The Rtt106 Histone Chaperone Is Functionally Linked to Transcription Elongation and Is Involved in the Regulation of Spurious Transcription from Cryptic Promoters in Yeast.\textsuperscript{[S]}\textsuperscript{[5]} \textsuperscript{*}

Rtt106 is a histone chaperone that has been suggested to play a role in heterochromatin-mediated silencing in \textit{Saccharomyces cerevisiae}. It interacts physically and functionally with the chromatin assembly factor-1 (CAF-1), which is associated with replication-coupled nucleosomal deposition. In this work, we have taken several approaches to study Rtt106 in greater detail and have identified a previously unknown function of Rtt106. We found genetic interactions between \textit{rtt106}\textsuperscript{A} and mutations in genes encoding transcription elongation factors, including Spt6, TFIIIS, and members of the PAF and yeast DSIF complexes. In addition, chromatin immunoprecipitation analysis indicates that Rtt106 is associated with transcribed regions of active genes. Furthermore, our results show that Rtt106 is required for the repression of transcription from a cryptic promoter within a coding region. This observation strongly suggests that Rtt106 is involved in the regulation of chromatin structure of transcribed regions. Finally, we provide evidence that Rtt106 plays a role in regulating the levels of histone H3 transcription-coupled deposition over transcribed regions. Taken together, our results indicate a direct link for Rtt106 with transcription elongation and the chromatin dynamics associated with RNA polymerase II passage.

In eukaryotic organisms, the chromatin structure has a major impact on important nuclear processes such as DNA replication or transcription. Most transcriptional regulation mechanisms involve chromatin modulation by histone-modifying enzymes or ATP-remodeling machines. However, another mechanism emerged from recent advances in chromatin studies that links nucleosomal assembly, disassembly, and histone dynamics to the control of transcription (1). At gene promoters, histone dynamics could modulate histone marks that play a critical role during activation or repression of genes (2).

As is the case for transcription initiation, chromatin structure is also a major obstacle to elongating RNA polymerase II (RNAP II)\textsuperscript{3} (3). However, cells have developed mechanisms involving different factors that deal with this challenge and allow RNAP II movement during elongation. Interestingly, these factors also play a role in chromatin refolding in the wake of transcription by promoting different mechanisms such as histone displacement, exchange, and redeposition (4).

The refolding of nucleosomes in the wake of RNAP II is of great importance to the cell because its absence has detrimental consequences. A defect in this process results in transcriptionally permissive chromatin along the transcribed regions (5). This permissive structure allows initiation of spurious transcription from cryptic sites within coding regions (5, 6). Importantly, recent observations show that this phenomenon is widespread in yeast.\textsuperscript{4} Repression of inappropriate transcription is very important, and key factors involved in this function are highly conserved and essential to cell survival. This includes histone chaperone proteins such as Spt6, Asf1, and the Facilitating Chromatin Transcription (FACT) complex (5–7).

In contrast to these factors, there is no evidence of the involvement of the replication-associated histone chaperone chromatin assembly factor-1 (CAF-1) in the repression of spurious transcription. This suggests the existence of a specific role for some histone chaperones associated with transcription. Interestingly, the newly discovered factor Rtt106 was exclusively linked to the CAF-1 function (8). Rtt106 possess a histone chaperone activity and interacts both functionally and physically with CAF-1 and histones H3/H4 (8). Moreover, Rtt106 and CAF-1 play an important role in heterochromatin silencing by controlling the spreading of the Sir proteins in yeast (9). Therefore, the new histone chaperone Rtt106 was proposed to connect the S-phase to epigenetic inheritance in yeast (8).

