δ or rtt106Δ cells, which would tend to argue against these chaperones operating in distinct parallel but functionally overlapping pathways. We thus propose (based on their co-purification, shared genetic relationships, and limited redundancy) that HIR1/HTB1 locus HIR binds the NEG-box DNA element to recruit Asf1 (or Asf1/H3-H4). Rtt106 then associates with Asf1 via the H3-H4 dimer (47) and binds DNA to stabilize its recruitment (Fig. 5D). But how does the HIR/Asf1/H3-H4/Rtt106 interaction group associate with other locations? Does Rtt106 use its dsDNA binding activity to recognize nucleosome-free DNA within transcriptionally active regions? Our findings provide the framework to address each of these questions.

In closing, we note our identification of a HIR/Asf1/H3-H4/Rtt106 (Mug183) protein interaction network in Schizosaccharomyces pombe (supplemental Table 5). Given this conserved physical interaction, we posit that the fission yeast RID chaperones also functionally co-operate as in budding yeast. Such conservation across ~380 million years of evolutionary distance (61) would further suggest that similar pathways may exist to regulate histone replacement and promoter fidelity in higher eukaryotes.

Acknowledgments—We thank Steve Buratowski, Brian Strahl, Jon Warner, and members of the Keogh and Mennella laboratories for helpful advice and comments.

REFERENCES

1. Heintzman, N. D., and Ren, B. (2007) The gateway to transcription: identifying, characterizing and understanding promoters in the eukaryotic genome. Cell. Mol. Life Sci. 64, 386–400

2. Bai, L., and Morozov, A. V. (2010) Gene regulation by nucleosome positioning. Trends Genet. 26, 476–483

3. Segal, E., and Widom, J. (2009) What controls nucleosome positions? Trends Genet. 25, 335–343

4. Segal, E., Fondufe-Mittendorf, Y., Chen, L., Thäström, A., Field, Y., Moore, I. K., Wang, J. P., and Widom, J. (2006) A genomic code for nucleosome positioning. Nature 442, 772–778

5. Schwabish, M. A., and Struhl, K. (2004) Evidence for eviction and rapid deposition of histones upon transcriptional elongation by RNA polymerase II. Mol. Cell. Biol. 24, 10111–10117

6. Workman, J. L. (2006) Nucleosome displacement in transcription. Genes Dev. 20, 2009–2017

7. Kaplan, C. D., Laprade, L., and Winston, F. (2003) Transcription elongation factors repress transcription initiation from cryptic sites. Science 301, 1096–1099

8. Cheung, V., Chua, G., Batada, N. N., Landry, C. R., Michnick, S. W., Hughes, T. R., and Winston, F. (2008) Chromatin- and transcription-related factors repress transcription from within coding regions throughout the Saccharomyces cerevisiae genome. PLoS Biol. 6, e277

9. Carrozza, M. J., Li, B., Florens, L., Suganuma, T., Swanson, S. K., Lee, K. K., Shaw, J. W., Anderson, S., Yates, J., Washburn, M. P., and Workman, J. L. (2005) Histone H3 methylation by Set2 directs deacetylation of coding regions by Rpd3S to suppress spurious intragenic transcription. Cell 123, 581–592

10. Fiedler, D., Braberg, H., Mehta, M., Chechik, G., Cagney, G., Mukherjee, P., Silva, A. C., Shales, M., Collins, S. R., van Wageningen, S., Kenmeren, P., Holstege, F. C., Weissman, J. S., Keogh, M. C., Koller, D., Shokat, K. M., and Krogan, N. J. (2009) Functional organization of the S. cerevisiae phosphorelay network. Cell 136, 952–963

11. Keogh, M. C., Podolny, V., and Buratowski, S. (2003) Buri kinase is required for efficient transcription elongation by RNA polymerase II. Mol. Cell. Biol. 23, 7005–7018

12. Keogh, M. C., and Buratowski, S. (2004) Using chromatin immunoprecipitation to map co-transcriptional mRNA processing in Saccharomyces cerevisiae. Methods Mol. Biol. 257, 1–16

