

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

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Shorter title: Ser7 of RNAPII-CTD promotes RNAi

Key words

heterochromatin; RNAi; non-coding RNA; RNA polymerase II.

Abstract

Some long non-coding 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, Ser7 of the C-terminal domain 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 (Chp1-CD), 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 co-transcriptional chromatin retention of hRNA by recruiting the RNA-chromatin connector protein Chp1, which facilitates RNAi-dependent heterochromatin formation. Our findings reveal a novel function of the CTD code: linking ncRNA transcription to RNAi for heterochromatin formation.

Significant statement

Some non-coding RNAs (ncRNA) 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 an area of great interest. Here, we found that in *S. pombe*, Ser7 of C-terminal domain (CTD) of RNAPII is involved in locus specific siRNA amplification within heterochromatin and also 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 novel principle of epigenetic regulation by ncRNAs that RNAPII-CTD links hRNA transcription to RNAi for heterochromatin formation.

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Introduction

Eukaryotic genomes encode large numbers of non-coding RNAs (ncRNAs) that are transcribed by RNA polymerase II (RNAPII). Some of these ncRNAs regulate gene expression by recruiting activating or repressive epigenetic modifiers; 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 process 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 bi-directionally at low level, which induces heterochromatin formation through the RNAi machinery (4-6).

The initial step of heterochromatin formation consists in the generation of double-stranded RNA (dsRNA) by hybridization with anti-sense transcripts and/or formation of hairpin structure, which are directly processed into primary small interfering RNAs (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 RNA dependent RNA polymerase complex (RDRC) onto heterochromatin which synthesizes dsRNA from hRNA (11). This dsRNA synthesis is coupled with secondary siRNA generation by Dcr1 around 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 co-transcriptionally act 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 in *A. thaliana*, *N. crassa*, *C. elegans*, *D. 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 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 unmodified state of each residue, collectively referred to as the CTD code, are deciphered by various interacting proteins to

regulate transcription and co-transcriptional events such as activating histone modifications and nascent RNA processing (19, 20).

Among various elements of the CTD code, unphosphorylated state at Ser2 and Ser5 is required for preinitiation complex formation and mediator recruitment at promoter (21-24). 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 promoter in mammals (26, 27). P-TEFb/Cdk9, Cdk12 and Cdk13 predominantly phosphorylate Ser7 pre-phosphorylated CTD (28-30). In *S. pombe*, Ser7 mutations in truncated CTD alter gene expression of phosphate homeostasis (32, 33) and subtelomeric genes (34). Meanwhile, with respect to cooperation of hRNA transcription and co-transcriptional hRNA processing, it has remained elusive whether the CTD codes contribute to exertion of RNAi-mediated heterochromatin formation.

Despite the importance of CTD codes, technical difficulties have precluded a comprehensive analysis of each residue: high copy repetitive units, substrate specificity of CTD kinases, and redundant phosphorylation by several kinases (35-37). Even more complicated, 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 non-phospho acceptor mutation but also perturb unphosphorylated state. Consequently, we found that Ser2 and Ser7 are responsible for 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 facilitate efficient secondary siRNA amplification. The cooperation was ensured by a Ser7-dependent association between RNAPII and RITS. Our findings reveal a novel 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 substituted with alanine that abrogates both phosphorylated and unphosphorylated states (*ctdS2A*, and *ctdS7A*; Fig. 1A) (42, 43). In addition to *ctdS2A* and *ctdS7A* mutant, we used *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. 1B). Repression of *otr::ade6⁺* by heterochromatin in the wild-type resulted in inhibited adenine biosynthesis, thereby formation of red colonies on medium containing a limiting amount of adenine (Low Ade). By contrast, heterochromatin mutants, such as *clr4Δ*, *dcr1Δ*, and *rpb2-m203*, formed white or pink colonies on Low Ade plates (Fig. 1C and Fig. S1A). All of the RNAPII mutants produced a mixture of red, pink, and white colonies with the exception of *rpb1-11* mutant (Fig. S1A). The variegated phenotype indicated the

silent and active states were metastable in the *ctdS2A* and *ctdS7A* mutants. Note that all of the RNAPII mutants proliferated almost normally under standard vegetative growth conditions (Fig. 1C). To obtain unambiguous results, white clones derived from each mutant were used in subsequent analyses. qRT-PCR of *ctdS2A* and *ctdS7A* mutants confirmed accumulation of hRNAs bi-directionally transcribed from *dh* pericentromeric repeats and forward transcripts of *otr::ade6⁺* (Fig. 1D and Fig. S1B).

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 to the corresponding signal in the wild-type cells. We found that *ctdS2A* affects expression of a subset of genes including meiotic genes and antisense RNAs as previously described (42). Similarly, *ctdS7A* caused change of a limited number of genes (Table S1), which include stress-response genes. In agreement with these results, *ctdS2A* and *ctdS7A* cells did not show obvious growth defect on a non-selective (N/S) plate (Fig. 1C). As expected from the results shown in Fig. 1 C and D, up-regulated genes more than 4-fold of each

mutant included a large population of pericentromeric ncRNAs (Table S1). The scatter plots comparing each mutant to the wild-type cells revealed that expression levels of some, but not all pericentromeric ncRNAs were markedly up-regulated in *ctdS2A* (4- to 8-fold) or *ctdS7A* (4- to 13-fold) cells (Fig. 1E). These results indicated that Ser2 and Ser7 are both 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 to that of *dcr1Δ* cells. Comparison by Venn diagrams and scatter plots showed that changes of gene expression in *dcr1Δ* cells were more similar to those in *ctdS7A* cells than those in *ctdS2A* cells (Fig. 1E , Fig. S1C, 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 2-fold changes in expression in the *ctdS2A* or *ctdS7A* mutants (four and three out of 115 genes, respectively (Table S2). Particularly, *ctdS7A* mutation did not to reduce the expression of gene participating in heterochromatic silencing. Hence, it is

unlikely that the CTD mutants impair heterochromatic silencing by affecting the expression of heterochromatic genes.

Ser2 and Ser7 of CTD play distinct roles for pericentromeric silencing

To assess the chromatin state around pericentromeric ncRNA genes in *ctdS2A* and *ctdS7A* cells, we used Chromatin immunoprecipitation (ChIP) assays to estimate the levels of repressive epigenetic marks: H3K9 dimethylation (H3K9me2), H3K9 trimethylation (H3K9me3), the HP1 family protein Swi6 (Fig. 2A and Fig. S1D). The truncated CTD mutant *rpb1-11* did not exhibit a remarkable reduction in H3K9me2 deposition nor Swi6 localization at either *dh* or *otr::ade6⁺*, except slight reduction of H3K9me3 at *dh* (Fig. 2A and Fig. S1D), indicating that the first 16 heptads are sufficient to form heterochromatin structure. Similarly, the *ctdS2A* mutation did not affect the enrichment of repressive epigenetic marks at *dh* repeats, albeit it moderately decreased them at *otr::ade6⁺* (Fig. 2A,B and Fig. S1D). Rik1, a component of the CLRC methyltransferase complex accumulated at high levels at *dh* and slightly decreased at *otr::ade6⁺* (Fig. S1E), indicating almost complete heterochromatin structure was retained in *ctdS2A* mutant. On this basis, we concluded that Ser2

does not contribute to 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 loss of silencing independently of heterochromatin structure.

In contrast to *ctdS2A*, *ctdS7A* mutation caused impairment of heterochromatin formation. In *ctdS7A* cells, levels of H3K9me_{2/3} and Swi6 were reduced (Fig. 2A and Fig. S1D): on *dh* repeats, they decreased by approximately 50%, while at *otr::ade6⁺*, they were markedly diminished to the nearly same extent as in *clr4Δ* and *dcr1Δ* cells (Fig. 2A, B and Fig. S1D, E). Residual H3K9me₂ on *dh* repeats is likely to be a common feature of RNAi mutants such as *dcr1Δ* (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

Similarity of the phenotypes between *ctdS7A* and *dcr1Δ* 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. 2C). In purified Ago1 complex 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 *clr4Δ*, *dcr1Δ*, and *ctdS7A* cells, indicating that siRNA generation was impaired in *ctdS7A* cells.

Furthermore, CHIP and RIP experiments revealed that RNAi machineries, such as Chp1, Ago1, and Rdp1 were not properly assembled at pericentromeric heterochromatin in *ctdS7A* cells, as were in *dcr1Δ* cells (Fig. 2B, 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* mutants cells

We next questioned whether the defects of *ctdS7A* are due to loss of phosphorylation at Ser7. To explore this, we tried to use phospho-mimetic mutants in which Ser7 was substituted with either Glutamic acid (*ctdS7E*) or Aspartic acid (*ctdS7D*) but failed to construct the full-length mutants, even in diploid cells. This suggested that the constitutive hyper-phosphorylation is toxic to cells and the balance of phosphorylation/dephosphorylation at Ser7 is important as reported in budding yeast and mammalian cells (45, 46). Since it was reported that truncated 18 repeats of *ctdS7E* (*ctdS7Ex18*) mutant in fission yeast 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 both *dh* and *otr::ade6⁺* (Fig. S3A). Interestingly, in contrast to the full length *ctdS7A* cells, the truncated *ctdS7Ax18* cells retained the substantial amount of H3K9me_{2/3} at both *dh* and *otr::ade6⁺* regions (Fig. S3A; 34). On the other hand, *ctdwtx18 dcr1Δ* and *ctdS7Ax18 dcr1Δ* double mutant

cells showed marked reduction at *dh* and *otr::ade6⁺* of H3K9me2/3 as nearly comparable level in *dcr1Δ* cells (Fig. S3A). Consistent with the ChIP assay, the *dg/dh* siRNAs in *ctdS7Ax18* were detected, while the siRNAs in full length mutant of *ctdS7A* cells were hardly detected (Fig. S3B). These observations indicated that truncation of CTD suppresses the failure in siRNA generation and heterochromatin formation seen in a full-length *ctdS7A* mutant. The difference of H3K9 methylation in *ctdS7A* between our data (Fig. 2A) and previously reported data (34) is most likely due to the length of CTD repeats, reinforcing the notion that truncated CTD mutants should be used with caution (43) and they are inappropriate to analyze the function of Ser7. Hereafter, we analyze the function of Ser7 using full length of CTD mutants.

Ser7 enables stable binding of hRNA to Chp1

All the data shown above strongly suggested that *ctdS7A* mutation abrogated pericentromeric heterochromatin formation through disturbance of 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. 3A). Silencing assay, qRT-PCR, and ChIP for H3, H3K9me2, and Chp1 confirmed that in *ctdS7A* and *dcr1Δ* cells, heterochromatin and silencing state at the *Kint2::ura4⁺* locus were not defective (Fig. S4A-E), supporting our assumption of Ser7 functions in 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 RNA immunoprecipitation (RIP) analysis. In the wild-type and even in *dcr1Δ* cells, Chp1 captured significant levels of *Kint2::ura4⁺* RNAs, but not *act1⁺* RNAs (Fig. 3B). By contrast, in *clr4Δ* cells, *Kint2::ura4⁺* transcripts were no longer captured by Chp1 (Fig. 3B). These results suggested that the interaction between hRNA and Chp1 does not depend on siRNA generation, but does on H3K9 methylation,

which may provide a scaffold for Chp1 to capture nascent hRNAs on chromatin. Interestingly, in *ctdS7A* cells, the amount of *Kint2::ura4⁺* transcripts captured by Chp1 was 3-fold lower than in the wild-type cells (Fig. 3B), despite the substantial enrichment of Chp1 at *Kint2::ura4⁺* in this mutant (Fig. S4E). These findings indicated that Ser7 plays a crucial role in the capture of hRNAs by RITS. Note that small amount of signals specifically found in heterochromatic *Kint2::ura4⁺* transcripts in the absence of Reverse Transcriptase (Figure 3B; RT-) are derived from DNA/RNA hybrid structure, which are significantly decreased when samples were treated with RNase H (17).

