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Ser7 of RNAPII-CTD facilitates heterochromatin formation by linking ncRNA to RNAi

Takuya Kajitani[a,b,1], Hiroaki Kato[c], Yuji Chikashige[d], Chihiro Tsutsumi[d], Yasushi Hiraoka[d,e], Hiroshi Kimura[f], Yasuyuki Ohkawa[g], Chikashi Obuse[h], Damien Hermand[i], and Yota Murakami[a,1]

[a]Graduate School of Science, Hokkaido University, Sapporo, Hokkaido 060-0810, Japan; [b]Graduate School of Medicine, Kyoto University, Kyoto 606-8507, Japan; [c]Department of Biochemistry, Shimane University School of Medicine, Izumo, Shimane 693-8501, Japan; [d]Frontier Research Laboratory, Advanced ICT Research Institute, National Institute of Information and Communications Technology, Kobe, Hyogo 651-2492, Japan; [e]Graduate School of Frontier Biosciences, Osaka University, Suita 565-0871, Japan; [f]Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, Yokohama 226-8501, Japan; [g]Division of Transcriptomics, Medical Institute of Bioregulation, Japan Science and Technology Agency-Core Research for Evolutional Science and Technology, Kyushu University, Fukuoka 812-8582, Japan; [h]Graduate School of Science, Osaka University, Toyonaka, Osaka 560-0043, Japan; and [i]Namur Research College, University of Namur, 5000 Namur, Belgium

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Some long noncoding RNAs (ncRNAs) transcribed by RNA polymerase II (RNAPII) are retained on chromatin, where they regulate RNAi and chromatin structure. The molecular basis of this retention remains unknown. We show that in fission yeast serine 7 (Ser7) of the C-terminal domain (CTD) of RNAPII is required for efficient siRNA generation for RNAi-dependent heterochromatin formation. Surprisingly, Ser7 facilitates chromatin retention of nascent heterochromatic RNAs (hRNAs). Chromatin retention of hRNAs and siRNA generation requires both Ser7 and an RNA-binding activity of the chromodomain of Chp1, a subunit of the RNA-induced transcriptional silencing (RITS) complex. Furthermore, RITS associates with RNAPII in a Ser7-dependent manner. We propose that Ser7 promotes cotranscriptional chromatin retention of hRNA by recruiting the RNA-chromatin connector protein Chp1, which facilitates RNAi-dependent heterochromatin formation. Our findings reveal a function of the CTD code: linking ncRNA transcription to RNAi for heterochromatin formation.

heterochromatin | RNAi | noncoding RNA | RNA polymerase II

Eukaryotic genomes encode large numbers of noncoding RNAs (ncRNAs) that are transcribed by RNA polymerase II (RNAPII). Some of these ncRNAs regulate gene expression by recruiting activating or repressive epigenetic modifiers, that is, histone modification enzymes and DNA methyltransferases (1). In this situation, ncRNAs are retained on chromatin at specific target loci where they act as scaffolds for the epigenetic modifiers. Since transcriptional regulation by ncRNAs is critically important in developmental processes and diseases, clarification of the principles ensuring locus-specific chromatin regulation by ncRNA is an area of great interest. In this respect, heterochromatin formation in *Schizosaccharomyces pombe* is a well-studied example. Transcription within pericentromeric repeats *dg* and *dh* is strictly repressed by heterochromatin characterized by H3K9 methylation and HP1 enrichment (2, 3). Meanwhile, RNAPII generates heterochromatic RNAs (hRNAs) as ncRNAs by transcribing the repeats bidirectionally at low level, which induces heterochromatin formation through the RNAi machinery (4–6).

The initial step of heterochromatin formation consists in the generation of dsRNA by hybridization with anti-sense transcripts and/or the formation of hairpin structures, which are directly processed into primary siRNAs by Dicer (Dcr1) (7, 8). The primary siRNAs are incorporated into the RNA-induced transcriptional silencing (RITS) complex, which contains the Argonaute protein Ago1, the chromodomain protein Chp1, and the WG/GW motif protein Tas3 (9). siRNA sequence complementarity mediates targeting of RITS to nascent hRNAs.

RITS acts as the hub of a protein complex that contributes to H3K9 methylation and secondary siRNA amplification. RITS interacts with the Clr4–Rik1–Cul4 (CLRC) complex via the LIM domain protein Stc1 (10). Clr4 deposits H3K9 methylation across pericentromeric repeats, and H3K9 methylation in turn serves as a platform for the HP1 proteins Swi6 and Chp2. RITS also interacts with the RNA-dependent RNA polymerase complex (RDRC) on heterochromatin which synthesizes dsRNA from hRNA (11). This dsRNA synthesis is coupled with secondary siRNA generation by Dcr1 around the nuclear periphery (11–14). In addition, H3K9 methylation is recognized by RITS via the Chp1 chromodomain (Chp1-CD), which is required for secondary siRNA amplification (15, 16). Therefore, H3K9 methylation and secondary siRNA amplification promote each other via RITS in a mutually dependent manner, forming a positive-feedback loop that assures maintenance of heterochromatin.

We showed previously that hRNAs in *S. pombe* are retained on chromatin (17) where hRNAs seem to act cotranscriptionally as a scaffold for RNAi machinery. However, the mechanistic details and the functions of this chromatin retention remain unknown. Since the similar RNAi-mediated heterochromatin formation is found in multiple species [e.g., in *Arabidopsis thaliana*, *Neurospora crassa*, *Caenorhabditis elegans*, *Drosophila melanogaster*, and mammals (18)], the underlying mechanisms coordinating transcription of hRNAs and RNAi should be investigated.

The largest subunit of RNAPII, Rpb1 contains evolutionarily conserved heptapeptide repetitive sequences (Tyr1–Ser2–Pro3–Thr4–Ser5–Pro6–Ser7) in its C-terminal domain (CTD). The CTDs

Significance

Some noncoding RNAs (ncRNAs) transcribed by RNA polymerase II (RNAPII) affect gene expression by altering chromatin structures. Since transcriptional regulation by ncRNA is critically important in developmental process and diseases, clarification of the principles ensuring the locus-specific chromatin regulation is of great interest. Here, we found that in *Schizosaccharomyces pombe* Ser7 of the C-terminal domain (CTD) of RNAPII is involved in locus-specific siRNA amplification within heterochromatin and facilitates heterochromatin formation. Ser7 and a chromodomain protein Chp1, which binds to H3K9 methylation and RNAs, cooperatively promote chromatin retention of the nascent heterochromatic RNAs (hRNAs) across heterochromatin. Our findings present a principle of epigenetic regulation by ncRNAs in which the RNAPII CTD links hRNA transcription to RNAi for heterochromatin formation.

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The authors declare no conflict of interest.

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Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, https://www.ncbi.nlm.nih.gov/geo (accession no. GEO53568).

[1]To whom correspondence may be addressed. Email: kajitani@sci.hokudai.ac.jp or yota@sci.hokudai.ac.jp.

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of fission yeast, budding yeast, and mammals consist of 29, 26, and 52 repeats, respectively. The CTD is highly phosphorylated during transcription. The patterns of phosphorylation, glycosylation, isomerization, and the unmodified state of each residue, collectively referred to as the "CTD code," are deciphered by various interacting proteins to regulate transcription and cotranscriptional events such as activating histone modifications and nascent RNA processing (19, 20).

Among various elements of the CTD code, the unphosphorylated state at Ser2 and Ser5 is required for preinitiation complex formation (21–23) and mediator recruitment at the promoter (24, 25). Phosphorylation at Ser5 (Ser5P) is a mark of transcription initiation, and phosphorylation at Ser2 (Ser2P) is a mark of transcription elongation and termination (19, 20). Phosphorylation at Ser7 promotes snRNA transcription by recruiting Integrator and RPAP2 at promoters in mammals (26, 27). P-TEFb/Cdk9 (28, 29), Cdk12 (30), and Cdk13 (31) predominantly phosphorylate the Ser7 prephosphorylated CTD. In *S. pombe*, Ser7 mutations in the truncated CTD alter gene expression of phosphate homeostasis (32, 33) and subtelomeric genes (34). However, with respect to cotranscriptional RNA processing of hRNAs, it has remained elusive whether the CTD codes are involved in RNAi-mediated heterochromatin formation.

Despite the importance of CTD codes, technical difficulties (i.e., high-copy-number repetitive units, substrate specificity of CTD kinases, and redundant phosphorylation by several kinases) have precluded a comprehensive analysis of each residue (35–37). Complicating matters further, CTD kinases phosphorylate and regulate each other (38–41). In this study, to manifest the functions of each residue, we used alanine-substitution mutants of CTD, which not only function as nonphosphorylated acceptor mutations but also perturb the unphosphorylated state. Consequently, we found that Ser2 and Ser7 are responsible for the repression of pericentromeric ncRNA expression. Ser2 played an important role in gene silencing, independently of heterochromatin structure, whereas Ser7 was required for siRNA generation and H3K9 methylation across pericentromeric heterochromatin. Interestingly, Ser7 and the RNA-binding activity of Chp1-CD cooperatively promoted chromatin retention of nascent hRNAs and facilitated efficient secondary siRNA amplification. The cooperation was ensured by a Ser7-dependent association between RNAPII and RITS. Our findings reveal a mode of coupling between the CTD codes and histone codes.