In this report, we describe a previously uncharacterized function of Rtt106 that is independent from its functional interaction with CAF-1. Our data demonstrate that Rtt106 is a histone chaperone involved in transcription elongation. Indeed, we found that Rtt106 interacts functionally and genetically with various elongation factors. Importantly, in the absence of Rtt106, we observe a derepression of spurious transcription from the cryptic promoter of the model gene \textit{FLO8}. This phenotype is generally associated with a defect in the adequate refolding of chromatin structure after RNAP II passage (5, 10). Therefore, both molecular evidence and genetic evidence link Rtt106 to the modulation of chromatin associated with elongation. In addition, ChIP assays clearly show that Rtt106 associ-
ates with transcribed regions of chromatin. Finally, using an in vivo histone deposition assay, we found that Rtt106 is important for the deposition of new histones in the transcribed regions of active genes. Altogether, our data show a new role for the histone chaperone Rtt106 in the chromatin modulations within transcribed regions.

**MATERIALS AND METHODS**

_Saccharomyces cerevisiae_ Strains, and Genetics Methods—All genotypes of the _S. cerevisiae_ strains used in this study are listed in supplemental Table S1. The synthetic genetic array screens were conducted as described in Ref. 11. Deletions or promoter replacement of genes were performed by homologous recombination and standard yeast genetics methods. The GALI-FLO8-HIS3 was described previously (12). Efficient G1 arrest (at least 95%) of cells was achieved by adding 500 ng/ml α-factor for 2–3 h. All oligonucleotide sequences are listed in supplemental Table S2.

_Chromatin Immunoprecipitation—_ChIP analyses were performed as described previously (12, 13). Northern blot analyses were performed as described in Ref. 12.

**RESULTS**

We showed that the high mobility group-like factor Spt2/Sin1 plays an important role in chromatin structure modifications during transcription elongation (12). We conducted a synthetic genetic array (SGA) using the _spt2A_ strain as the query strain (12). This method allowed us to construct and analyze double mutants in which _spt2A_ was combined with deletions in most of the non-essential genes of _S. cerevisiae_. We found several candidates, among which was the gene encoding the histone chaperone Rtt106 (supplemental Fig. S1). It has specific affinity toward histone H3 and H4 and was suggested to function in the S-phase (8). In vivo, Rtt106 interacts physically with the CAF-1 that is involved in chromatin assembly coupled to replication and also in heterochromatin silencing in yeast (8, 9). Since Spt2 function is tightly associated with transcription elongation, our finding of synthetic interaction between _spt2A_ and the mutation in _RTT106_ could suggest a specific replication-independent role of this new histone chaperone.

To get more insights into the biological function of Rtt106, we conducted another SGA screen using, this time, _rtt106A_ as the query strain. In Fig. 1A, we show all the potential interactions of Rtt106 identified in our SGA screen. We found 58 candidates representing different cellular functions. Interestingly, our analysis of gene ontology terms revealed an enrichment of genes involved in DNA replication and CAF-1 function (p value of 0.00015). This is consistent with previous reports showing a functional link between Rtt106 and CAF-1 (8, 14). Importantly, we also found an enrichment of genes involved in transcription by RNAP II (Fig. 1A, genes in _green_). It is interesting to note that this group is significantly enriched in factors that are mainly involved in the elongation step (p value of 0.003). As shown in Fig. 1A (in _green_), Rtt106 interacts with Spt4, which is a subunit of the elongation factor DSIF, the PAF elongation complex component Leo1, elongation factor 1 (Elf1), and the Ctk1 kinase, which controls the transition from transcription initiation to elongation through the phosphorylation of the largest RNAP II subunit Rpb1 C-terminal domain repeats on the serine 2 residue. These data suggest a potential role of Rtt106 in transcription that is independent from its function with CAF-1 in both chromatin assembly coupled to DNA replication and heterochromatin silencing.