Previous work showed that Chp1-CD is not only 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-amut1* mutant that specifically loses the ability to interact with RNA (16). We first realized the phenotypic similarity between *ctdS7A* and *chp1-amut1* mutants in terms of the silencing at *otr::ade6⁺*; *chp1-amut1* cells as well as *ctdS7A* cells showed variegated phenotype of *otr::ade6⁺* gene expression on Low Ade plate (Fig. 3C and Fig.

S5A, B). The frequencies of appearance of red, pink, or white colonies in *chp1-amut1* cells and *ctdS7A* cells in the absence of any selection (Fig. S5B) and after isolation of white or red clones (Fig. 3C) were quite similar, which suggested *ctdS7A* and *chp1-amut1* mutants have similar silencing defects. In agreement with this, patterns of H3K9me2 in *chp1-amut1* cells and in *ctdS7A* cells were nearly comparable (Fig. S5C). Hereafter, we used white clones of *chp1-amut1* mutant for further analyses.

Next, we analyzed the effect of *chp1-amut1* mutation on the capture of hRNA by RITS. Remarkably, the *chp1-amut1* mutation caused a reduction in the interaction level between Chp1 and *Kint2::ura4⁺* transcripts to the same extent as the *ctdS7A* mutation (Fig. 3B). In *ctdS7A chp1-amut1* double mutant, the Chp1 RIP signal of *Kint2::ura4⁺* transcripts was comparable to that in either single mutant (Fig. 3B). 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 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. 2C) and promotes the interaction between hRNA and Chp1-CD (Fig. 3B). Furthermore, the bivalent function of Chp1-CD in H3K9 methylation binding 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 generation of siRNAs on chromatin.

To test this hypothesis, we analyzed the effect of *ctdS7A* and *chp1-amut1* mutants on the chromatin retention of heterochromatic *Kint2::ura4⁺* transcripts by histone H3 RIP analysis (17). H3 RIP signals in both *ctdS7A* and *chp1-amut1* cells, which reflect the amount of chromatin retained *Kint2::ura4⁺* transcripts, declined by approximately 4-fold relative to the wild-type, suggesting that Ser7 and the RNA-binding activity of Chp1-CD play critical roles in proper chromatin retention of hRNAs (Fig. 3D). Furthermore, the *ctdS7A chp1-amut1* double

mutant also exhibited marked reduction to the similar level as either single mutation alone (Fig. 3D). These results suggested that Ser7 and the RNA-binding activity of Chp1-CD play important role(s) in ensuring chromatin retention of hRNAs (Fig. 3B and D). Note that the reduction of H3 RIP signal in *ctdS7A* cells is due to a decrease of the chromatin retention of *Kint2::ura4⁺* transcripts, but not due to an increase of total transcripts, because the *Kint2::ura4⁺* transcript was repressed in *ctdS7A* cells to the same level as in the wild-type and *dcr1Δ* cells (Fig. S4B). In H3-RIP assay, significant amount of RNA bound to H3 in *dcr1Δ* cells, in which no siRNA were formed. In *ctdS7A* cells and *chp1-amut1* cells, the amount of H3-bound RNA was much lower than that in *dcr1Δ* cells, indicating that low siRNA was not the reason of defects in chromatin retention of *Kint2::ura4⁺* RNA in these mutants (Fig. 3D and E). By contrast, *clr4Δ* severely impaired chromatin retention of hRNA (Fig. 3E), indicating that H3K9 methylation is essential for stable chromatin retention of hRNA.

Considering the interaction between Chp1-CD and H3K9 methylation, chromatin retention seems to assure maintenance of heterochromatin by activated siRNA generation at once established locus.

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 4-fold lower than the wild-type level) (Fig. 3E), suggesting that an intact RITS complex mediates chromatin retention of hRNA though siRNA is not required for the retention.

Dcr1 overexpression rescues primary siRNA generation in *ctdS7A* and *chp1-amut1*

The initial step of heterochromatin formation is the primary siRNA generation from intrinsic hairpin structure of the 5' region of *dg/dh* RNAs and/or annealed bi-directional *dg/dh* transcripts. Subsequently, RITS-RDRC-Dcr1 interaction promotes the secondary siRNA amplification (12). Regarding the primary siRNA biogenesis, others and we previously indicated that overexpression of *dcr1*⁺ the mutant cells of RITS, RDRC or CLRC caused marked siRNA generation (14, 49, 50; Fig. 4A and Fig. S6B), indicating that Dcr1 can produces primary siRNA without RITS, RDRC or CLRC. Then, we next investigated whether Ser7 and the RNA-binding activity of Chp1-CD affect this Dcr1-dependend primary siRNA generation. We performed *dcr1*⁺ overexpression under the control of the *urg1* promoter (Fig. S6A) in various mutants including *ctdS7A*. In *clr4* Δ cells,

substantial restoration of pericentromeric siRNA generation (to nearly wild-type levels) was observed, showing H3K9 methylation is not required for primary siRNA biogenesis (Fig. 4A and Fig.S6B). In *ctdS7A* cells, *dcr1*⁺ overexpression resulted in significant recovery of pericentromeric siRNA generation (Fig. 4A and Fig.S6B), showing that *ctdS7A* cells can still generate Dcr1-dependent primary siRNAs. Similar recovery of siRNA synthesis was also observed in *ago1Δ* and *chp1Δ* cells. Note that *dcr1*⁺ overexpression promoted more efficient secondary siRNA amplification in *ctdS7A*, *ago1Δ*, and *chp1Δ* cells than in *rdp1Δ* 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 RDRC (12).

Next, we examined whether siRNA recovery upon *dcr1*⁺ overexpression would be sufficient to promote H3K9 methylation. In *rdp1Δ* cells, *dcr1*⁺ overexpression resulted in slight accumulation of H3K9me2 at *otr::ade6*⁺ (Fig. 4B), reflecting the small amount of siRNA produced by *dcr1*⁺ overexpression. By contrast, in *ago1Δ* and *chp1Δ* cells, despite significant recovery of pericentromeric siRNAs upon *dcr1*⁺ overexpression, the level of H3K9me2 at *otr::ade6*⁺ was not elevated (Fig. 4B), indicating that 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. 4B). 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-amut1* single mutants, and the *ctdS7A chp1*Δ and *ctdS7A chp1-amut1* double mutants. siRNA production under *dcr1*⁺ overexpression in each single mutant was restored to the wild-type levels (Fig. 4C). Interestingly, the *ctdS7A chp1-amut1* double mutants generated smaller amount of siRNAs with *dcr1*⁺ overexpression than that in the single mutants (Fig. 4C) which is contrary to the results of Chp1-RIP and H3 RIP assay; 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). Similar reduction of siRNA was observed in *ctdS7A chp1*Δ double mutant (Fig. 4C). 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-amut1* cells (Fig. 4D). Furthermore, the *ctdS7A chp1-amut1* double mutant exhibited moderate H3K9me2 deposition under *dcr1*⁺ overexpression, reflecting the low levels of recovered siRNAs (Fig. 4C 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. 4B), 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. 5A). In brief, overexpression of *dcr1*⁺ supplies the primary siRNA from *dg/dh* repeats even in mutant cells. Insertion of 3 copies of the complementally sequence of

siRNA hotspot into 3' UTR of *ura4*⁺ (*ura4-3ERIS*) promotes ectopic RITS targeting to the *ura4-3ERIS* transcripts, resulting in generation of the secondary siRNA corresponding to *ura4*⁺. Silencing assay, H3K9me2 ChIP, and northern blot indicated that in wild-type cells, this system mediates heterochromatin formation at *ura4-3ERIS* locus according to secondary siRNA production (Fig. 5 B-D). On the other hand, *ctdS7A* cells as well as *dcr1Δ* and *rdp1Δ* 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 amount of primary siRNA (Fig. 5C and Fig. S6C), indicating that Ser7 facilitates secondary siRNA amplification. Considering the facts that RITS complex directs secondary siRNA amplification via RITS-RDRC-Dcr1 interaction (11, 12) and Ser7 enhances RITS targeting to the nascent RNA (Fig. 3B), Ser7 would promotes RITS targeting to *ura4-3ERIS* RNA. Notably, the secondary *ura4* siRNA was not produced in *clr4Δ* cells, despite the fact that Chp1 captures *ura4-3ERIS* RNA at the same level as that of wild-type cells, indicating that H3K9 methylation is important for primary siRNA to secondary siRNA transition.

Ser7 is required for the assembly of RNAPII-RITS complex

The experiments described above showed cooperative role of Ser7 and RITS particularly 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 RNAPII (Ser2P, Ser5P, and Ser7P) with antibodies specifically recognizing each phosphorylated form of CTD (Fig. S7A), Chp1 was co-immunoprecipitated with each form (Fig. 6A). Note that immunoprecipitants with anti-Ser7P had the highest Chp1 signal (more than 5-fold greater than the others). Moreover, Ago1 was also associated with the Ser7P form of RNAPII (Fig. 6A). Importantly, Chp1 and Ago1 signals were not detected in cells that express *ctdS7A*. These results suggested that RNAPII co-immunoprecipitates 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. S7B) and compared the CTD signals with those prepared in the absence of Benzonase®. Consistent with the former experiment, RNAPII was

associated with RITS (Fig. 6B). 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 RNAPII-RITS complex and 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. S7C.). Since these factors function with transcribing RNAPII, this result suggests that RNAPII-RITS assembly occurs co-transcriptionally. Besides, Chp1 interacted with silencing factors related to histone deposition (51, 52) and IPI complex (53), implying the possibility that RITS composes multiple silencing complex contributing to RNAi-dependent and-independent mechanisms.

We next investigated whether the integrity of the RITS components could affect the RNAPII-RITS co-immunoprecipitation. In *ago1Δ* and *stc1Δ* cells, RNAPII was immunoprecipitated with Chp1 as efficiently as in the wild-type cells (Fig. S7D). By contrast, in *tas3Δ* cells, as in *ctdS7A* cells, signals of RNAPII were

indistinguishable from the background (*chp1Δ*) (Fig. S7D). Because Tas3 directly interacts with Chp1 independently of Ago1 (54), the Chp1-Tas3 subcomplex is likely to be preferentially associated with RNAPII. Consistent with this, the *chp1-amut1* mutation did not severely affect the interaction between RNAPII and Chp1 (Fig. 6D), because the Chp1-Tas3 subcomplex is maintained in *chp1-amut1* cells *in vivo* (16). In summary, Ser7 is required for the association between RNAPII and RITS (particularly Chp1), which co-transcriptionally ensures chromatin retention of hRNAs to facilitate RNAi-dependent heterochromatin formation.