Results

Mutations at Ser2 and Ser7 of CTD Cause Loss of Pericentromeric Silencing. The RNAPII-CTD of fission yeast contains 29 heptapeptide repeats. To investigate the function of each residue of CTD at Ser2 and Ser7, full-length mutants have been generated in which Ser2 or Ser7 in all heptads are replaced by alanine, which abrogates both the phosphorylated and unphosphorylated states (*ctdS2A* and *ctdS7A*) (Fig. 1*A*) (42, 43). In addition to *ctdS2A* and *ctdS7A* mutants, we used the *rpb1-11* mutant, which encodes a protein with a short CTD containing only 16 repeats (Fig. 1*A*). First, we tested the expression level of *ade6*$^+$ inserted into the outer repeat of pericentromeric repeats (*otr::ade6*$^+$) (Fig. 1*B*). Repression of *otr::ade6*$^+$ by heterochromatin in the wild type resulted in inhibited adenine biosynthesis, resulting in the formation of red colonies on medium containing a limiting amount of adenine (low-adenine condition). By contrast, heterochromatin mutants, such as *clr4*Δ, *dcr1*Δ, and *rpb2-m203*, formed white or pink colonies on low-adenine plates (Fig. 1*C* and Fig. S1*A*). All the RNAPII mutants produced a mixture of red, pink, and white colonies with the exception of the *rpb1-11* mutant (Fig. S1*A*). The variegated phenotype indicated that the silent and active states were metastable in the *ctdS2A* and *ctdS7A*mutants. Note that all the RNAPII mutants proliferated almost normally under standard vegetative growth conditions (Fig. 1*C*). To obtain unambiguous results, white clones derived from each mutant were used in subsequent analyses. qRT-PCR of *ctdS2A* and *ctdS7A* mutants confirmed the accumulation of hRNAs bidirectionally transcribed from *dh* pericentromeric repeats and forward transcripts of *otr::ade6*$^+$ (Fig. 1*D* and Fig. S1*B*).

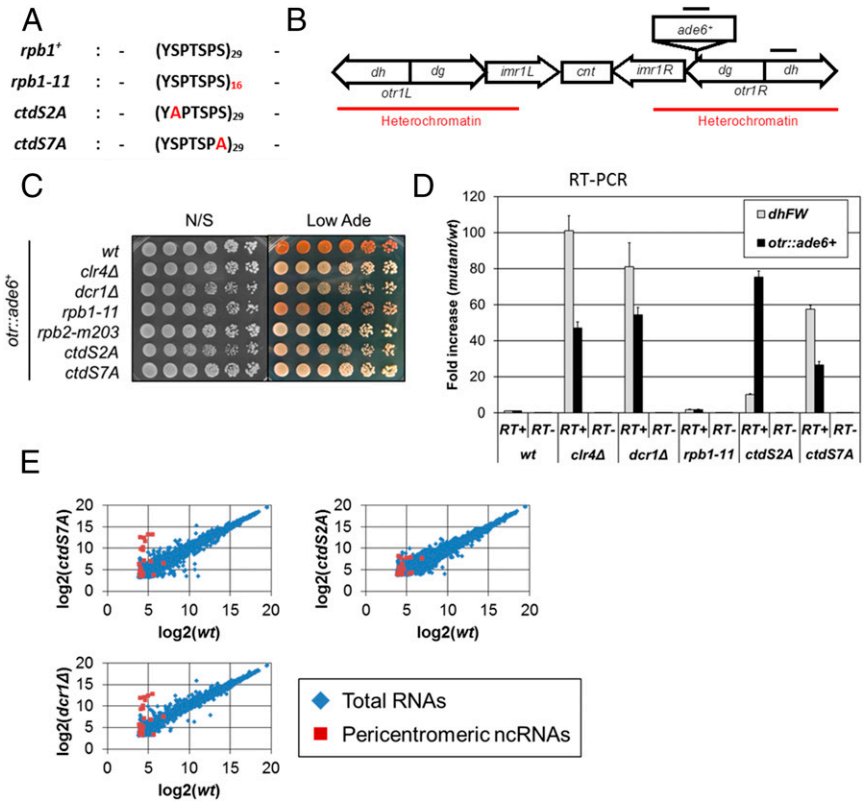


Fig. 1. Unphosphorylatable mutations of CTD Ser2 and Ser7 cause loss of pericentromeric silencing. (*A*) Amino acid sequences of the CTD in the wild type and mutants. (*B*) Schematic representation of the pericentromeric regions of the strains used in the silencing assay. The *ade6*$^+$ gene was inserted into the *dg* repeat in the outermost repeats (*otr*) on the right side of the pericentromeric region of chromosome I (*otr::ade6*$^+$). Bars show the position of the *otr::ade6*$^+$ and *dh* regions amplified in qRT-PCR, ChIP-qPCR, and RIP-qPCR. (*C*) Silencing assay of the *otr::ade6*$^+$ reporter gene. Cells were spotted in fivefold serial dilutions on nonselective (N/S) medium or on YES medium without adenine (Low Ade). (*D*) Real-time qRT-PCR of *otr::ade6*$^+$ and *dh* forward transcripts (*dh*FW) relative to the wild type. Primers for cDNA synthesis are gene-specific reverse-strand primers. Primer pairs for qPCR are site-specific primers at *dh*, *otr::ade6*$^+$ at the pericentromere, and *act1*$^+$. qRT-PCR signals were normalized against *act1*$^+$ and are presented as the fold increase relative to the wild type (defined as 1). Error bars indicate the SEM from three biologically independent experiments. (*E*) Scatter plot comparing gene expression in *ctdS2A*, *ctdS7A*, or *dcr1Δ* cells with that in the wild-type cells. Blue rhombuses indicate expression levels of all transcripts, and red squares indicate expression levels of pericentromeric ncRNA, calculated as the $\log_2$ ratio.

Global Expression Profiles of Mutants of CTD Ser2 and Ser7. To characterize the global gene-expression profiles in *ctdS2A* or *ctdS7A* mutant cells, we compared the level of the microarray signal of each transcript in the CTD mutants with the corresponding signal in the wild-type cells. We found that *ctdS2A* affects the expression of a subset of genes, including meiotic genes and antisense RNAs, as previously described (42). Similarly, *ctdS7A* caused change in a limited number of genes (Table S1), including stress-response genes. In agreement with these results, *ctdS2A* and *ctdS7A* cells did not show obvious growth defects on a nonselective (N/S) plate (Fig. 1*C*). As expected from the results shown in Fig. 1 *C* and *D*, genes that were up-regulated more than in fourfold each mutant included a large population of pericentromeric ncRNAs (Table S1). The scatter plots comparing each mutant with the wild-type cells revealed that expression levels of some, but not all, pericentromeric ncRNAs were markedly up-regulated in *ctdS2A* (four- to eightfold) or *ctdS7A* (four- to 13-fold) cells (Fig. 1*E*). These results indicated that both Ser2 and Ser7 are required for strict repression of pericentromeric ncRNAs.

Because previous studies revealed that the RNAi machinery represses the expression of pericentromeric ncRNAs, we compared the transcriptome in *ctdS2A* and *ctdS7A* cells with that of *dcr1*Δ cells. Comparison by Venn diagrams and scatter plots showed that changes in gene expression in *dcr1*Δ cells were more similar to those in *ctdS7A* cells than to those in *ctdS2A* cells (Fig. 1*E* and Fig. S1*C* and Table S1). These data suggested that Ser7 and Dcr1 silence pericentromeric ncRNAs via the same pathway. Only a small number of genes related to heterochromatic silencing exhibited more than twofold changes in expression in the *ctdS2A* or *ctdS7A* mutants (4 and 3 of 115 genes, respectively) (Table S2). Particularly, *ctdS7A* mutation did not reduce the expression of genes participating in heterochromatic silencing. Hence, it is unlikely that the CTD mutants impair heterochromatic silencing by affecting the expression of heterochromatic genes.