_Genetic Evidence That Rtt106 Interacts with Transcription Elongation Factors—_To examine this possibility further, we tested the interaction between _rtt106A_ and the _rpb1Δ-104_ mutant, which is deleted in several C-terminal domain repeats. As shown in Fig. 1B, the double mutant _rtt106Δ rpb1Δ-104_ grew more slowly than either single mutant. This synthetic phenotype confirms a link between Rtt106 and the transcription process. In addition, we found that _rtt106A_ strains containing the suppressor of Ty insertion (SPT) phenotype reporter _lys2-1288_ are also able to grow on medium lacking lysine, indicating an SPT phenotype of these mutants (Fig. 1C). An SPT phenotype is presumably associated with genes that have a clear connection to transcription and chromatin structure regulation. It is interesting to note that in contrast to _rtt106Δ_ and in parallel to the wild type strain, the CAF-1 subunit mutant _cac1Δ_ is not able to grow on medium lacking lysine (Fig. 1C). This further indicates that Rtt106 possesses a specific function in transcription that is independent of the CAF-1 factor.
Rtt106 Role in Transcription

To confirm a potential Rtt106 function in elongation, we studied the possible interaction with the PAF complex members. The PAF complex plays an important role in chromatin modulation associated with transcription elongation. It coordinates the recruitment of different epigenetic marks that are of great importance for the regulation of chromatin structure during elongation (reviewed in Ref. 4). We constructed pairwise double mutants of rtt106Δ and each of the PAF genes (Fig. 1D). In addition to Leo1 that was identified in our SGA screen, we found that the rtt106Δ paf1Δ and rtt106Δ ctr9Δ grew more slowly than each single mutant. Moreover, the double mutants did not grow at 33 °C, indicating a strong synthetic phenotype.

The genetic interactions between Rtt106, Spt2, Spt4, PAF, and Elf1 factors indicate a strong link between Rtt106 and the transcription elongation process. This is further supported by our observation that rtt106Δ genetically interacts with mutations in genes encoding several other elongation factors, including TFIIIS, the DSIF subunit Spt5, and the histone H3 Lys-36 methyltransferase gene Set2 (supplemental Fig. S2).

Rtt106 Is Recruited to Transcribed Chromatin Regions—Taken together, the results described above strongly indicate that Rtt106 is involved in transcription elongation. To test whether this new Rtt106 function is direct, we asked whether this factor is physically associated with actively transcribed regions. To this end, we analyzed by ChIP assays the association of Rtt106 across the constitutively active gene PMA1 and to a non-transcribed locus (intergenic region of chromosome V, hereafter called NoORF). As shown in Fig. 2A, after immunoprecipitation with an antibody directed against Rtt106-Myc, we observed a significant enrichment at both the indicated transcription initiation sequence (probe B) and the 3′-untranslated region (probe D) versus the non-transcribed region (NoORF:probe A). In contrast, we did not observe a significant enrichment at the non-transcribed upstream activation sequence of PMA1 (probe B).

FIGURE 2. Rtt106 is functionally and physically associated with transcription elongation. A, Rtt106 is associated with the transcribed regions of PMA1. Chromatin immunoprecipitations were performed using anti-Myc antibody to immunoprecipitate Rtt106-13Myc. The horizontal bars in the diagram represent the fractions analyzed by quantitative PCR; each reaction analyzing PMA1 and intergenic V regions (NoORF) was separated on a polyacrylamide gel. A representative of duplicate experiments is shown. UAS, upstream activation sequence. B, Rtt106 is associated with the transcribed regions of several genes. ChIP assays were performed as described in A except that the DNA was quantified using real-time PCR. The chromatin used as a control was extracted from an untagged strain, and the values shown represent the average and standard error of three independent experiments. C, Rtt106 is essential to the inhibition of transcription initiation from the cryptic promoter of the pGAL1::FLO8:HIS3 reporter gene. The reporter construct is diagrammed in the top part of the figure. Shown below are serial dilutions of wild type (WT), spt2Δ, and rtt106Δ strains containing the pGAL1::FLO8:HIS3 reporter construct that were grown on synthetic complete medium (SC) or medium lacking histidine and containing galactose as the carbon source (SC-His-Gal). D, rtt106Δ interacts genetically with Spt6. Cells from wild type, rtt106Δ, spt6-1004, and rtt106Δ spt6-1004 were grown on rich medium (YPD) for 3 days at the indicated temperature. E, Rtt106 inhibits transcription initiation from the FLO8-HIS3 cryptic promoter. Total RNAs from wild type and rtt106Δ strains were analyzed by a Northern blot with a probe for HIS3. SCR1 served as a loading control. F, Rtt106 and Spt6 collaborate to inhibit transcription initiation from the FLO8 cryptic promoter. Total RNAs from wild type, rtt106Δ, spt6-1004, rtt106Δ spt6-1004, and rtt106Δ spt6-1004 strains grown at 25 °C were analyzed by a Northern blot with a probe for FLO8. SCR1 served as a loading control.
we analyzed by ChIP assay the association of Rtt106 with the coding regions of several genes in G1 arrested cells but did not find it to be significantly different from that seen in exponentially growing cells (supplemental Fig. S3). Therefore, our data show association of Rtt106 with the coding region of active genes and exclude the possibility that this recruitment is linked to the role of Rtt106 during S-phase.