Discussion

Our findings demonstrated that Ser2 and Ser7 of RNAPII-CTD have novel but distinct functions of CTD code in co-transcriptional gene silencing within heterochromatic regions. Ser2 is involved in the heterochromatic silencing, but

does not participate in heterochromatin formation, while Ser7 is involved in the RNAi-dependent heterochromatin formation. The major findings of our study about the function of Ser7 are as follows: (1) Ser7 is critical for efficient generation of siRNA which directs subsequent RNAi-dependent heterochromatin formation; (2) Ser7 ensures chromatin retention of nascent hRNAs redundantly with the RNA-binding activity of Chp1-CD; (3) Ser7 directs RITS targeting to the nascent RNA, facilitating secondary siRNA amplification; (4) Ser7 promotes assembly of RNAPII-RITS complex.

Based on these findings, we propose a novel model of co-transcriptional chromatin retention of hRNA by the CTD Ser7 and the RNA-binding activity of Chp1-CD for the secondary siRNA amplification (Fig. 7). Firstly, Ser7-dependent RNAPII-RITS complex accompanied with transcription machineries is assembled, and subsequently hRNAs are transcribed by the RNAPII-RITS complex (Fig. 7; Step1). Secondly, the RNAPII-associated RITS co-transcriptionally captures nascent hRNAs through the RNA-binding motif of Chp1-CD (Fig. 7; Step2). The interaction of Chp1-CD with H3K9 methylation further reinforces chromatin retention of hRNAs (Fig. 7; Step3). The retained hRNAs are likely to be a preferential target of siRNA-containing RITS (Fig. 7;

Step4). Finally, hRNAs retained on chromatin provide scaffolds for assembly of factors promoting secondary siRNA amplification (Fig. 7; Step5) 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 RNAPII-RITS complex at heterochromatin (Fig. 7; Step1). In addition, since H3K9 methylation enhances association between Chp1-CD and RNAs (16), accelerated secondary siRNA amplification seems to occur only at heterochromatin, resulting 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 co-transcriptionally, because we identified components of the transcription machinery as Ser7-dependent Chp1 interactors (Fig. 6C). The hRNAs captured

by RITS on chromatin, in turn, may partly contribute to the protein-protein interaction between RNAPII and RITS (Fig. 6B). 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 molecular basis of linking hRNA transcription to secondary siRNA biogenesis on chromatin. Similar to the interaction between RNAPII and RITS in *S. pombe*, interaction between RNAPII and Argonaute family proteins is evolutionally conserved in *H. 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 in multiple species on RNAi and heterochromatin formation.

Possible mechanisms of Ser7 residues

We revealed the important role of Ser7 in connecting RNAPII to RNAi. There exist two possible mechanisms that Ser7 itself (possibly unphosphorylated state) or phosphorylation at Ser7 is important for the function at this stage. Our observation that Ser7 phosphorylated CTD efficiently associated with RITS (Fig. 6A) appears to support the importance of phosphorylation at Ser7. In addition,

inhibition of Cdk9 that would be responsible for Ser7 phosphorylation resulted in loss of hRNA silencing and siRNA production (61). We did confirm that inhibition of Cdk9 decreases the amount of phosphorylation at Ser7 as well as at Ser2 (data not shown). However, it is difficult to conclude that phosphorylation at Ser7 by Cdk9 is a single cause of recruiting RNAi factors, because Cdk9 is a multi-target kinase. Alternately, it is also possible that amino acid residue Ser7 itself is crucial for the function. This possibility is supported by the recent observation in *A. thaliana*: an atypical RNAPII-like polymerase Pol V interacts with AGO4 via CTD of the largest subunit of Pol V independently on phosphorylation of CTD (58, 62)

With respect to linking transcription to RNAi, RITS complex may recognize Ser7 or phosphorylated Ser7. Alternately, unknown factors (factor X in Fig. 7) connect RNAPII to RITS in Ser7-dependent manner. Importantly, the suppressive phenotypes of the truncated *ctdS7Ax18* (Fig. S3A and B) raise a 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

co-transcriptionally, which ultimately promotes co-transcriptional formation of heterochromatin. Further biochemical analysis will reveal the role of Ser7 in RNAPII-RITS interaction.

Materials and Methods

Strain construction

S. pombe strains used in this study are listed in Supplemental Table S3.

Construction of *ctdS2A* and *ctdS7A* was described previously (24).

Silencing assay

Silencing assay was performed as described previously (63).

RNA immunoprecipitation (RIP) assay

RNA immunoprecipitation was performed as described previously (17).

Microarray analysis

RNA preparation, labeling, hybridizations, and scanning were performed as described previously (64). Sequences of probes and the original data from the

microarray experiments were deposited in GEO

(<http://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 can be found with this article online at *SI*.

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Figure Legends

Fig. 1. Unphosphorylatable mutations of CTD Ser2 and Ser7 cause loss of pericentromeric silencing

(A) Amino acid sequences of 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 5-fold serial dilutions on YES (non-selective, N/S) or YES without Adenine (Low Ade).

(D) Quantitative real-time RT-PCR (qRT-PCR) of *otr::ade6⁺* and *dh* forward transcripts (*dhFW*) 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⁺*. Signals of qRT-PCR were normalized against *act1⁺* and are presented as the fold increase relative to

the wild-type (defined as 1). Error bars indicate S.E.M from three biologically independent experiments.

(E) Scatter plot comparing gene expression in *ctdS2A*, *ctdS7A*, or *dcr1Δ* cells with that of the wild-type cells. Blue rhombuses and red square boxes indicate expression levels of all transcripts or pericentromeric ncRNA, respectively, calculated as the $\log_2$ (ratio).

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 % Input.

Error bars indicate S.E.M from three independent experiments.

(C) Northern blot detecting siRNA derived from pericentromeric *dg* and *dh* transcripts. Upper panel: 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 panel: 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 % Input. RIP signals of *act1⁺* are negative control. Error bars indicate S.E.M from three independent experiments.

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 *K* region. The small bar indicates the position of the primer set used for the 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. 2E using site-specific primers for *Kint2::ura4⁺*. Error bars indicate S.E.M from three biologically independent experiments.

(C) Stability of the variegated phenotype of *otr::ade6⁺* silencing. White and red colonies were picked and re-streaked onto Low Ade plates, and colonies with the indicated colors were counted in 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 were identical to those in Fig. 3B.

Fig. 4. Mutation at Ser7 and RNA-binding activity of Chp1-CD was rescued by *dcr1⁺* overexpression

(A and C) Northern blot 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 were identical to those in Fig. 2C. Untrimmed raw data of (A) is Fig. S6B.

(B and D) ChIP assay 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 were identical to those in Fig. 2A. Error bars indicate S.E.M from three biologically independent experiments.

Fig. 5. Ser7 promotes secondary siRNA amplification.

(A) Schematic diagram of the artificial heterochromatin formation assay. 3 copies of DNA sequences ERIS, which Dcr1-dependent primary siRNAs preferentially targets to, were inserted into 3' UTR of endogenous *ura4⁺* gene. To reinforce the primary siRNA generation, *dcr1⁺* were overexpressed under *urg1⁺* promoter. The primary siRNA induces the secondary siRNA which are corresponding to *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-FOA. Silencing states of *otr::ade6⁺* was simultaneously monitored on 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 were identical to those in Fig. 2A. Error bars indicate S.E.M from three biologically independent experiments.

(D) Northern blot of the indicated strains in the presence (+) or absence (-) of *dcr1*⁺ overexpression (*dcr1-o/e*) in the indicated strains. Details of the northern blot were identical to those in Fig. 4A. Untrimmed raw data of (D) is Fig. S6C.

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 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 Mg²⁺ 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. Red 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 IPI complex.

(D) Western blot analysis of Ser2P associated with Chp1-13Myc in *chp1-amut1* mutants. RITS mutants (*chp1* Δ , *ago1* Δ , and *tas3* Δ) and *stc1* Δ (C) or (D).

Fig. 7. Model of chromatin retention of hRNA mediated by Ser7, Chp1-CD, and generation of siRNA in *cis*

Stepwise explanation of Ser7 function in siRNA biogenesis at pericentromeric repeats. See text for details.

A

<i>rpb1</i> ⁺	:	-	(YSPTSPS) ₂₉	-
<i>rpb1-11</i>	:	-	(YSPTSPS) ₁₆	-
<i>ctdS2A</i>	:	-	(YAPTSPS) ₂₉	-
<i>ctdS7A</i>	:	-	(YSPTSPA) ₂₉	-