CTD Ser2 and Ser7 Play Distinct Roles for Pericentromeric Silencing. To assess the chromatin state around pericentromeric ncRNA genes in *ctdS2A* and *ctdS7A* cells, we used ChIP assays to estimate the levels of repressive epigenetic marks: H3K9 dimethylation (H3K9me2), H3K9 trimethylation (H3K9me3), and the HP1 family protein Swi6 (Fig. 2*A* and Fig. S1*D*). The truncated CTD mutant *rpb1-11* did not exhibit a remarkable reduction in H3K9me2 deposition or Swi6 localization at either *dh* or *otr::ade6*$^{+}$, except for a slight reduction of H3K9me3 at *dh* (Fig. 2*A* and Fig. S1*D*), indicating that the first 16 heptads are sufficient to form the heterochromatin structure. Similarly, the *ctdS2A* mutation did not affect the enrichment of repressive epigenetic marks at *dh* repeats, although it moderately decreased them at *otr::ade6*$^{+}$ (Fig. 2 *A* and *B* and Fig. S1*D*). Rik1, a component of the CLRC methyltransferase complex, accumulated at high levels at *dh* and slightly decreased levels at *otr::ade6*$^{+}$ (Fig. S1*E*), indicating that almost the complete heterochromatin structure was retained in *ctdS2A* mutant. On this basis, we concluded that Ser2 does not contribute to the formation of heterochromatin structure. Meanwhile, activating histone modifications found on euchromatin, such as H3K4 dimethylation (H3K4me2), H3K4 trimethylation (H3K4me3), H3K9 acetylation (H3K9Ac), and H3K14 acetylation (H3K14Ac) were obviously elevated at *otr::ade6*$^{+}$ (Fig. S2), which might be related to a loss of silencing that is independent of heterochromatin structure.

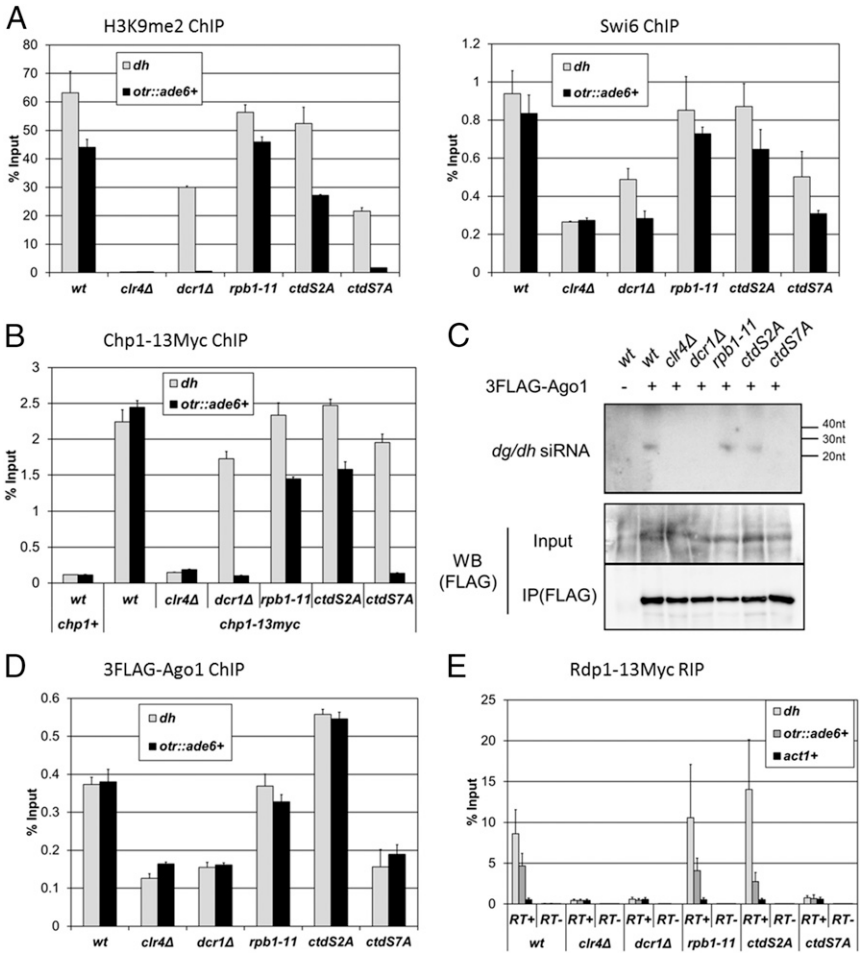


Fig. 2. CTD Ser7 is required for heterochromatin formation via efficient generation of siRNA. (*A* and *B*) ChIP assay of H3K9me2 and Swi6 (*A*) and Chp1-13Myc (*B*). H3K9me2, Swi6, and Chp1 were precipitated, and the associated DNAs were quantified by qPCR. Primer pairs for qPCR were site-specific primers at *dh*, *otr::ade6*$^{+}$ at the pericentromere. The results of ChIP are presented as percent input. Error bars indicate SEM from three independent experiments. (*C*) Northern blot detecting siRNA derived from pericentromeric *dg* and *dh* transcripts. (*Upper*) Northern blot of Ago1-bound mature siRNAs. Ago1-bound siRNA was isolated by phenol/chloroform treatment. siRNAs were detected by $^{32}P^{-}$labeled oligo-DNA probes. (*Lower*) Western blot of Input and immunoprecipitated Ago1 using anti-FLAG antibody. (*D*) ChIP assay of Ago1. 3FLAG-Ago1 was immunoprecipitated, and the associated DNAs were quantified by qPCR. Details of qPCR are identical to *A*. (*E*) RIP assay of Rdp1. Rdp1-13Myc was immunoprecipitated, and the associated RNAs were analyzed by qRT-PCR. Primers for cDNA synthesis were gene-specific reverse-strand primers. Primer pairs for qPCR were site-specific primers for *dh*, *otr::ade6*$^{+}$ at the pericentromere, and *act1*$^{+}$. RIP signals of each strain are presented as percent input. RIP signals of *act1*$^{+}$ are the negative control. Error bars indicate the SEM from three independent experiments.

In contrast to *ctdS2A*, *ctdS7A* mutation caused impairment of heterochromatin formation. In *ctdS7A* cells, levels of H3K9me2/3 and Swi6 were reduced (Fig. 2*A* and Fig. S1*D*): On *dh* repeats, they decreased by ~50%, while at *otr::ade6*$^{+}$, they were markedly diminished, nearly to the same extent as in *clr4Δ* and *dcr1Δ* cells (Fig. 2 *A* and *B* and Fig. S1 *D* and *E*). Residual H3K9me2 on *dh* repeats is likely to be a common feature of RNAi mutants such *as dcr1*Δ (10, 44). In accordance with the decrease in repressive histone modifications in *ctdS7A* cells, levels of activating histone modifications such as H3K4 methylation and histone acetylations were significantly elevated at both *dh* and *otr::ade6*$^{+}$ (Fig. S2). In particular, obvious hyperacetylation occurred at *dh* in *ctdS7A* cells. These phenotypes of *ctdS7A* cells were quite similar to those of *dcr1Δ* cells. Since Ser7 is involved in heterochromatin formation, we focused on the function of Ser7 in subsequent experiments.

Ser7 Is Required for Heterochromatin Formation via Efficient siRNA Generation. The similarity of the phenotypes in *ctdS7A* and *dcr1*Δ mutants suggested that Ser7 is involved in siRNA generation for heterochromatin formation. To test this, we next analyzed Ago1-bound mature siRNAs derived from pericentromeric ncRNAs by Northern blotting (Fig. 2*C*). In Ago1 complex purified from wild-type, *rpb1-11*, and *ctdS2A* cells, *dg*/*dh* siRNAs were clearly detected at comparable signal intensities. However, siRNA signal was not detected in *clr4*Δ, *dcr1*Δ, and *ctdS7A* cells, indicating that siRNA generation was impaired in *ctdS7A* cells. Furthermore, ChIP and RNA immunoprecipitation (RIP) experiments revealed that RNAi machineries, such as Chp1, Ago1, and Rdp1, were not properly assembled at pericentromeric heterochromatin in *ctdS7A* cells, as well as in *dcr1*Δ cells (Fig. 2 *B*, *D*, and *E*). These results suggest that Ser7 is required for proper assembly of RNAi machineries at pericentromeric repeats for siRNA generation.

Truncated CTD Mutation Rescues the Defect in siRNA Generation in ctdS7A-Mutant Cells. We next questioned whether the defects of *ctdS7A* are due to the loss of phosphorylation at Ser7. To explore this, we tried to use phospho-mimetic mutants in which Ser7 was replaced with either glutamic acid (*ctdS7E*) or aspartic acid (*ctdS7D*), but we failed to construct the full-length mutants even in diploid cells. This suggested that the constitutive hyper-phosphorylation is toxic to cells and that the balance between phosphorylation and dephosphorylation at Ser7 is important, as reported in budding yeast and mammalian cells (45, 46). Since it was reported that in fission yeast the truncated mutant with 18 repeats of *ctdS7E* (*ctdS7Ex18*) was viable (47), we first examined the heterochromatin defects and siRNA generation in truncated-CTD mutant (*ctdwtx18*) cells and truncated-*ctdS7A* mutant (*ctdS7Ax18*) cells.