13. Paffl, M. W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, e45

14. Janke, C., Magiera, M. M., Rathfelder, N., Taxis, C., Reber, S., Maekawa, H., Moreno-Borchart, A., Doenges, G., Schwob, E., Schiebel, E., and Knop, M. (2004) A versatile toolbox for PCR-based tagging of yeast genes: new fluorescent proteins, more markers and promoter substitution cassettes. Yeast 21, 947–962

15. Winzeler, E. A., Shoemaker, D. M., Astromoff, A., Liang, H., Anderson, K., Andre, B., Bangham, R., Benito, R., Boeke, J. D., Bussey, H., Chu, A. M., Connelly, C., Davis, K., Dietrich, F., Dow, S. W., El Bakkoury, M., Foury, F., Friend, S. H., Gentenla, E., Giaever, G., Hegemann, J. H., Jones, T., Laub, M., Liao, H., Liebundguth, N., Lockhart, D. J., Luca-Daniela, A., Lussier, M., M’Rabet, N., Menard, P., Mittmann, M., Pai, C., Rebischung, C., Reveu, J. L., Riles, L., Roberts, C. J., Ross-MacDonald, P., Scherens, B., Snyder, M., Sookhai-Mahadeo, S., Storms, R. K., Véronneau, S., Voet, M., Volckaert, G., Ward, T. R., Wysocki, R., Yen, G. S., Yu, K., Zimmermann, K., Philipsen, P., Johnston, M., and Davis, R. W. (1999) Functional characterization of the S. cerevisiae genome by gene deletion and parallel analysis. Science 285, 901–906

16. Breslow, D. K., Cameron, D. M., Collins, S. R., Schuldiner, M., Stewart-Ornstein, J., Newman, H. W., Braun, S., Madhani, H. D., Krogan, N. J., and Weissman, J. S. (2008) A comprehensive strategy enabling high-resolution functional analysis of the yeast genome. Nat. Methods 5, 711–718

17. Mehta, M., Braberg, H., Wang, S., Lozsa, A., Shales, M., Solache, A., Krogan, N. J., and Keogh, M. C. (2010) Individual lysine acetylations on the N terminus of Saccharomyces cerevisiae H2A.Z are highly but not differentially regulated. J. Biol. Chem. 285, 39855–39865

18. Kim, H. S., Vanoosthuyse, V., Fillingham, J., Roguev, A., Watt, S., Kulis, J.,
T. Treyer, A. Carpenter, L. R. Bennett, C. S. Emili, A. Greenblatt, J. F., Hardwick, K. G., Krogan, N. J., Bühler, J., and Keogh, M. C. (2009) An acetylated form of histone H2A.Z regulates chromosome architecture in *Schizosaccharomyces pombe*. Nat. Struct. Mol. Biol. **16**, 1286–1293

19. Holstege, F. C., Jennings, E. G., Wyrick, J. I., Lee, T. I., Hengartner, C. J., Green, M. R., Golub, T. R., Lander, E. S., and Young, R. A. (1998) Dissecting the regulatory circuitry of a eukaryotic genome. *Cell* **95**, 717–728

20. Pattenden, S. G., Gogol, M. M., and Workman, J. L. (2010) Features of cryptic promoters and their varied reliance on bromodomain-containing factors. *PloS One* 5, e12927

21. Tong, A. H., and Boone, C. (2006) Synthetic genetic array analysis in *Saccharomyces cerevisiae*. Methods Mol. Biol. **313**, 171–192

22. Keogh, M. C., Kurdistani, S. K., Morris, S. A., Ahn, S. H., Podolny, V., Collins, S. R., Schuldiner, M., Chin, K., Punta, T., Thompson, N. J., Boone, C., Emili, A., Weissman, J. S., Hughes, T. R., Strahl, B. D., Grunstein, M., Greenblatt, J. F., Buratowski, S., and Krogan, N. J. (2005) Co-transcriptional set2 methylation of histone H3 lysine 36 recruits a repressive Rpd3 complex. *Cell* **123**, 593–605

23. Joshi, A. A., and Struhl, K. (2005) Eaf3 chromodomian interaction with methylated H3-K36 links histone deacetylation to Pol II elongation. *Mol. Cell* **20**, 971–978

24. Chu, Y., Simic, R., Warner, M. H., Arndt, K. M., and Prelich, G. (2007) Regulation of histone modification and cryptic transcription by the Bur1 and Pafl complexes. *EMBO J.* **26**, 4646–4656