B

C

D

RT-PCR

E

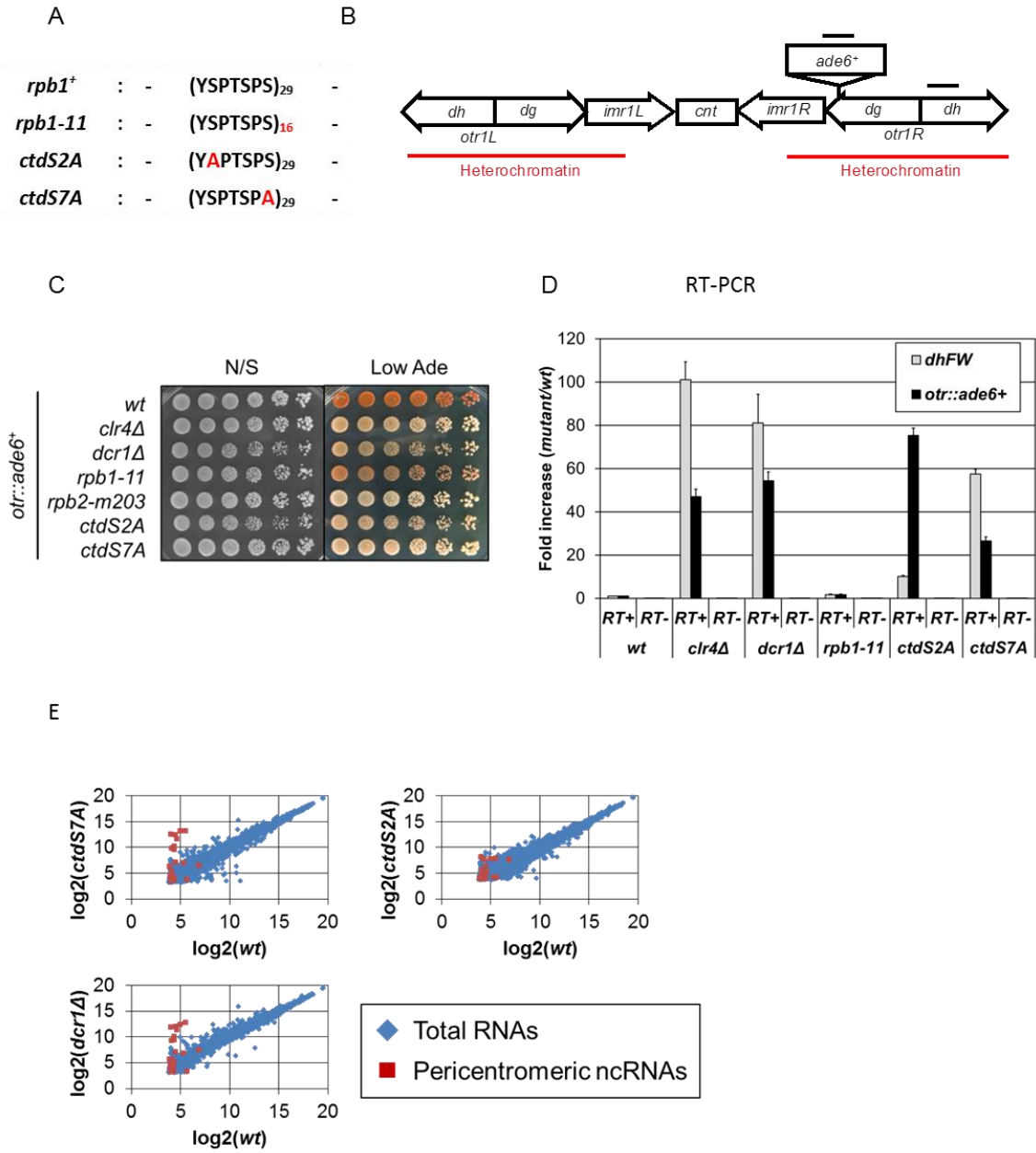


Fig. 2

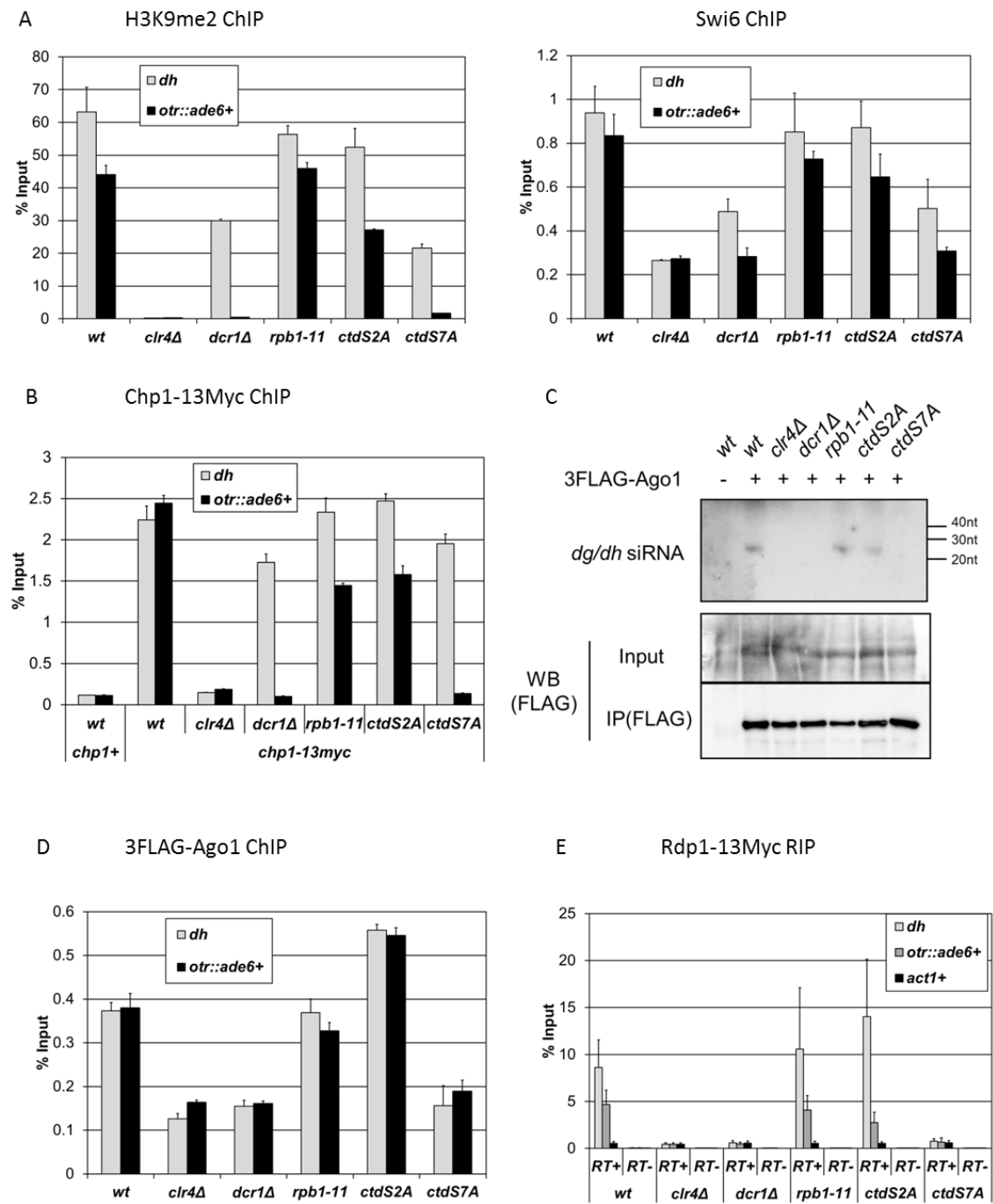


Fig. 3

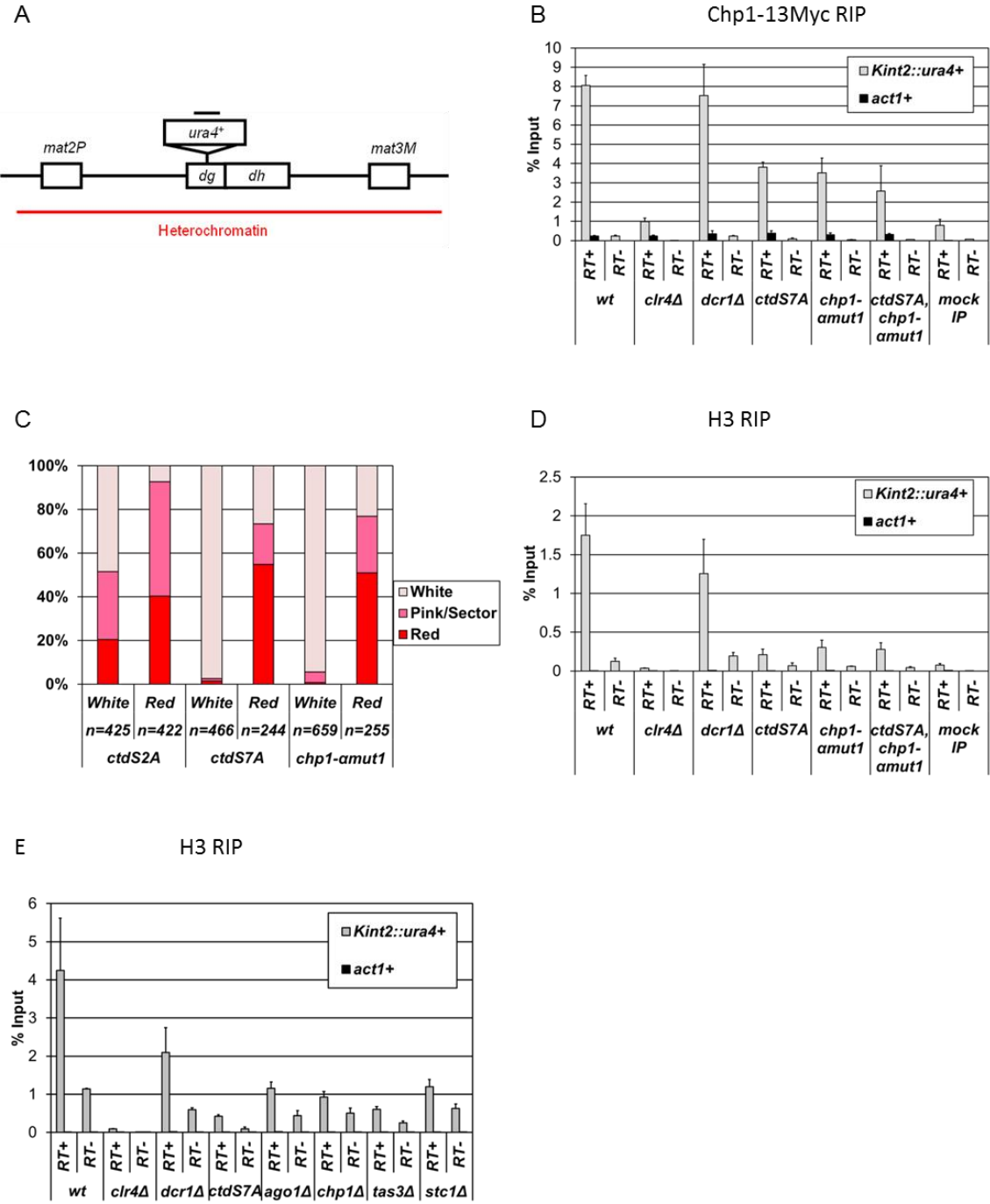


Fig. 4

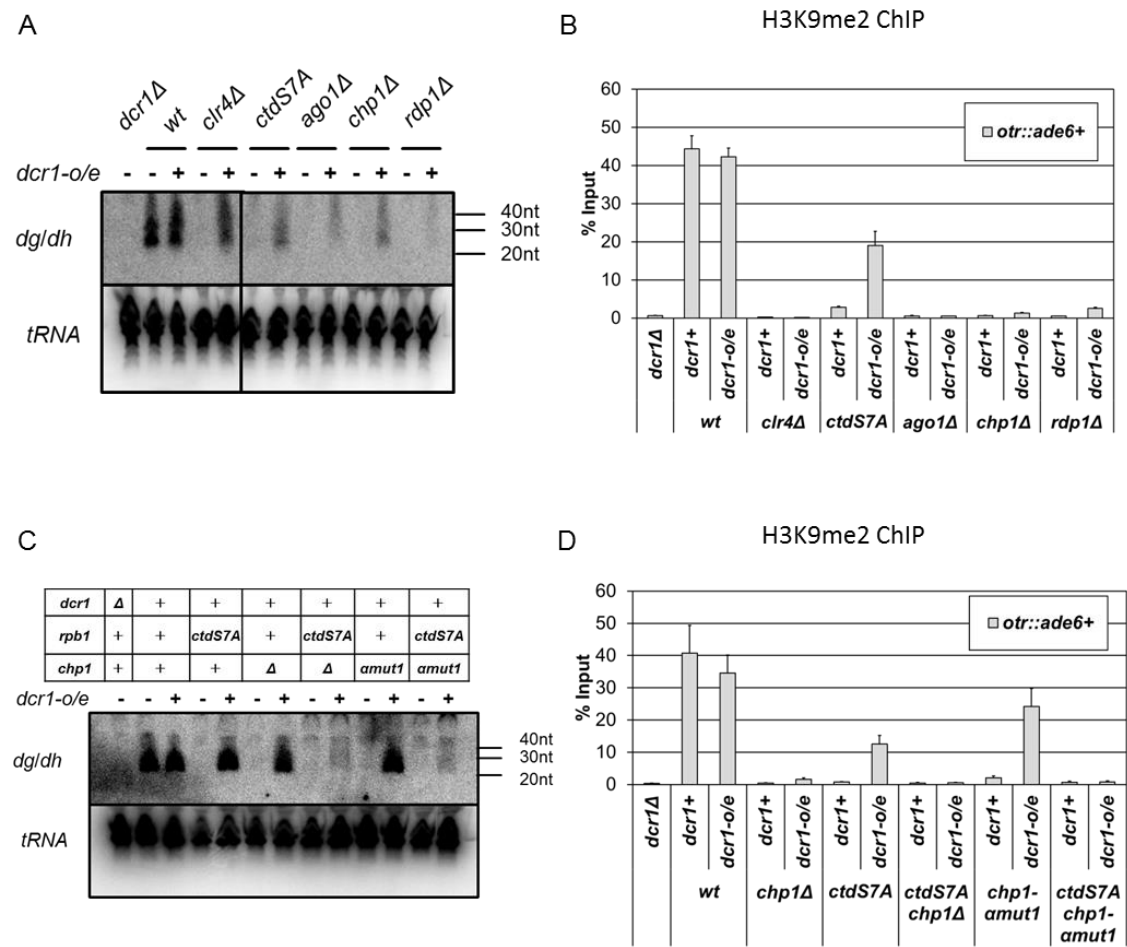
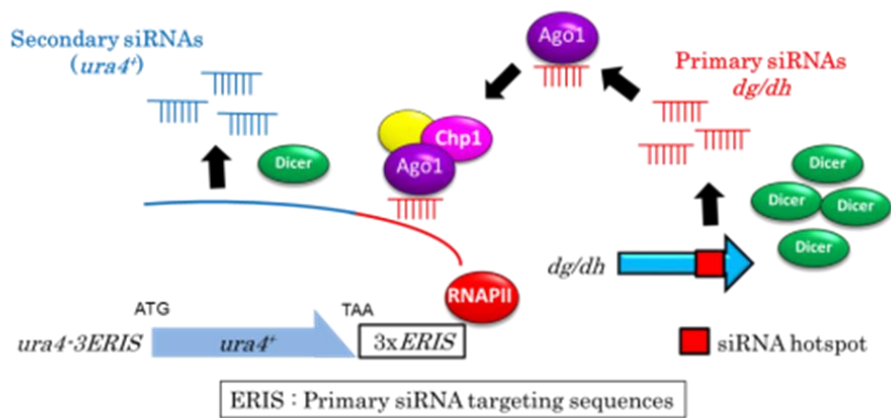
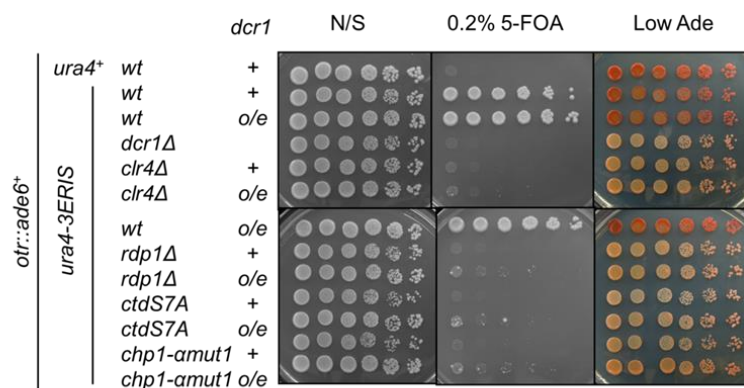


Fig. 5

A

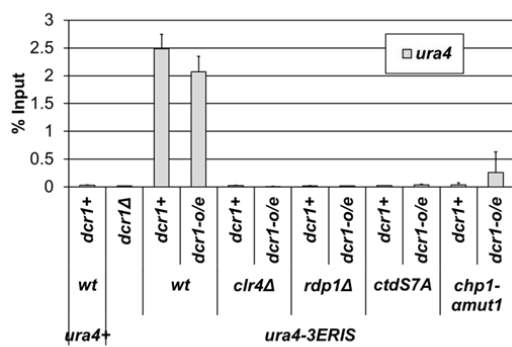


B



C

H3K9me2 ChIP



D

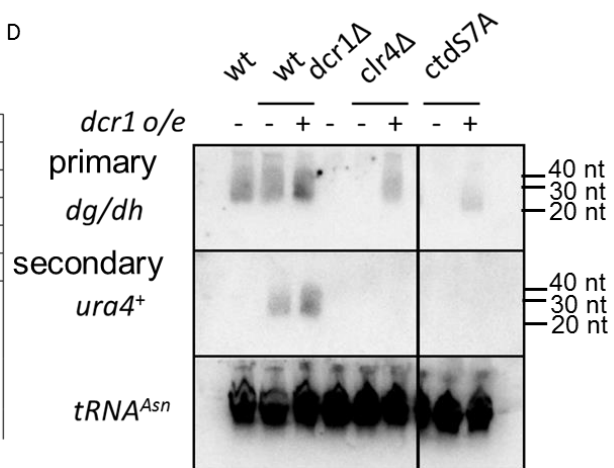


Fig. 6

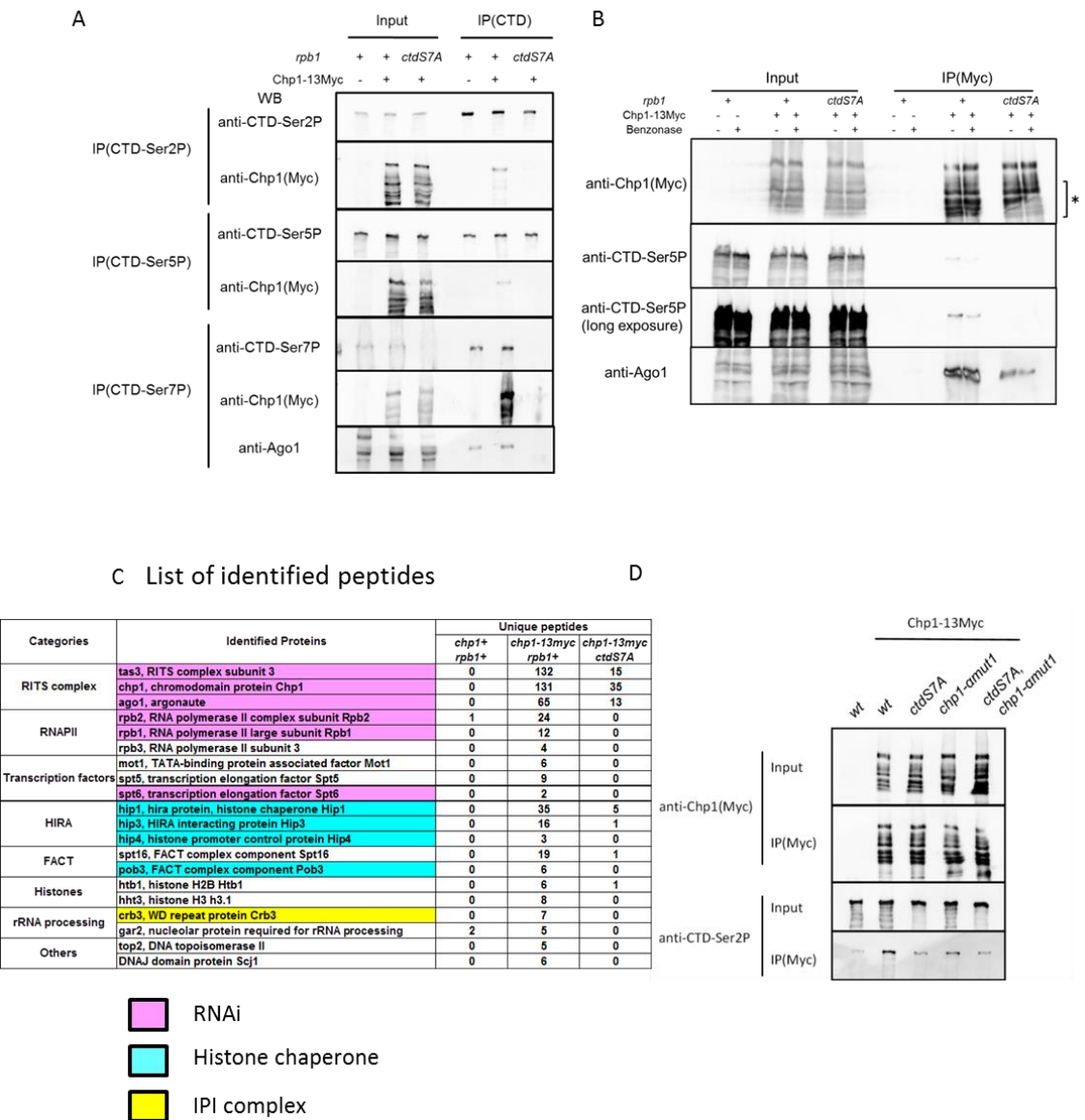
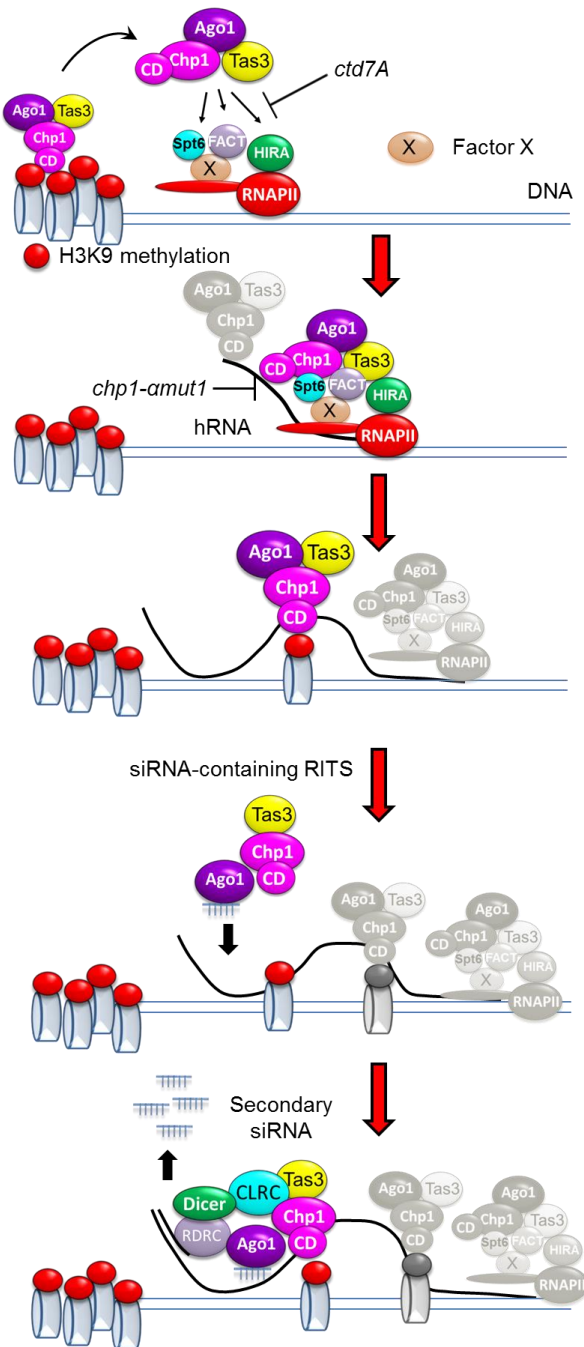


Fig. 7



1. Transcription of hRNAs by RNAPII-RITS complex

2. Ser7-dependent capture of nascent hRNAs by Chp1-CD

3. Chromatin retention of hRNAs

4. Primary siRNA-dependent RITS targeting

5. Secondary siRNA amplification within heterochromatin

Supporting Information

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

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SI Materials and Methods

Strain construction

S. pombe strains used in this study are listed in Table S3. Standard procedures (1) were used for cultivation of yeast cells. Gene targeting and C-terminal and N-terminal tagging were accomplished by PCR-based methods (2).