Rtt106 Is Critical for the Repression of Spurious Transcription from Cryptic Promoter—To test further the role of Rtt106 in elongation, we used a reporter system sensitive to transcription elongation defects in vivo. This reporter is based on previous studies showing that cryptic promoters exist within the coding regions of certain genes and can become active in particular transcription elongation mutants (5). This phenotype has been most extensively characterized for the FLO8 gene (5, 10, 12, 15).

To test whether an rtt106Δ mutation allows cryptic initiation, we used a reporter for FLO8 cryptic initiation in which the 3′-coding region of FLO8 has been replaced with the HIS3 coding region such that HIS3 is only expressed when the FLO8 cryptic promoter is active (12). Using this reporter, we tested the expression of FLO8-HIS3 in wild type, rtt106Δ, and spt2Δ mutant strains by assaying growth on medium lacking histidine. Our results (Fig. 2C) show that, similar to spt2Δ, rtt106Δ allows growth on medium lacking histidine, indicating HIS3 expression from the FLO8 cryptic promoter. After that, we tested cryptic initiation at FLO8-HIS3 more directly and performed Northern hybridization analysis using a HIS3 probe. In addition to the full-length FLO8-HIS3 transcript, we observed a short HIS3 transcript only in the rtt106Δ mutant (Fig. 2E). This observation confirms that in the absence of Rtt106, the repression of cryptic transcription is impaired and suggests that Rtt106 plays a direct role in regulation of chromatin structure during elongation.

Rtt106 Cooperates with the Essential Elongation Factor Spt6 to Inhibit Spurious Transcription—Recent studies show that Spt6 plays a central role in the reformation of normal chromatin in the wake of transcription. It controls the level of histone H4 over transcribed regions and also modulates the histone H3 and H4 acetylation through the control of H3 Lys-36 methylation (5, 10). Our results indicate that Rtt106 may play an important role in the reformation of repressing chromatin structure at the transcribed region of active genes and suggest a potential functional link between Rtt106 and the essential elongation factor Spt6. To address this possibility, we constructed the double mutant rtt106Δ spt6-1004 and assayed growth in different conditions. We observed synthetic growth defects in the double mutant at 33°C (Fig. 2D). This phenotype could suggest that both factors are required to regulate chromatin structure at the transcribed regions and thereby inhibit spurious transcription. We addressed this possibility by asking whether the double mutant rtt106Δ spt6-1004 produces higher levels of transcripts from cryptic promoters. For that, we performed Northern blot analyses using a FLO8 probe to study the endogenous FLO8 gene transcripts in different strains (Fig. 2F). We found that both rtt106Δ and spt6-1004 produce short transcript indicative of spurious transcription. However, the level of FLO8 short transcript is significantly increased in the double mutant rtt106Δ spt6-1004, suggesting that the two factors cooperate to inhibit cryptic promoters and spurious transcription.