Similar to *rpb1-11* cells, *ctdwtx18* cells did not show any marked reduction of H3K9 methylation at either *dh* or *otr::ade6*$^{+}$ (Fig. S3*A*). Interestingly, in contrast to the full-length *ctdS7A* cells, the truncated *ctdS7Ax18* cells retained a substantial amount of H3K9me2/3 at both the *dh* and *otr::ade6*$^{+}$ regions (Fig. S3*A*) (34). On the other hand, *ctdwtx18 dcr1*Δ and *ctdS7Ax18 dcr1*Δ double-mutant cells showed a marked reduction of H3K9me2/3at *dh* and *otr::ade6*$^{+}$ nearly comparable to the level in *dcr1*Δ cells (Fig. S3*A*). Consistent with the ChIP assay, *dg*/*dh* siRNAs were detected in *ctdS7Ax18*, while the siRNAs were hardly detected in full-length mutant *ctdS7A* cells (Fig. S3*B*). These observations indicated that truncation of CTD suppresses the failure in siRNA generation and

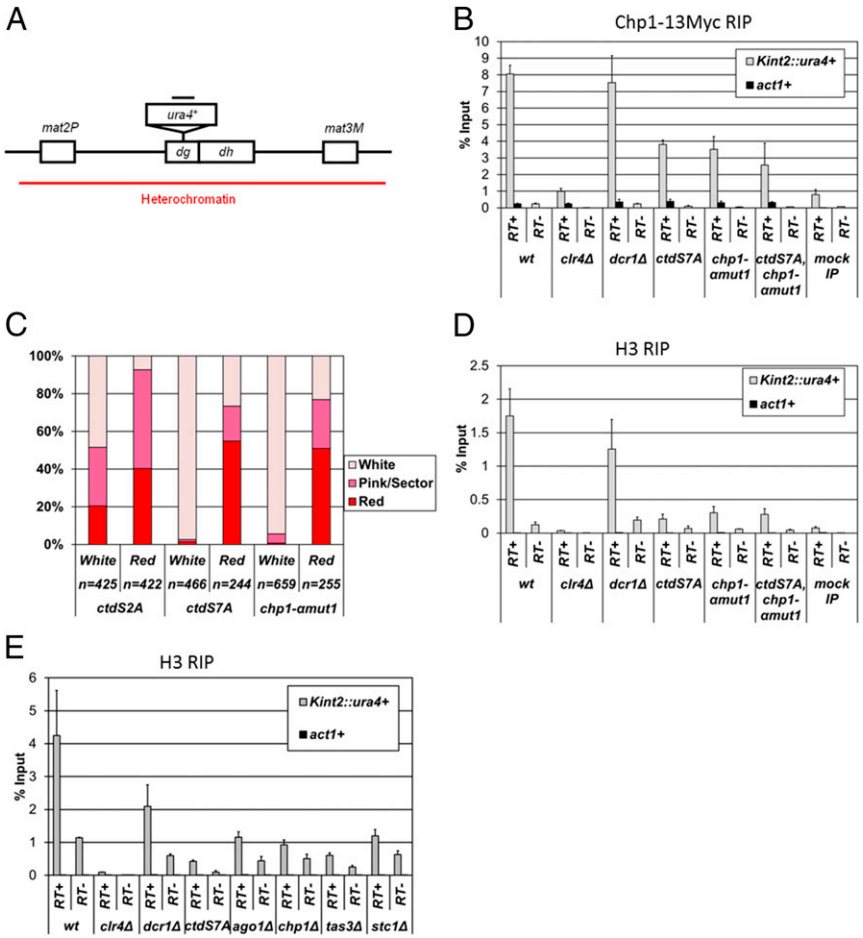


Fig. 3. CTD Ser7 and RNA-binding activity of Chp1-CD cooperatively ensure chromatin retention of hRNA. (*A*) Schematic diagram showing heterochromatin at the mating-type locus. The *ura4*$^{+}$ gene was inserted into a *dg*-like sequence of the *K* region. The small bar indicates the position of the primer set used for qRT-PCR, ChIP-qPCR, and RIP-qPCR. (*B*) RIP assay for Chp1 evaluating the association between *Kint2::ura4*$^{+}$ RNA and Chp1. Chp1 was immunoprecipitated, and the associated RNAs were analyzed by qRT-PCR as in Fig. 2*E* using site-specific primers for *Kint2::ura4*$^{+}$. Error bars indicate the SEM from three biologically independent experiments. (*C*) Stability of the variegated phenotype of *otr::ade6*$^{+}$ silencing. White and red colonies were picked and restreaked onto low-adenine plates, and colonies with the indicated colors were counted in the indicated strains. (*D* and *E*) RIP assay for histone H3 showing that RNA transcribed from *Kint2::ura4*$^{+}$ associates with histone H3. Histone H3 was immunoprecipitated, and the associated RNAs were analyzed by qRT-PCR. Details of qRT-PCR are identical to those in *B*.

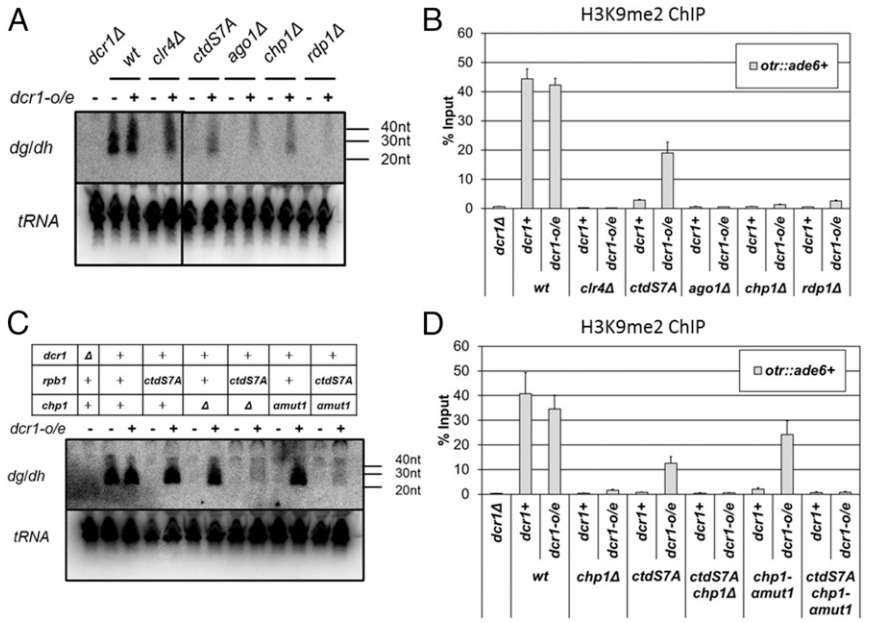


Fig. 4. Mutation at Ser7 and RNA-binding activity of Chp1-CD was rescued by *dcr1*$^+$ overexpression. (*A* and *C*) Northern blots of the indicated strains in the presence (+) or absence (–) of *dcr1*$^+$ overexpression (*dcr1-o/e*) in RNAi mutants (*A*) and *chp1*$^+$ mutants (*C*). Small RNAs were selectively purified from total RNA using the mirVana miRNA Isolation Kit. Signals of *tRNA*^{Asn} are loading control. Details of siRNA detection are identical to those in Fig. 2*C*. Untrimmed raw data of *A* are shown in Fig. S6*B*. (*B* and *D*) ChIP assays showing H3K9me2 levels in the indicated mutants in the presence (+) or absence (–) of *dcr1*$^+$ overexpression (*dcr1-o/e*) at *otr::ade6*$^+$ in RNAi mutants (*B*) and *chp1*$^+$ mutants (*D*). Details are identical to those in Fig. 2*A*. Error bars indicate the SEM from three biologically independent experiments.

heterochromatin formation seen in a full-length *ctdS7A* mutant. The difference in H3K9 methylation in *ctdS7A* between our data (Fig. 2*A*) and previously reported data (34) is most likely due to the length of the CTD repeats, reinforcing the notion that truncated CTD mutants should be used with caution (43) and that they are inappropriate for analyzing the function of Ser7. Hereafter, we analyze the function of Ser7 using full-length CTD mutants.

Ser7 Enables Stable Binding of hRNA to Chp1. All the data shown above strongly suggested that the *ctdS7A* mutation abrogated pericentromeric heterochromatin formation by disturbing siRNA generation. Since siRNA generation and heterochromatin formation at pericentromeric repeats are mutually dependent, it is difficult to determine whether the defects in siRNA generation observed in *ctdS7A* cells were a direct consequence of the mutation or a secondary effect of the loss of heterochromatin due to the mutation. Because two redundant pathways, RNAi and Atf1/Pcr1, establish heterochromatin at the mating-type locus (48), heterochromatin structure is preserved even in siRNA-deficient cells. Therefore, we decided to investigate the function of Ser7 at the mating-type locus and constructed strains in which a *ura4*$^+$ reporter gene was inserted into the *K* region of the mating-type locus (*Kint2::ura4*$^+$) (Fig. 3*A*). A silencing assay, qRT-PCR, and ChIP for H3, H3K9me2, and Chp1 confirmed that heterochromatin and the silencing state at the *Kint2::ura4*$^+$ locus were not defective in *ctdS7A* and *dcr1*Δ cells (Fig. S4), supporting our assumption that Ser7 functions in the RNAi pathway.