Antibodies

Mouse monoclonal antibodies against the histone modifications H3K9me2 (m5.1.1) (3), H3K9me3 (CMA318) (4), H3K9Ac (CMA310) (4), and H3K14Ac (7G8), and the epitope tags Myc (Millipore, 4A6) and FLAG (Sigma, M2), were used for ChIP and RIP assays. Rabbit polyclonal antiserum against H3 was

purchased from Millipore (07-690), and used for ChIP and RIP assays. Mouse monoclonal antibodies against the CTD of RNAPII were anti-CTD phosphorylated at Serine-2 (CMA603) (5), and anti-CTD phosphorylated at Serine-5 (Pd76D6). Rat monoclonal antibody against CTD phosphorylated at Serine-7 (3A4D12) (6) was also used. Rabbit polyclonal antibodies against Ago1 (abcam, ab18190) was purchased from the indicated supplier. anti-Swi6 rabbit polyclonal antibodies were prepared using bacterially expressed full-length GST-Swi6 protein and affinity purified.

Silencing assay

Silencing assay was performed as described previously (7). Briefly, each strain was precultured in 5 ml of liquid YES medium (yeast extract containing 225 mg/l of adenine, uracil, histidine, leucine, and lysine). Cells were washed twice with 1 ml of deionized H₂O and suspended in 20% glycerol. The most dense spot contained 2.0×10^7 cells/ml. Five-fold serial dilutions were prepared, and 5 μ l of each cell suspension was spotted on indicator plates: YES (non-selective, N/S), YES without Adenine (Low Ade), and YES containing 0.1% or 0.2% 5-FOA (0.1% or 0.2% 5-FOA). Spotted cells were cultured at 32°C for 3–5 days.

RT-PCR

Each strain was cultured in 25 ml of liquid YES to 5×10^6 cells/ml, harvested by centrifugation, and washed with 10 ml of PBS. Cell pellets were suspended in buffer containing 1% SDS and acid phenol. Total RNAs were extracted by three rounds of freezing/thawing in liquid N₂ and a 65°C water bath. Total RNA (1 µg) was treated with 25 units of recombinant DNase I (Takara Bio) at 37°C. cDNAs were reverse transcribed with 200 units of PrimeScript Reverse Transcriptase (Takara Bio) at 42°C with 3 pmol of target-specific reverse primers. Quantitative PCR was performed with PCR cocktails containing SYBR Dye on a Thermal Cycler Dice Real-Time System (Takara Bio). Signal intensity was calculated by the $\Delta\Delta C_t$ method. The signals were normalized to *act1*⁺ and represent fold increase as compared to the signal of wild-type. Primer sets used for quantitative PCR are listed in Table S4. In each graph, error bars show the standard error of the mean obtained from multiple biological replicates. (n = 3).

Chromatin immunoprecipitation (ChIP) assay

Chromatin immunoprecipitation was performed as described previously (8) with modifications. Cells were cultured in 50 ml of liquid YES to 1×10^7 cells/ml. Cells were crosslinked with 1% formaldehyde at 30°C for 30 min. For Swi6 ChIP, cells were shifted to 18°C for 120 min and subsequently crosslinked with 1% formaldehyde at 25°C for 30 min. Cells were suspended in lysis buffer (50 mM HEPES-KOH [pH 7.5], 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% DOC-Na, 2 mM PMSF, 1/50 volume of Protease Inhibitors cocktail [Nacalai Tesque] and 1/100 volume of PhosSTOP [Roche]). Cell lysates were prepared by vigorous vortexing with zirconia beads (Biospec Products) in a Multi-beads shocker (Yasui Kikai) at 0°C. Chromatin in crude lysates was digested by sonication using a Bioruptor UCD-250 (Cosmo Bio). Cell debris was precipitated by centrifugation at 15,000 rpm for 15 min at 4°C. Supernatants were incubated with Dynal M-280 magnetic beads conjugated to sheep anti-mouse, rabbit, or rat IgG (Veritas) at 4°C for 4 hours. Magnetic beads were washed twice with lysis buffer, lysis buffer containing 500 mM NaCl, and LiCl wash Buffer (10 mM Tris-HCl [pH 8.0], 250 mM LiCl, 0.5% NP-40, 0.5% DOC-Na, 1 mM EDTA, 2 mM PMSF, and 1/50 volume of Protease Inhibitors cocktail). Magnetic beads were treated with 100 µg/ml of RNase A (Wako) at 37°C for 15 min and 500 µg/ml of

Proteinase K at 45°C for 60 min, and then crosslinks were reversed at 65°C for more than 9 hours. DNAs were isolated by phenol/chloroform extraction and isopropanol precipitation. DNA pellets were resuspended in TE. Quantitative PCR was performed as described in RT-PCR. Signal intensity was calculated by the $\Delta\Delta\text{Ct}$ method. Primer sets used for quantitative PCR are listed in Table S4. qPCR signals were represented as % Input. In each graph, error bars show the standard error of the mean obtained from multiple biological replicates. (n = 3).

RNA immunoprecipitation (RIP) assay

RNA immunoprecipitation was performed as described previously (9). Briefly, cells were cultured in 50 ml of liquid YES to 1×10^7 cells/ml. Lysates were prepared as described above for the ChIP assay, except that RNasin Plus (Promega) was added to buffers and the EDTA in LiCl wash buffer was replaced with EGTA. Immunoprecipitated RNAs and Input RNAs were treated with 500 $\mu\text{g/ml}$ of Proteinase K at 45°C for 60 min, and crosslinks were reversed at 65°C for more than 9 hours in TE containing 2% SDS and 80 units of RNasin Plus (Promega). Immunoprecipitated RNA was isolated by two rounds of acid-phenol extraction and isopropanol precipitation. DNA was removed by addition of

excess (60 units) of recombinant DNase I (Takara Bio) and incubation at 37°C for 60 min. cDNAs were reverse transcribed with 480 units of PrimeScript Reverse Transcriptase (Takara Bio) at 42°C with 7.2 pmol of target gene-specific reverse primers. Primer sets are listed in Table S4. Quantitative PCR was performed as described above for qRT-PCR. Signal intensity was calculated by the $\Delta\Delta C_t$ method. Primer sets used for quantitative PCR are listed in Table S4. RIP signals were represented as % Input. In each graph, error bars show the standard error of the mean obtained from multiple biological replicates (n = 3).

Northern blot for small RNAs

Cells were cultured in 50 ml of liquid YES to 1×10^7 cells/ml. Total small RNAs were isolated using the *mirVana* miRNA Isolation Kit (Ambion). RNAs were separated on 15% polyacrylamide gels containing 8 M urea. RNAs were blotted onto Hybond N+ nylon transfer membrane (GE Healthcare) and UV-crosslinked to the membrane. The membrane was baked at 42°C for 60 min. The membrane was prehybridized with 10 ml of Ultrahyb-Oligo Hybridization Buffer (Ambion) at 42°C for 30 min. DNA oligo-probes corresponding to *dg/dh* or *tRNA^{Asn}* were

labeled with 3,000 Ci/mmol of [γ - 32 P] ATP using 10 units of T4 polynucleotide kinase (TOYOBO) at 37°C for 60 min and purified using Illustra ProbeQuant G-50 MicroColumns (GE Healthcare). Radioisotope-labeled oligo-probes were hybridized at 42°C for 2 days, and then washed three times with 2×SSC containing 0.1% SDS. To detect *dg/dh*, or *tRNA^{Asn}* signals, membranes were exposed for 4 days (*dg/dh*) or 1 day (*tRNA^{Asn}*). Radioisotope signals were visualized on a BAS-300 (FUJIFILM). DNA sequences of the oligo-probes are listed in Table S4.

Microarray analysis

RNA preparation, labeling, hybridizations, and scanning were performed as described previously (10). The scanned data were processed as follows: 5589 probes (including 60 control probes) were spotted multiply to fill 15744 spots in each array. The geometric mean of *gProcessedsignals* of the spots for each probe was calculated to generate a data set for 5589 probes. The geometric mean was normalized by the 75th percentile method (the value at 75% was set to 2500) to obtain the processed values. When the average of the *gIsWellAboveBG* of the spots for each probe was higher than 0.55, the probe

signal was considered to be above the detection limit (*gProcessedsignals* and *gIsWellAboveBG* are defined by the Feature Extraction software). Using the processed values, the geometric mean and expression ratio were calculated. Sequences of probes and the original data from the microarray experiments were deposited in GEO (<http://www.ncbi.nlm.nih.gov/geo>) with accession number GEO53568. Based on the expression ratio of each mutant relative to the wild-type cells, up-regulated genes and down-regulated genes were defined as those with $\log_2(\text{ratio}) > 2$ and < -2 , respectively. Venn diagrams comparing differentially regulated genes in *ctdS2A*, *ctdS7A*, and *dcr1Δ* cells were produced using the Venn diagram generator (<http://jura.wi.mit.edu/bioc/tools/venn.php>). The *P*-value of overlapping genes was calculated by Fisher's exact test.

Immunoprecipitations and western blotting

Immunoprecipitation was performed as described previously (11) with several modifications. Cells were cultured in 600 ml of YES to 1×10^7 cells/ml and harvested by centrifugation. Cell pellets were suspended in Buffer B (100 mM KOAc [pH 7.5], 100 mM Tris-acetate [pH 7.8], 20% glycerol, 1 mM EDTA, 1 mM EGTA, 0.4% NP-40, 2 mM DTT, 1 mM PMSF, 1/50 volume of Protease Inhibitors

cocktail, and 1/100 volume of PhosSTOP [Roche]). Cell lysates were prepared by gentle vortexing with zirconia beads (Biospec products) in a Multi-beads shocker (Yasui Kikai) at 0°C. Crude lysates were cleared by two-step centrifugation at 2,330×*g* for 15 min and 15,000×*g* for 10 min at 4°C. Protein concentration was measured by Bio-Rad protein assay (Bio-Rad Laboratories) and normalized with Buffer B. Immunoprecipitation was performed by rocking at 4°C for 4 hours with Dynabeads M-270 Epoxy to which anti-Myc or anti-CTD antibodies were conjugated. Dynabeads were washed nine times with Buffer B and transferred to new tubes every three washes, and then the Dynabeads were boiled at 98°C for 10 min. Immunoprecipitated proteins were separated on 6% acrylamide gels and blotted to Immobilon-P transfer membranes (Millipore) in a Transblot SD cell (Bio-Rad Laboratories). For blocking, membranes were soaked in 3% skim milk or 5% BSA in PBST for 30 min at room temperature, and then rinsed three times in PBST by gentle soaking. Membranes were incubated overnight with 1 µg of primary antibodies diluted with Can Get signal immunoreaction enhancer solution 1 (TOYOBO). Secondary antibodies were HRP-conjugated goat anti-mouse or goat anti-rat IgG (Jackson ImmunoResearch). Immobilon Western chemiluminescent HRP substrate

(Millipore) or ECL Select (GE Healthcare) was used as the substrate for HRP. Chemiluminescent measurement and signals were visualized on a LAS-3000 (FUJIFILM). Antibodies used for detection of phosphorylated CTD were described above.