Rtt106 Plays an Important Role in Chromatin Assembly Coupled to Transcription—These genetic and molecular data suggest a role for Rtt106 in the regulation of chromatin structure during transcription elongation. To gain additional insights into the role of Rtt106 in this process, we wanted to know whether this histone chaperone is involved in the histone dynamics associated with transcription elongation. To address this, we asked whether the deletion of RTT106 would affect the levels of histone H3 exchange in the coding region of the inducible GAL1 or the constitutive PMA1 genes. We used a histone exchange assay previously described by us and others (13, 16, 17). In this system, there are two different sources of histone H3 in the cell, the endogenous histone tagged with the Myc epitope and a galactose-inducible form fused to the FLAG tag coexpressed with histone H4. To eliminate the contribution of DNA replication-dependent histone deposition, exponentially growing cells containing the double histone H3 tag system are blocked in G1 with α-factor. After incubation with α-factor, cells are either fixed or induced to express FLAG-H3 prior to formaldehyde treatment. ChIP assays were then performed using anti-FLAG, anti-histone H3, and anti-Rpb1 antibodies. The values reported for the incorporation of new H3 represent the percentage of IP relative to histone H3 occupancy. The value of the wild type after galactose induction was set to 1. All values shown represent the average and standard error of three independent experiments.

![FIGURE 3. Rtt106 contributes to the transcription-dependent histone H3 deposition in transcribed regions of active genes.](image-url)
level of incorporation at both GAL1 and PMA1 coding regions in wild type strain. These levels are consistent with those previously reported (13). Importantly, although we do not observe a significant change in Rpb1 association (Fig. 3B), deletion of RTT106 results in a significantly reduced incorporation of histone H3 at the coding regions of GAL1 and PMA1. It is interesting to note that in the absence of Rtt106, histone deposition at the non-transcribed intergenic V region (NoORF) is not decreased. These observations clearly indicate that in the absence of Rtt106, new histone deposition in the wake of the RNAII is reduced. Therefore, our data strongly suggest that the Rtt106 histone chaperone regulates transcription-dependent histone H3 deposition during elongation to ensure that transcribed regions regain normal chromatin structure in the wake of transcription.

**DISCUSSION**

Previous studies have suggested that Rtt106 is a histone chaperone protein that plays a role in heterochromatin silencing through a link to the S-phase and DNA replication-dependent chromatin assembly (8, 9). The present study shows several new results that strongly indicate an important role of Rtt106 during transcription elongation. First, our synthetic lethal screen using rtt106A and the yeast deletion library uncovered several genes involved in transcription and, more specifically, in transcription elongation. Second, a direct test of genetic interactions between rtt106Δ and mutants of genes encoding elongation factors confirmed a strong genetic link between this histone chaperone and transcription elongation. Third, our ChIP data clearly show that Rtt106 is physically associated with transcribed regions. Fourth, in the absence of Rtt106, we observe a derepression of spurious transcription from the FLO8 cryptic promoter. This provides strong evidence that Rtt106 is required for chromatin structure refolding in the wake of transcription elongation. Finally, we show that Rtt106 is important for normal transcription-dependent histone H3 deposition at active genes. This result indicates that Rtt106 is involved in the histone dynamics that are tightly associated with an elongating RNA polymerase II.

We have previously shown that in highly expressed genes, newly synthesized histone H3 is incorporated in the transcribed regions of those genes (13). This observation suggested that nucleosomes are disassembled in front of RNAII and reassembled in its wake using new histones at these locations. Our new data clearly indicate that Rtt106 is important for the deposition of new histones in the highly transcribed genes GAL1 and PMA1. This effect is similar to the one we observed in asf1Δ in a previous study (13). Therefore, it is possible that Rtt106 provides new histones H3/H4 to the machinery that reassemble nucleosomes in the wake of transcription at highly transcribed genes. Alternatively, Rtt106 could deposit histones directly after the RNAII passage. Regardless of the exact mechanism, redeposition of nucleosomes in the wake of RNAII is impaired in the absence of Rtt106, and this provides strong evidence that this factor plays an important function in transcription-coupled chromatin assembly.