As RITS targeting to nascent hRNAs is critical for RNAi-dependent heterochromatin formation, we next evaluated the interaction of hRNAs (*Kint2::ura4*$^+$ transcript) and euchromatic RNAs (*act1*$^+$ transcripts) with Chp1 by RIP analysis. In wild-type and even in *dcr1*Δ cells, Chp1 captured significant levels of *Kint2::ura4*$^+$ RNAs but not *act1*$^+$ RNAs (Fig. 3*B*). By contrast, in *clr4*Δ cells, *Kint2::ura4*$^+$ transcripts were no longer captured by Chp1 (Fig. 3*B*). These results suggested that the interaction between hRNA and Chp1 does not depend on siRNA generation but does depend on H3K9 methylation, which may provide a scaffold for Chp1 to capture nascent hRNAs on chromatin. Interestingly, the amount of *Kint2::ura4*$^+$ transcripts captured by Chp1 was threefold lower in *ctdS7A* cells than in wild-type cells (Fig. 3*B*), despite the substantial enrichment of Chp1 at *Kint2::ura4*$^+$ in this mutant (Fig. S4*E*). These findings indicated that Ser7 plays a crucial role in the capture of hRNAs by RITS. Note that small amounts of signals specifically found in heterochromatic *Kint2::ura4*$^+$ transcripts in the absence of reverse transcriptase (Fig. 3*B*; RT–) are derived from the DNA/RNA hybrid structure and were significantly decreased when samples were treated with RNase H (17).

Previous work showed that Chp1-CD not only is a reader of H3K9 methylation but also has a unique RNA-binding activity that is critical for RNAi-dependent heterochromatin formation at pericentromeric repeats (16). Since we speculated that the RNA-binding activity of Chp1-CD is involved in the Ser7-dependent capture of hRNA by RITS, we used the *chp1-αmut1* mutant that specifically loses the ability to interact with RNA (16). We first realized the phenotypic similarity between *ctdS7A* and *chp1-αmut1* mutants in terms of the silencing at *otr::ade6*$^+$; the *chp1-αmut1* cells as well as *ctdS7A* cells showed the variegated phenotype of *otr::ade6*$^+$ gene expression on low-adenine plates (Fig. 3*C* and Fig. S5 *A* and *B*). The frequencies of the appearance of red, pink, or white colonies in *chp1-αmut1* cells and *ctdS7A* cells in the absence of any selection (Fig. S5*B*) and after isolation of white or red clones (Fig. 3*C*) were quite similar, suggesting that *ctdS7A* and *chp1-αmut1* mutants have similar silencing defects. In agreement with this, patterns of H3K9me2 in *chp1-αmut1* cells and in *ctdS7A* cells were nearly comparable (Fig. S5*C*). Hereafter, we used white clones of the *chp1-αmut1* mutant for further analyses.

Next, we analyzed the effect of the *chp1-αmut1* mutation on the capture of hRNA by RITS. Remarkably, the *chp1-αmut1* mutation reduced the interaction between Chp1 and *Kint2::ura4*$^+$ transcripts to the same extent as the *ctdS7A* mutation (Fig. 3*B*). In the *ctdS7A chp1-αmut1* double mutant, the Chp1 RIP signal of *Kint2::ura4*$^+$ transcripts was comparable to the signal in either single mutant (Fig. 3*B*). This result suggested that Ser7 is required for the capture of hRNA by RITS via the RNA-binding surface of Chp1-CD. These results support the notion that Ser7 and the RNA-binding activity of Chp1-CD act in the RITS–hRNA interaction.

Ser7 and the RNA-Binding Activity of Chp1-CD Ensure Chromatin Retention of hRNA. In *S. pombe*, hRNAs that induce RNAi-dependent heterochromatin formation should be retained at specific locations on heterochromatin to exert their function. We previously showed that hRNAs of *S. pombe* are indeed retained on heterochromatin (17). In addition, we showed that Ser7 is required for siRNA generation (Fig. 2*C*) and promotes the interaction between hRNA and Chp1-CD (Fig. 3*B*). Furthermore, the bivalent function of Chp1-CD in H3K9 methylation binding

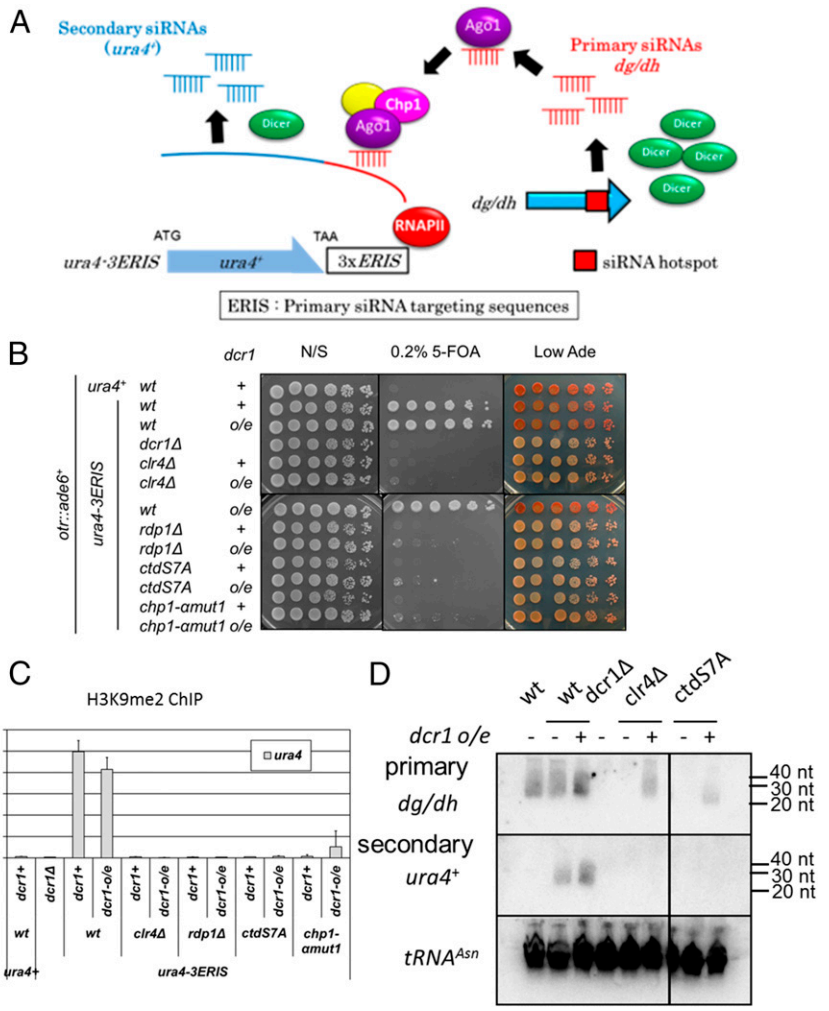


Fig. 5. Ser7 promotes secondary siRNA amplification. (*A*) Schematic diagram of the artificial heterochromatin formation assay. Three copies of ERIS DNA sequences, which Dcr1-dependent primary siRNAs preferentially target, were inserted into the 3′ UTR of endogenous *ura4*$^+$ gene. To reinforce the primary siRNA generation, *dcr1*$^+$ was overexpressed under the *urg1*$^+$ promoter. The primary siRNA induces the secondary siRNA, which corresponds to the *ura4*$^+$ sequence. (*B*) Silencing assay of the *ura4-3ERIS* experiment using the indicated strains. Silencing states of *ura4*$^+$ *or ura4-3ERIS* expression were monitored by viability on plates containing 0.2% of 5-fluoroorotic acid (5-FOA). Silencing states of *otr::ade6*$^+$ were simultaneously monitored on low-adenine (Low Ade) plates. (*C*) ChIP assay showing H3K9me2 levels at *ura4-ERIS* in the indicated mutants in the presence (+) or absence (–) of *dcr1*$^+$ overexpression (*dcr1-o/e*). Details are identical to those in Fig. 2*A*. Error bars indicate the SEM from three biologically independent experiments. (*D*) Northern blot of the indicated strains in the presence (+) or absence (–) of *dcr1*$^+$ overexpression (*dcr1-o/e*). Details of the Northern blot were identical to those in Fig. 4*A*. Untrimmed raw data of *D* are shown in Fig. S6*C*.

and RNA binding implies that Chp1-CD connects hRNAs with heterochromatin structure. In this respect, we hypothesized that Ser7 mediates chromatin retention of nascent hRNAs, in cooperation with the RNA-binding activity of Chp1-CD, to stimulate the generation of siRNAs on chromatin.