Immunoaffinity purification and LC-MS/MS analysis

Cells were cultured in 1,200 ml of YES. Immunoaffinity purifications were performed using the procedure described above for immunoprecipitation. Precipitated Dynabeads were boiled at 98°C for 10 min in 40 µl of 1× Laemmli buffer. Purified proteins were precipitated by addition of ice-cold 100% TCA with 10% DOC and centrifugation at 15,000 rpm for 30 min. Protein pellets were washed with ice-cold acetone and dried. Pellets were resuspended in 1× Laemmli buffer containing 8 M urea. Protein complexes were separated on 10% acrylamide gel and stained with CBB. LC-MS/MS analysis was performed as described previously (12).

Supplemental Figure Legends

Fig. S1. Ser2 and Ser7 are required for repression of pericentromeric ncRNA expression.

(A) Variegation of silencing of *ade6⁺* inserted into pericentromeric repeat (*otr::ade6⁺*) was observed in *rpb2-m203*, *ctdS2A*, and *ctdS7A* cells, but not in *clr4Δ*, *dcr1Δ*, or *rpb1-11* cells. The repression of *ade6⁺* caused formation of red colonies on Low Ade plates, whereas derepression of *ade6⁺* caused formation of white or pink colonies.

(B) The amount of reverse transcripts from *dh* repeats was measured in the indicated strains by quantitative RT-PCR. *ctdS2A* or *ctdS7A* mutation caused accumulation of transcripts. Primers for cDNA synthesis were *dh*-specific forward-strand primer and *act1⁺* specific reverse-strand primers. Primer pairs for quantitative PCR were site-specific primers for *dh* at the pericentromere and *act1⁺*. Details of qPCR were identical to those in Fig. 1D.

(C) The Venn diagrams showing the number of transcripts whose expression levels were more than 4-fold higher in mutants than in the wild-type. *P*-values were calculated using Fisher's exact test. Left panel: transcripts of *ctdS2A* mutant (left circle) vs. *ctdS7A* mutant (right circle); middle panel: *ctdS2A* mutant

(left circle) vs. *dcr1* Δ mutant (right circle); right panel: *ctdS7A* mutant (left circle) vs *dcr1* Δ mutant (right circle).

(D) H3K9me3 ChIP assay showing that *ctdS7A* but not *ctdS2A* cells failed to form proper heterochromatin structure like *dcr1* Δ cells. ChIP was performed on the indicated strains. Details of qPCR were identical to those in Fig. 2A.

(E) ChIP assay for Rik1, a component of CLRC, showed that *ctdS7A* mutation reduced localization of Rik1 at *dh* repeats and *otr::ade6⁺*, whereas *ctdS2A* mutation did not. ChIP was performed on the indicated strains expressing Rik1-13Myc. Details of qPCR were identical to those in Fig. 2A.

Fig. S2. Measurement of active histone modifications across heterochromatin.

(A) ChIP assay using anti-H3K9Ac, anti-H3K14Ac, and anti-H3 antibodies showing the effects of *ctdS2A* and *ctdS7A* mutation on pericentromeric ncRNA silencing. ChIP signal of H3K9Ac or H3K14Ac was normalized against that of H3 in order to eliminate effects due to variation in histone density. Acetylation at both residues exhibited similar patterns. In *ctdS2A* cells, H3K9Ac and H3K14Ac were moderately elevated at *otr::ade6⁺* but not at *dh*. In *ctdS7A* cells, H3K9Ac and H3K14Ac were moderately elevated at *otr::ade6⁺*, but markedly enriched at *dh*. Details of qPCR except data presentation were identical to those in Fig. 2A.

(B) ChIP assay using anti-H3K4me2, anti-H3K4me3, and anti-H3 antibodies showing the effects of *ctdS2A* and *ctdS7A*. *ctdS2A* caused marked enrichment of H3K4me2 and H3K4me3 at *otr::ade6⁺* but not at *dh*. *ctdS7A* resulted in moderate accumulation of H3K4me2 and H3K4me3 at *dh* and *otr::ade6⁺*. Details of qPCR including data presentation were identical to those in (A). *: $P < 0.05$. P -values of H3K4me3 in *ctdS7A* cells at *dh* and *otr::ade6⁺* are 0.0106, 0.0178, respectively. P -values were determined using a two-sided Student's t -test.

Fig. S3. Truncated CTD mutation rescues the defect in siRNA generation in *ctdS7A* mutant cells.

(A) H3K9me2/3 across pericentromeric repeats are analyzed in indicated strains by ChIP assay, suggesting that *ctdS7Ax18* mutation does not affect enrichment of H3K9me2/3. H3K9me2/3 were precipitated using antibody against H3K9me2 and H3K9me3, and the associated DNAs were calculated by qPCR. Details of qPCR were identical to those in Fig. 2A.

(B) Northern blot detecting siRNA derived from pericentromeric transcripts, suggesting that siRNA is efficiently generated in *ctdS7Ax18* mutant cells as comparable levels to that in wild-type cells. siRNAs were purified by *mirVana* miRNA isolation kit. siRNAs were detected by ³²P labeled oligo-DNA probes. *tRNA^{Asn}* is a loading control.

Fig. S4. Ser2 and Ser7 do not affect heterochromatin formation at the mating-type locus.

(A) Silencing assay of indicated strains showing that the *ura4⁺* gene inserted into the *K* region of the mating-type locus (*Kint2::ura4⁺*) is still repressed in *ctdS2A* and *ctdS7A* cells, as well as in *dcr1Δ* cells, despite derepression of *otr::ade6⁺*.

Endogenous *ura4⁺* strain without the *Kint2::ura4⁺* reporter gene served as a control for *ura4⁺* expression. Cells were spotted in 5-fold serial dilutions on YES (non-selective, N/S), 0.1% 5-FOA (FOA), EMM lacking uracil (-Ura), or YES without Adenine (Low Ade).

(B) qRT-PCR of *Kint2::ura4⁺* transcript showing that *Kint2::ura4⁺* expression is repressed in *ctdS2A* and *ctdS7A* cells, as well as in *dcr1Δ* cells. Primers for cDNA synthesis were gene-specific reverse-strand primers. Details of qPCR were identical to those in Fig. 1D.

(C) Histone H3 ChIP assay showing that H3 occupancy at *Kint2::ura4⁺* is not altered in the indicated mutants relative to the wild-type cells. Signals of ChIP-qPCR at *Kint2::ura4⁺* were normalized against those at *act1⁺*. Details of qPCR were identical to those in Fig. 2A.

(D and E) ChIP assay of H3K9me2 (D), and Chp1-13Myc (E) at *Kint2::ura4⁺* showing that normal heterochromatin structure at *Kint2::ura4⁺* is maintained in *ctdS2A* and *ctdS7A* cells, as well as in *dcr1Δ* cells. ChIP was performed in the indicated strains expressing Chp1-13Myc. Details of qPCR were identical to those in Fig. 2A.

Fig. S5. Ser7 and RNA-binding activity of Chp1-CD play similar roles in silencing.

(A) Variegation of *otr::ade6⁺* silencing was observed on Low Ade plates in *ctdS7A*, *chp1-amut1*, and *chp1Δ* cells, but not in *ctdS7A chp1-amut1* double mutant cells. Cells were streaked on Low Ade plate without any selection.

(B) Comparison of primary colony color ratio in *ctdS2A*, *ctdS7A*, *chp1-amut1*, *chp1Δ*, and *ctdS7A chp1-amut1* cells grown on Low Ade plates. Colony color was classified in three categories, white, pink/sector, and red, and the proportions of colonies of each color were calculated. White or red colonies in this assay were picked and re-streaked on Low Ade plates to monitor stability of *otr::ade6⁺* silencing in Fig. 3C. (C) H3K9me2 ChIP assay at *dh* and *otr::ade6⁺* showing that both *chp1-amut1* and *ctdS7A* cells exhibit *dcr1Δ*-like impairment of pericentromeric heterochromatin. H3K9me2 at *dh* was partially maintained, but not at *otr::ade6⁺* in *chp1-amut1*, *ctdS7A*, and *ctdS7A chp1-amut1* double mutant cells. Details of qPCR were identical to those in Fig. 2A.

Fig. S6. Dcr1 overexpression partially restores pericentromeric ncRNA silencing.

(A) qRT-PCR of *dcr1*⁺ showing that *dcr1*⁺ overexpression under the control of the *urg1* promoter results in a 20-fold increase in the *dcr1*⁺ mRNA level relative to expression from the endogenous *dcr1*⁺ promoter. Total RNAs were isolated from *dcr1*⁺ and *Purg1-3flag-dcr1* (*dcr1-o/e*) cells. Total RNA (1 µg) was used for this assay. Primers for cDNA synthesis are *dcr1*⁺ gene-specific reverse-strand primers. qRT-PCR signals were normalized against *act1*⁺ and are presented as the fold increase relative to cells endogenously expressing *dcr1*⁺ (defined as 1). Details of qPCR were identical to Fig. 1D.

(B) Raw data of Fig. 4A.

(C) Raw data of Fig. 6D.

Fig. S7. LC-MS/MS analysis of immunoaffinity purification of Chp1-13Myc.

(A) Evaluation of the reactivity of antibodies against anti-Ser2P, Ser5P, and Ser7P. Cell lysates indicated strains were prepared by boiling with 1xlaemmli buffer containing 4 M urea. Lsk1 is a Ser2P kinase and Csk1 is a kinase activating Lsk1. Lysates were separated on 6% acrylamide gels and transferred to PDVF membranes. Membranes were blocked in 5% BSA and 2 µl of each antibody was used for western blot. anti-TAT1 was used as a loading control.

(B) Optimization of Benzonase® nuclease treatment. Crude cell lysates were prepared as described in immunoprecipitations. Each concentration of Benzonase® and 2 mM of MgCl₂ were added and incubated at 4°C for 30 min. The lysates were centrifuged and the supernatants were treated with Proteinase K at 45°C for 60 min. Nucleic acids were purified by Phenol/Chloroform twice and Ethanol precipitation. Nucleic acids were loaded on a 1% agarose gel and stained with ethidium bromide.

(C) Silver-stained SDS-PAGE gels of Chp1-13Myc purification. Chp1-13Myc was purified from the indicated strains. Purified proteins were concentrated by TCA precipitation and separated on 5–20% acrylamide gradient gels. Molecular

size marker was loaded on each side of the lane of acrylamide gel.

Silver-staining was performed using SilverQuestTM Staining Kit (Invitrogen).

(D)Western blot analysis of Ser2P associated with Chp1-13Myc in RITS mutants (*chp1* Δ , *ago1* Δ , and *tas3* Δ).