25. Li, B., Jackson, J., Simon, M. D., Fleharty, B., Gogol, M., Seidel, C., Workman, J. L., Shilatifard, A. (2009) Histone H3 lysine 36 dimethylation (H3K36me2) is sufficient to recruit the Rpd3s histone deacetylase complex and to repress spurious transcription. *J. Biol. Chem.* **284**, 7970–7976

26. Krogan, N. J., Dover, J., Wood, A., Schneider, J., Heidt, J., Boateng, M. A., Clarke, D. S., Gleason, B. G., and Shilatifard, A. (2003) The Pafl complex is required for histone H3 methylation by COMPASS and Dot1p: linking transcriptional elongation to histone methylation. *Mol. Cell* **11**, 721–729

27. Hwang, W. W., Venkatasesubrahmanym, S., Iancu, V. A., Krogan, N. J., and Madhani, H. D. (2003) A conserved RING finger protein required for histone H2B monoubiquitation and cell size control. *Mol. Cell* **11**, 261–266

28. Pinskiya, M., Gourvenec, S., and Morillon, A. (2009) Histone H3 lysine 4 di- and tri-methylation deposited by cryptic transcription attenuates promoter activation. *EMBO J.* **28**, 1697–1707

29. Xie, L., Pelz, C., Wang, W., Bashar, A., Varlamova, O., Shadle, S., and Impey, S. (2011) KDM5B regulates embryonic stem cell self-renewal and represses cryptic intragenic transcription. *Science* **332**, 587–596

30. Fillingham, J., Kainth, P., Lambert, J. P., van Bakel, H., Tsui, K., Peña-Castillo, L., Nislow, C., Figyeś, D., Hughes, T. R., Greenblatt, J., and Andrews, B. J. (2009) Two-color cell array screen reveals interdependent roles for histone chaperones and a chromatin boundary regulator in histone gene repression. *Mol. Cell* **35**, 340–351

31. Mousson, F., Ochsenschlager, K. M., and Mann, C. (2007) The histone chaperone Asf1 at the crossroads of chromatin and DNA checkpoint pathways. *Chromosoma* **116**, 79–93

32. Office, C., Keogh, M. C., and Greenblatt, J. F. (2008) Chaperone complex assembly.

33. Mousson, F., Ochsenschlager, K. M., and Mann, C. (2007) The histone chaperone Asf1 at the crossroads of chromatin and DNA checkpoint pathways. *Chromosoma* **116**, 79–93

34. Office, C., Keogh, M. C., and Greenblatt, J. F. (2008) Chaperone complex assembly.
S. J., Stadler, S., Dewell, S., Law, M., Guo, X., Li, X., Wen, D., Chappier, A., DeKelver, R. C., Miller, J. C., Lee, Y. L., Boydston, E. A., Holmes, M. C., Gregory, P. D., Greally, J. M., Rafii, S., Yang, C., Scambler, P. J., Garrick, D., Gibbons, R. J., Higgs, D. R., Cristea, I. M., Urnov, F. D., Zheng, D., and Allis, C. D. (2010) Distinct factors control histone variant H3.3 localization at specific genomic regions. Cell 140, 678–691

59. Drané, P., Ouararhni, K., Depaux, A., Shuaib, M., and Hamiche, A. (2010) The death-associated protein DAXX is a novel histone chaperone involved in the replication-independent deposition of H3.3. Genes Dev. 24, 1253–1265

60. Lewis, P. W., Elsaesser, S. J., Noh, K. M., Stadler, S. C., and Allis, C. D. (2010) Daxx is an H3.3-specific histone chaperone and cooperates with ATRX in replication-independent chromatin assembly at telomeres. Proc. Natl. Acad. Sci. U.S.A. 107, 14075–14080

61. Sipiczki, M. (2000) Where does fission yeast sit on the tree of life? Genome Biol. 1, REVIEW1011

62. Osley, M. A., Gould, J., Kim, S., Kane, M. Y., and Hereford, L. (1986) Identification of sequences in a yeast histone promoter involved in periodic transcription. Cell 45, 537–544

63. Osley, M. A., and Lycan, D. (1987) Trans-acting regulatory mutations that alter transcription of Saccharomyces cerevisiae histone genes. Mol. Cell. Biol. 7, 4202–4210