To test this hypothesis, we analyzed the effect of *ctdS7A* and *chp1-αmut1* mutants on the chromatin retention of heterochromatic *Kint2::ura4*$^+$ transcripts by histone H3 RIP analysis (17). H3 RIP signals in both *ctdS7A* and *chp1-αmut1* cells, which reflect the amount of chromatin retained by *Kint2::ura4*$^+$ transcripts, declined by approximately fourfold relative to the wild type cells, suggesting that Ser7 and the RNA-binding activity of Chp1-CD play critical roles in proper chromatin retention of hRNAs (Fig. 3*D*). Furthermore, the *ctdS7A chp1-αmut1* double mutant also exhibited marked reduction to a level similar to that of either single mutation alone (Fig. 3*D*). These results suggested that Ser7 and the RNA-binding activity of Chp1-CD play important role(s) in ensuring chromatin retention of hRNAs (Fig. 3 *B* and *D*). Note that the reduction of the H3 RIP signal in *ctdS7A* cells is due to a decrease in the chromatin retention of *Kint2::ura4*$^+$ transcripts but is not due to an increase of total transcripts, because the *Kint2::ura4*$^+$ transcript was repressed in *ctdS7A* cells to the same level as in wild-type and *dcr1*Δ cells (Fig. S4*B*). In the H3-RIP assay, a significant amount of RNA bound to H3 in *dcr1*Δ cells in which no siRNA were formed. In *ctdS7A* cells and *chp1-αmut1* cells, the amount of H3-bound RNA was much lower than that in *dcr1*Δ cells, indicating that low siRNA was not the reason for the defects in chromatin retention by *Kint2::ura4*$^+$ RNA in these mutants (Fig. 3 *D* and *E*). By contrast, *clr4*Δ severely impaired the chromatin retention of hRNA (Fig. 3*E*), indicating that H3K9 methylation is essential for stable chromatin retention of hRNA. Given the interaction between Chp1-CD and H3K9 methylation, chromatin retention seems to assure maintenance of heterochromatin by the generation of activated siRNA once a locus had been established.

As in *ctdS7A* cells, the RITS mutants *ago1*Δ, *chp1*Δ, and *tas3*Δ and *stc1*Δ cells also exhibited reduced H3 RIP signals (about fourfold lower than the wild-type level) (Fig. 3*E*), suggesting that an intact RITS complex mediates the chromatin retention of hRNA although siRNA is not required for the retention.

Dcr1 Overexpression Rescues Primary siRNA Generation in *ctdS7A* and *chp1-αmut1*. The initial step in heterochromatin formation is the generation of primary siRNA from the intrinsic hairpin structure of the 5′ region of *dg*/*dh* RNAs and/or annealed bidirectional *dg*/*dh* transcripts. Subsequently, the RITS–RDRC–Dcr1 interaction promotes the secondary siRNA amplification (12). Regarding the primary siRNA biogenesis, others (49, 50) and we (14) previously indicated that overexpression of *dcr1*$^+$ in mutant cells lacking RITS, RDRC, or CLRC caused marked siRNA generation (Fig. 4*A* and Fig. S6*B*), indicating that Dcr1 can produce primary siRNA without RITS, RDRC, or CLRC. Next, we investigated whether Ser7 and the RNA-binding activity of Chp1-CD affect this Dcr1-depenndent primary siRNA generation. We performed *dcr1*$^+$ overexpression under the control of the *urg1* promoter (Fig. S6*A*) in various mutants, including *ctdS7A*. In *clr4*Δ cells, substantial restoration of pericentromeric siRNA generation (to nearly wild-type levels) was observed, showing

that H3K9 methylation is not required for primary siRNA biogenesis (Fig. 4*A* and Fig. S6*B*). In *ctdS7A* cells, *dcr1*$^+$ overexpression resulted in significant recovery of pericentromeric siRNA generation (Fig. 4*A* and Fig. S6*B*), showing that *ctdS7A* cells can still generate Dcr1-dependent primary siRNAs. Similar recovery of siRNA synthesis was also observed in *ago1*Δ and *chp1*Δ cells. Note that *dcr1*$^+$ overexpression promoted more efficient secondary siRNA amplification in *ctdS7A*, *ago1*Δ, and *chp1*Δ cells than in *rdp1*Δ cells. This may indicate that *dcr1*$^+$ overexpression stimulates the production of dsRNA in these mutants, probably by the enhanced catalytic activity of Rdp1 due to the increased interaction between Dcr1 and the RDRC (12).

Next, we examined whether siRNA recovery upon *dcr1*$^+$ overexpression would be sufficient to promote H3K9 methylation. In *rdp1*Δ cells, *dcr1*$^+$ overexpression resulted in a slight accumulation of H3K9me2 at *otr::ade6*$^+$ (Fig. 4*B*), reflecting the small amount of siRNA produced by *dcr1*$^+$ overexpression. By contrast, in *ago1*Δ and *chp1*Δ cells, despite significant recovery of pericentromeric siRNAs upon *dcr1*$^+$ overexpression, the level of H3K9me2 at *otr::ade6*$^+$ was not elevated (Fig. 4*B*), indicating that the RITS complex is essential for siRNA-dependent deposition of H3K9me2. Unlike these RNAi mutants, *ctdS7A* cells exhibited striking deposition of H3K9me2 under *dcr1*$^+$ overexpression, at levels comparable to that in the wild-type cells (Fig. 4*B*). These results suggested that under *dcr1*$^+$ overexpression in *ctdS7A* cells, primary siRNA production by Dcr1 is sufficient for H3K9 methylation, and Ser7 is not essential for H3K9 methylation by Clr4 after primary siRNA production.

To gain the further insight into the importance of chromatin retention of hRNA on siRNA generation, we measured the amount of siRNAs in *ctdS7A*, *chp1*Δ, and *chp1-αmut1* single mutants and in the *ctdS7A chp1*Δ and *ctdS7A chp1-αmut1* double mutants. With *dcr1*$^+$ overexpression, siRNA production in each single mutant was restored to the wild-type levels (Fig. 4*C*). Interestingly, the *ctdS7A chp1-αmut1* double mutants generated smaller amounts of siRNAs with *dcr1*$^+$ overexpression than did the single mutants (Fig. 4*C*), which is contrary to the results of the Chp1-RIP and H3 RIP assays; Ser7 and Chp1-CD appear to function in the same pathway for capture of hRNA by Chp1 and retention of hRNA on chromatin (Fig. 3 *B* and *D*). A similar reduction of siRNA was observed in the *ctdS7A chp1*Δ double mutant (Fig. 4*C*). These results revealed that Ser7 and the RNA-binding activity of Chp1-CD have redundant functions in Dcr1-dependent primary siRNA generation, probably in the step of Dcr1 loading on hRNAs.

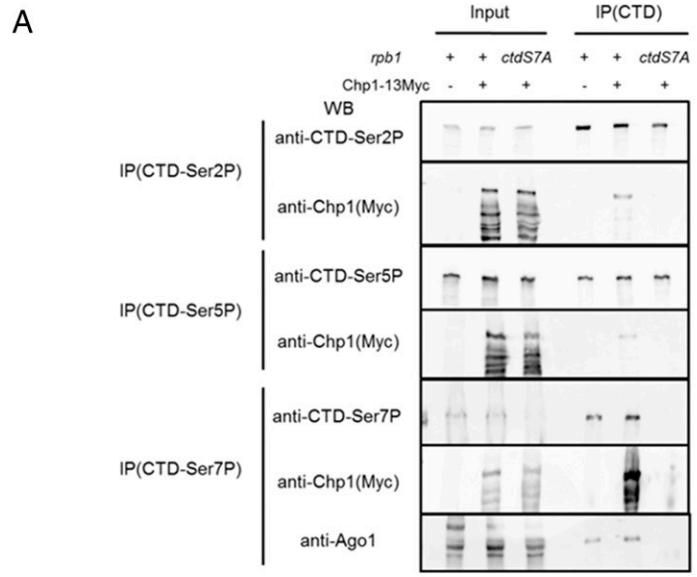
ChIP assays revealed that *dcr1*$^+$ overexpression facilitates proper H3K9me2 deposition in *ctdS7A* and *chp1-αmut1* cells (Fig. 4*D*). Furthermore, the *ctdS7A chp1-αmut1* double mutant exhibited moderate H3K9me2 deposition under *dcr1*$^+$ overexpression, reflecting the low levels of recovered siRNAs (Fig. 4 *C* and *D*). These results suggest that Ser7 and the RNA-binding activity of Chp1-CD contribute to H3K9 methylation by redundantly facilitating efficient primary siRNA generation. By contrast, cells lacking *chp1*$^+$ did not exhibit any restoration of H3K9me2 (Fig. 4*B*), confirming that an intact RITS complex is necessary to connect siRNA generation to H3K9 methylation, probably via the RITS–CLRC interaction.