The Spt6 histone chaperone is essential for the maintenance of histone H4 over transcribed regions and thereby for normal nucleosomal occupancy (5). Moreover, Spt6 is required for methylation of histone H3 on lysine 36, which mediates subsequent deacetylation of histone H3 and H4 by Rpd3 at coding regions (10). This deacetylation plays an essential role in stabilizing the chromatin structure after RNAII passage, resulting in inhibition of cryptic promoters (4). Our genetic and molecular data show that Rtt106 cooperates with Spt6 to repress the FLO8 cryptic promoter. It is possible that Rtt106 plays a significant role in the Spt6 pathway described above. It may cooperate with Spt6 to maintain a normal level of histones and, therefore, adequate nucleosomal occupancy. Alternatively, similarly to other histone chaperones that were shown to be required for some histone modifications, Rtt106 may stimulate the histone methyltransferase activity of Set2 and therefore regulate indirectly the acetylation status of histones H3 and H4. A future study distinguishing between these possible mechanisms will be undoubtedly interesting.

Acknowledgments—We thank Nikita Avvakumov for helpful comments and advice on the manuscript. We are grateful to Fred Winston for donation of yeast strains and sharing of unpublished observations.

**REFERENCES**

1. Williams, S. K., and Tyler, J. K. (2007) *Curr. Opin. Genet. Dev.* 17, 88–93
2. Workman, J. L. (2006) *Genes Dev.* 20, 2009–2017
3. Izban, M. G., and Luse, D. S. (1991) *Genes Dev.* 5, 683–696
4. Li, B., Carey, M., and Workman, J. L. (2007) *Cell* 128, 707–719
5. Kaplan, C. D., Laprade, L., and Winston, F. (2003) *Science* 301, 1096–1099
6. Mason, P. B., and Struhl, K. (2003) *Mol. Cell. Biol.* 23, 8323–8333
7. Schwabish, M. A., and Struhl, K. (2006) *Mol Cell* 22, 415–422
8. Huang, S., Zhou, H., Katzmann, D., Hochstrasser, M., Atanasova, E., and Zhang, Z. (2005) *Proc. Natl. Acad. Sci. U. S. A.* 102, 14922–14927
9. Carrozza, M. J., Li, B., Florens, L., Suganuma, T., Swanson, S. K., Lee, K. K., Shia, W. L., Anderson, S., Yates, J., Washburn, M. P., and Workman, J. L. (2005) *Cell* 123, 581–592
10. Tong, A. H., Evangelista, M., Parsons, A. B., Xu, H., Bader, G. D., Page, N., Robinson, M., Raghibizadeh, S., Hogue, C. W., Bussey, H., Andrews, B., Tyers, M., and Boone, C. (2001) *Science* 294, 2364–2368
11. Nourani, A., Robert, F., and Winston, F. (2006) *Mol. Cell. Biol.* 26, 1496–1509
12. Ruijani, A., Jacques, P. E., Bhat, W., Robert, F., and Nourani, A. (2007) *Mol. Cell* 27, 393–405
13. Collins, S. R., Miller, K. M., Maas, N. L., Roguev, A., Fillingham, J., Chu, C. S., Schultz, M., Gebbia, M., Recht, J., Shales, M., Ding, H., Xu, H., Han, J., Ingvarsdottir, K., Cheng, B., Andrews, B., Boone, C., Berger, S. L., Hieter, P., Zhang, Z., Brown, G. W., Ingles, C. J., Emili, A., Allis, C. D., Toczyski, D. P., Weissman, J. S., Greenblatt, J. F., and Krogan, N. J. (2007) *Nature* 446, 806–810
14. Prather, D., Krogan, N. J., Emili, A., Greenblatt, J. F., and Winston, F. (2005) *Mol. Cell. Biol.* 25, 10122–10135
15. Schermer, U. J., Korber, P., and Horz, W. (2005) *Mol Cell* 19, 279–285
16. Dion, M. F., Kaplan, T., Kim, M., Buratowski, S., Friedman, N., and Rando, O. I. (2007) *Science* 315, 1405–1408