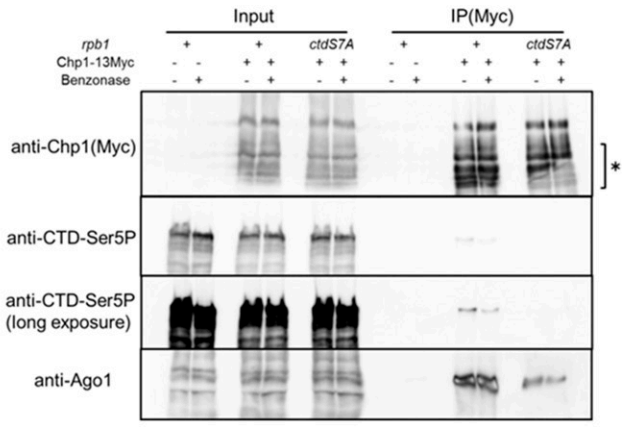
Ser7 Facilitates Secondary siRNA Amplification. Next, to examine whether Ser7 is involved in secondary siRNA amplification, we designed an artificial siRNA generation system dissecting siRNA synthesis into Dcr1-dependent primary siRNA and RITS-mediated secondary siRNA (Fig. 5*A*). In brief, overexpression of *dcr1*$^+$ supplies the primary siRNA from *dg*/*dh* repeats even in mutant cells. Insertion of three copies of the complemental sequence of the siRNA hotspot into the 3′ UTR of *ura4*$^+$ (*ura4-3ERIS*) promotes ectopic RITS targeting to the *ura4-3ERIS* transcripts, resulting in the generation of the secondary siRNA corresponding to *ura4*$^+$. The silencing assay, H3K9me2 ChIP, and Northern blot indicated that in wild-type cells this system mediates heterochromatin formation at the *ura4-3ERIS* locus according to secondary siRNA production (Fig. 5 *B*–*D*). On the other hand, *ctdS7A* cells as well as *dcr1*Δ and *rdp1*Δ cells could not form heterochromatin in this system (Fig. 5 *B*–*D*). Northern blot analysis indicated that *ctdS7A* cells fail to produce the secondary siRNAs (*ura4* siRNA) even though they produce substantial amounts of primary siRNA (Fig. 5*C* and Fig. S6*C*), indicating that Ser7 facilitates secondary siRNA amplification. Given that RITS complex directs secondary siRNA amplification via RITS–RDRC–Dcr1 interaction (11, 12) and Ser7 enhances RITS targeting to the nascent RNA (Fig. 3*B*), Ser7 would promote RITS targeting to *ura4-3ERIS* RNA. Notably, the secondary *ura4* siRNA was not produced in *clr4*Δ cells, indicating that H3K9 methylation is important for the transition from primary siRNA to secondary siRNA.

Ser7 Is Required for the Assembly of the RNAPII–RITS Complex. The experiments described above showed cooperative roles for Ser7 and RITS, particularly in the RNA-binding activity of Chp1-CD. To explore the molecular basis of this cooperative function, we investigated an interaction between RNAPII and RITS by immunoaffinity purification. When we immunoprecipitated Ser2-, Ser5-, or Ser7-phosphorylated (Ser2P, Ser5P, and Ser7P) RNAPII with antibodies specifically recognizing each phosphorylated form of CTD (Fig. S7*A*), Chp1 was coimmunoprecipitated with each form (Fig. 6*A*). Note that immunoprecipitants with anti-Ser7P had the highest Chp1 signal (more than fivefold greater than the others). Moreover, Ago1 was also associated with the Ser7P form of RNAPII (Fig. 6*A*). Importantly, Chp1 and Ago1 signals were not detected in cells that express *ctdS7A*. These results suggested that RNAPII coimmunoprecipitates with RITS in a Ser7-dependent manner.

To confirm this result, we conversely immunoprecipitated Chp1. In addition, to determine whether the interaction was mediated by nucleic acid, we treated crude lysates with Benzonase endonuclease, which removes both DNA and RNA (Fig. S7*B*), and compared the CTD signals with those prepared in the absence of Benzonase. Consistent with the former experiment, RNAPII was associated with RITS (Fig. 6*B*). Following Benzonase treatment, the RNAPII signal was still detectable, albeit weaker, demonstrating that the RNAPII–RITS assembly is partly dependent on nucleic acids. In light of our other findings, the indirect interaction appears to be mediated by RNAs retained on chromatin. Thus, the assembly of the RNAPII–RITS complex and the chromatin retention of nascent hRNAs might mutually promote each other. LC-MS/MS analysis of Chp1-interacting proteins revealed that Chp1 is further associated with components of the transcription machinery, such as components of HIRA, FACT, Mot1, Spt5, and Spt6, whose interaction decreased in *ctdS7A*-mutant cells (Fig. 6*D* and Fig. S7*C*). Since these factors function with transcribing RNAPII, this result suggests that RNAPII–RITS assembly occurs cotranscriptionally. Besides, Chp1 interacted with silencing factors related to histone deposition (51, 52) and the trimeric complex involved in processing of ITS2 (IPI complex) (53), implying that RITS composes multiple silencing complexes contributing to RNAi-dependent and -independent mechanisms.

We next investigated whether the integrity of the RITS components could affect RNAPII–RITS coimmunoprecipitation. RNAPII was immunoprecipitated with Chp1 in *ago1*Δ and *stc1*Δ cells as efficiently as in wild-type cells (Fig. S7*D*). By contrast, in *tas3*Δ cells, as in *ctdS7A* cells, RNAPII signals were indistinguishable from the background (*chp1*Δ) (Fig. S7*D*). Because Tas3 interacts with Chp1 directly, independently of Ago1 (54), the Chp1–Tas3 subcomplex is likely to be preferentially associated with RNAPII. Consistent with this, the *chp1-αmut1* mutation did not severely affect the interaction between RNAPII and Chp1 (Fig. 6*D*), because the Chp1–Tas3 subcomplex is maintained in *chp1-αmut1* cells in vivo (16). In summary, Ser7 is required for the association between RNAPII and RITS (particularly Chp1), which cotranscriptionally ensures chromatin retention of hRNAs to facilitate RNAi-dependent heterochromatin formation.





C List of identified peptides

Categories	Identified Proteins	Unique peptides		
		chp1+ rpb1+	*chp1-13myc rpb1+*	*chp1-13myc ctdS7A*
RITS complex	tas3, RITS complex subunit 3	0	132	15
	chp1, chromodomain protein Chp1	0	131	35
	ago1, argonaute	0	65	13
RNAPII	rpb2, RNA polymerase II complex subunit Rpb2	1	24	0
	rpb1, RNA polymerase II large subunit Rpb1	0	12	0
	rpb3, RNA polymerase II subunit 3	0	4	0
Transcription factors	mot1, TATA-binding protein associated factor Mot1	0	6	0
	spt5, transcription elongation factor Spt5	0	9	0
	spt6, transcription elongation factor Spt6	0	2	0
HIRA	hip1, hira protein, histone chaperone Hip1	0	35	5
	hip3, HIRA interacting protein Hip3	0	16	1
	hip4, histone promoter control protein Hip4	0	3	0
FACT	spt16, FACT complex component Spt16	0	19	1
	pob3, FACT complex component Pob3	0	6	0
Histones	htb1, histone H2B Htb1	0	6	1
	hht3, histone H3 h3.1	0	8	0
rRNA processing	crb3, WD repeat protein Crb3	0	7	0
	gar2, nucleolar protein required for rRNA processing	2	5	0
Others	top2, DNA topoisomerase II	0	5	0
	DNAJ domain protein Scj1	0	6	0

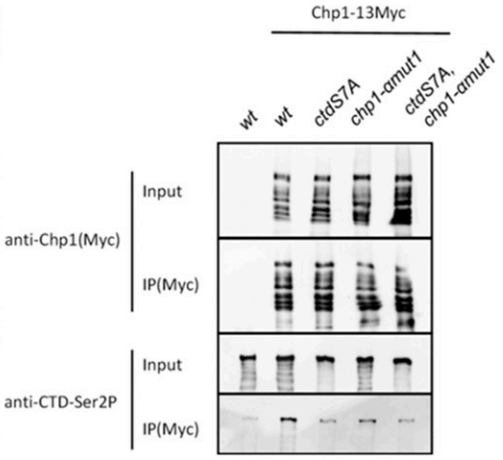


Fig. 6. Ser7 is required for the interaction between RNAPII and RITS. (*A*) Western blot analysis of Chp1-13Myc and Ago1 associated with RNAPII. Each phosphorylated form of the CTD was immunoprecipitated with anti-Ser2P, -Ser5P, and -Ser7P antibodies and then blotted with anti-Myc or anti-Ago1 antibodies. (*B*) Western blot analysis of Ser5P and Ago1 associated with Chp1-13Myc. Crude lysates were treated with (+) or without (–) 300 U/mL Benzonase endonuclease in 2 mM Mg^{2+} for 30 min, and then Chp1-13Myc was immunoprecipitated. An asterisk indicates degradation products of Chp1-13Myc. (*C*) List of peptides identified by LC-MS/MS analysis. Magenta shading indicates silencing factors related to RNAi function. Blue shading indicates characterized silencing factors related to histone deposition. Yellow shading indicates silencing factors related to the IPI complex. (*D*) Western blot analysis of Ser2P associated with Chp1-13Myc in *ctdS7A* and *chp1-αmut1* mutants.

Discussion

Our findings demonstrated that Ser2 and Ser7 of the RNAPII-CTD have novel but distinct CTD code functions in cotranscriptional gene silencing within heterochromatic regions. Ser2 is involved in heterochromatic silencing but does not participate in heterochromatin formation, while Ser7 is involved in RNAi-dependent heterochromatin formation. The major findings of our study regarding the function of Ser7 are as follows: (*i*) Ser7 is critical for the efficient generation of siRNA, which directs subsequent RNAi-dependent heterochromatin formation; (*ii*) Ser7 ensures chromatin retention of nascent hRNAs redundantly with the RNA-binding activity of Chp1-CD; (*iii*) Ser7 directs RITS targeting to the nascent RNA, facilitating secondary siRNA amplification; (*iv*) Ser7 promotes assembly of the RNAPII–RITS complex.

Based on these findings, we propose a model of cotranscriptional chromatin retention of hRNA by CTD Ser7 and the RNA-binding activity of Chp1-CD for the secondary siRNA amplification (Fig. 7). First, the Ser7-dependent RNAPII–RITS complex accompanied by

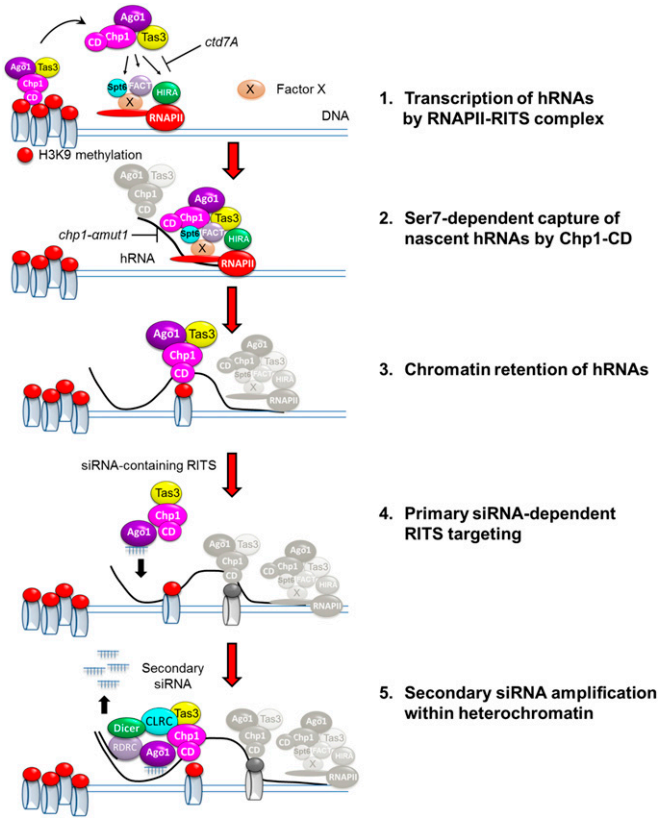


Fig. 7. Model of chromatin retention of hRNA mediated by Ser7, Chp1-CD, and the generation of siRNA in *cis*. The figure presents a stepwise explanation of Ser7 function in siRNA biogenesis at pericentromeric repeats. See text for details.

transcription machineries is assembled, and subsequently hRNAs are transcribed by the RNAPII–RITS complex (Fig. 7, step1). Second, the RNAPII-associated RITS cotranscriptionally captures nascent hRNAs through the RNA-binding motif of the Chp1-CD (Fig. 7, step 2). The interaction of Chp1-CD with H3K9 methylation further reinforces chromatin retention of hRNAs (Fig. 7, step 3). The retained hRNAs are likely to be a preferential target of siRNA-containing RITS (Fig. 7, step 4). Finally, hRNAs retained on chromatin provide scaffolds for the assembly of factors promoting secondary siRNA amplification (Fig. 7, step 5), and the following siRNA-dependent RITS–CLRC interaction leads to locus-specific H3K9 methylation. Besides, once established, H3K9 methylation acts as storage of Chp1, enabling efficient formation of the RNAPII–RITS complex at heterochromatin (Fig. 7, step 1). In addition, since H3K9 methylation enhances the association between Chp1-CD and RNAs (16), accelerated secondary siRNA amplification seems to occur only at heterochromatin, resulting in the maintenance of heterochromatin. In *ctdS7A* cells, RNAPII cannot form a complex with RITS, whereas in *chp1-amut1* cells, the Chp1-CD cannot capture the hRNAs even though the RNAPII–RITS complex is assembled.

Ser7 Links hRNA Transcription to Secondary siRNA Amplification. In our model, Ser7 facilitates heterochromatin formation by linking hRNA transcription to secondary siRNA amplification. The RNAPII–RITS assembly and accompanying chromatin retention of hRNAs appear to occur cotranscriptionally, because we identified components of the transcription machinery as Ser7-dependent Chp1 interactors (Fig. 6*C*). The hRNAs captured by RITS on chromatin, in turn, may partly contribute to the protein–protein interaction between RNAPII and RITS (Fig. 6*B*). The RNAPII–RITS association presumably mediates the stable assembly of the RITS–RDRC–Dcr1–hRNA complex in collaboration with a connector protein, Dsh1 (14). The hRNAs in the complex are close to Dcr1, thus achieving efficient secondary siRNA synthesis. In this way, Ser7 provides the molecular basis for linking hRNA transcription to secondary siRNA biogenesis on chromatin. Similar to the interaction between RNAPII and RITS in *S. pombe*, the interaction between RNAPII and Argonaute family proteins is evolutionally conserved in *Homo sapiens* (55), *D. melanogaster* (56), and *A. thaliana* (57). In *A. thaliana*, Pol V interacts with Argonaute proteins (58–60). Therefore, it would be worthwhile to test the effect of Ser7 on RNAi and heterochromatin formation in multiple species.

Possible Mechanisms of Ser7 Residues. We revealed the important role of Ser7 in connecting RNAPII to RNAi. There are two possible mechanisms through which Ser7 itself (possibly in the unphosphorylated state) or phosphorylation at Ser7 is important for the function at this stage. Our observation that the Ser7P CTD associated efficiently with RITS (Fig. 6*A*) appears to support the importance of phosphorylation at Ser7. In addition, the inhibition of Cdk9 that would be responsible for Ser7 phosphorylation resulted in the loss of hRNA silencing and siRNA production (61). In mammals, besides Ser5, P-TEFb/Cdk9 phosphorylates Ser7 (31, 62). However, it is difficult to conclude that phosphorylation at Ser7 by Cdk9 is a single cause of recruiting RNAi factors, because Cdk9 is a multitarget kinase. Alternately, it is also possible that the amino acid residue Ser7 itself is crucial for the function. This possibility is supported by a recent observation in *A. thaliana*: An atypical RNAPII-like polymerase Pol V interacts with AGO4 via the CTD of the largest subunit of Pol V independently of CTD phosphorylation (58, 63).

With respect to linking transcription to RNAi, the RITS complex may recognize Ser7 or Ser7P. Alternately, unknown factors (factor X in Fig. 7) may connect RNAPII to RITS in a Ser7-dependent manner. Importantly, the suppressive phenotypes of the truncated *ctdS7Ax18* cells (Fig. S3 *A* and *B*) raise the possibility that Ser7 is not a direct binding target of RITS but changes the state of transcribing RNAPII to facilitate the RNAPII–RITS interaction. Although there are many possible mechanisms for the interaction between RNAPII and RITS (particularly Chp1), the important notion in this study is that Ser7 mediates the association cotranscriptionally, which ultimately promotes the cotranscriptional formation of heterochromatin. Further biochemical analysis will reveal the role of Ser7 in the RNAPII–RITS interaction.

Materials and Methods

Strain Construction. *S. pombe* strains used in this study are listed in Table S3. Construction of *ctdS2A* and *ctdS7A* was described previously (24).

Silencing Assay. Silencing assay was performed as described previously (64).

RIP Assay. RIP was performed as described previously (17).

Microarray Analysis. RNA preparation, labeling, hybridizations, and scanning were performed as described previously (65). Sequences of probes and the original data from the microarray experiments were deposited in the Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/geo/) with accession number GEO53568.

Northern Blot for Small RNAs. Northern blot analysis was performed as described previously (14) with modifications.

Supporting Information. Supporting Information includes *SI Materials and Methods*, supporting references, seven figures, and four tables.

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