Table S1. List of genes up-regulated and down-regulated in *ctdS2A*, *ctdS7A*, and *dcr1Δ* cells ($\log_2(\text{ratio}) > 2$, $\log_2(\text{ratio}) < -2$)

Up-regulated and down-regulated genes in *ctdS2A*, *ctdS7A*, and *dcr1Δ* cells are listed. Genes up-regulated in each mutant are more than 4-fold as compared to those in wild-type cells. Genes down-regulated in each mutant are less than 1/4-fold as compared to those in wild-type cells. Up-regulated pericentromeric ncRNAs are indicated as red characters. Down-regulation of *rpb1* in *ctdS2A* and *ctdS7A* cells was omitted, because replacement of CTD sequences and marker insertion at 3'UTR presumably impaired the affinity of *rpb1* mRNA with the microarray probe. The relative expression levels are presented as $\log_2(\text{ratio})$.

Table S2. Changes in silencing-related gene expression in *ctdS2A* and *ctdS7A* mutants.

Expression levels of heterochromatic silencing-related genes in *ctdS2A* and *ctdS7A* cells are listed. Dark colored columns indicate up- or down-regulation of gene expression more than 2-fold or less than 1/2-fold, respectively. The relative expression levels are presented as $\log_2(\text{ratio})$.

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Fig. S1

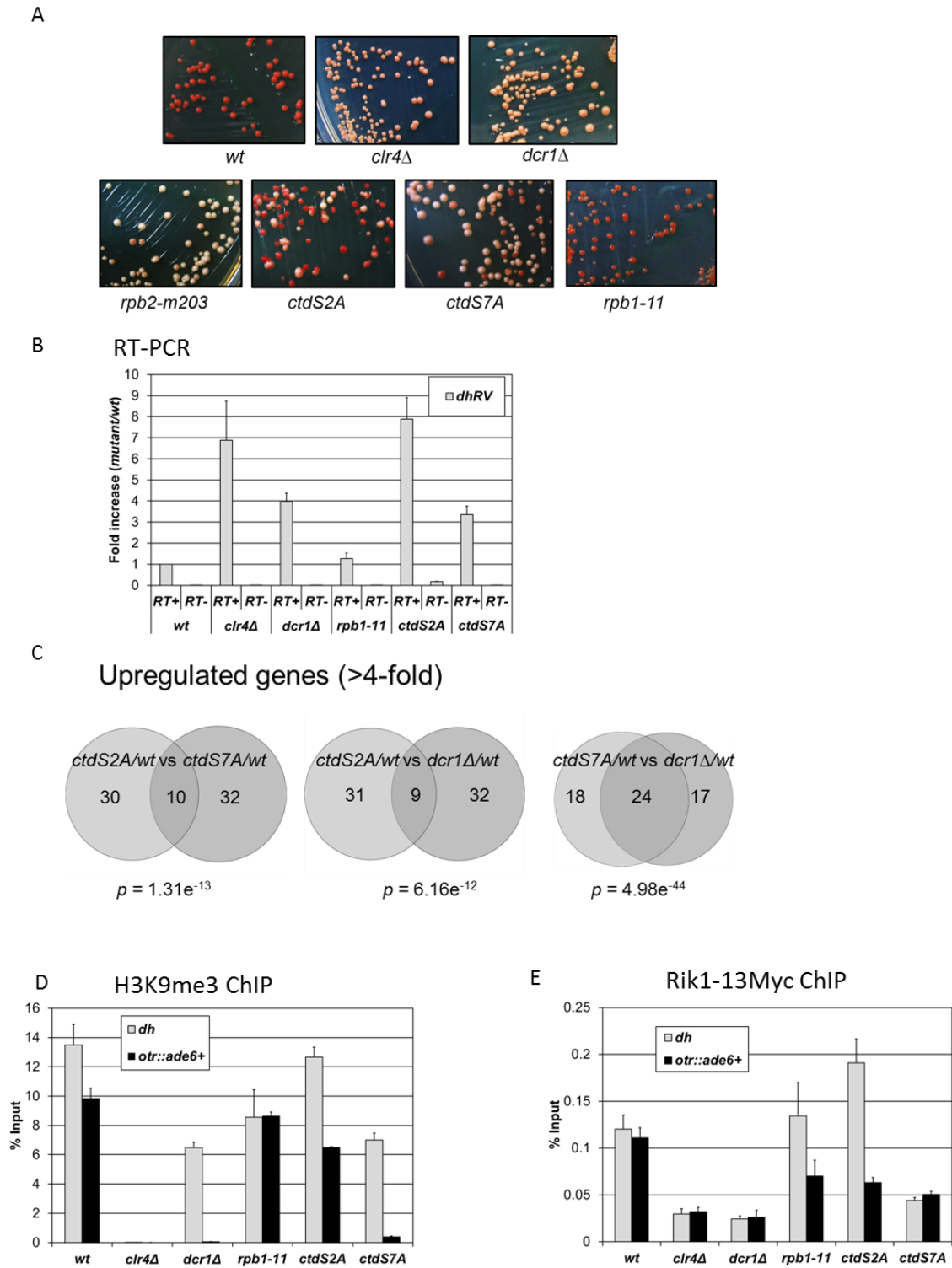


Fig. S2

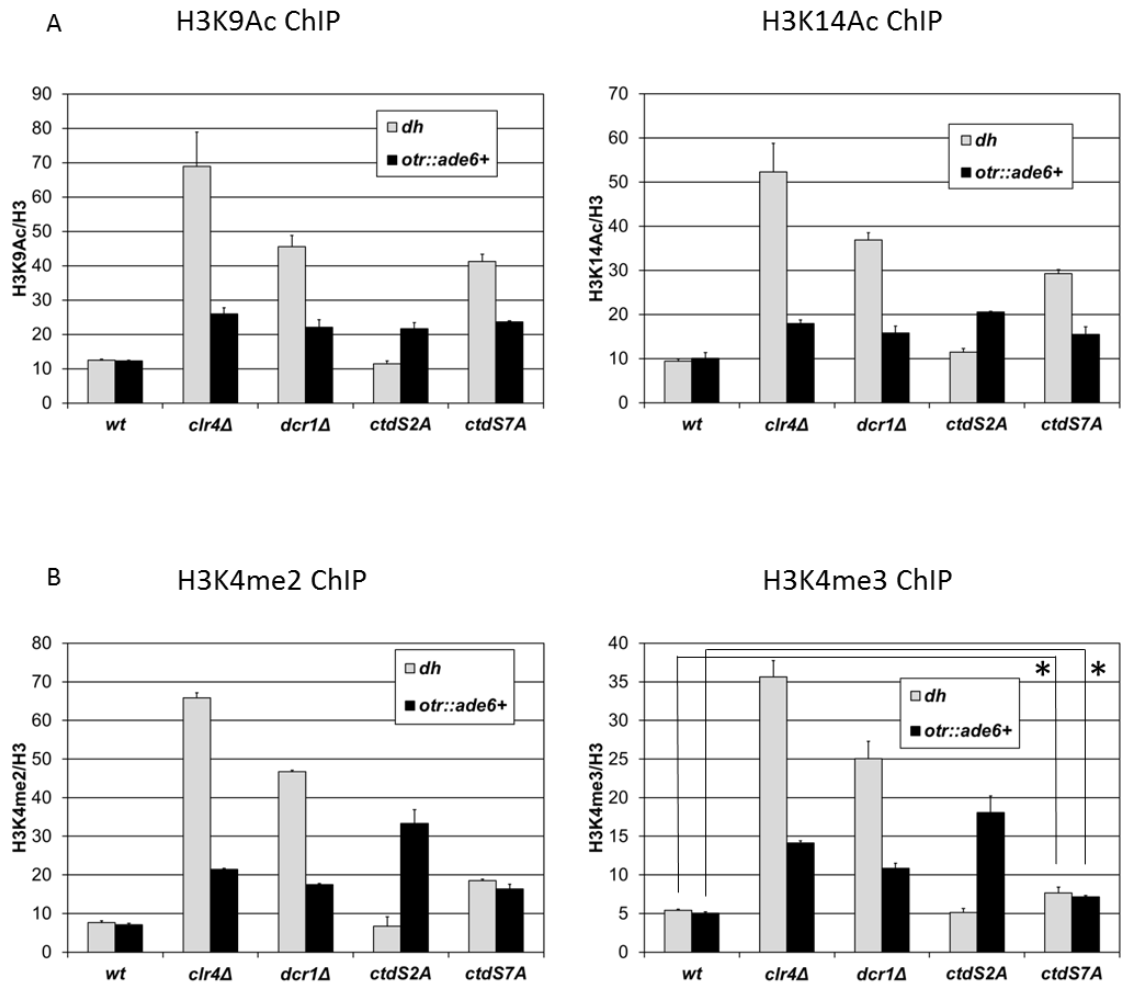
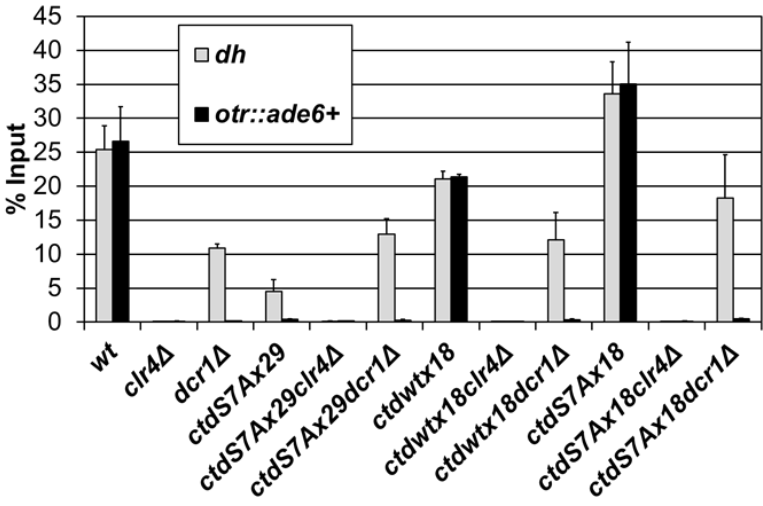


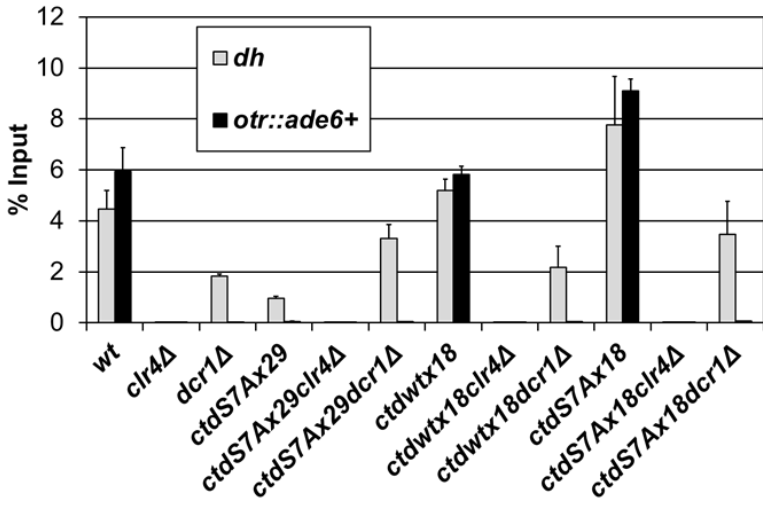
Fig. S3

A

H3K9me2 ChIP



H3K9me3 ChIP



B

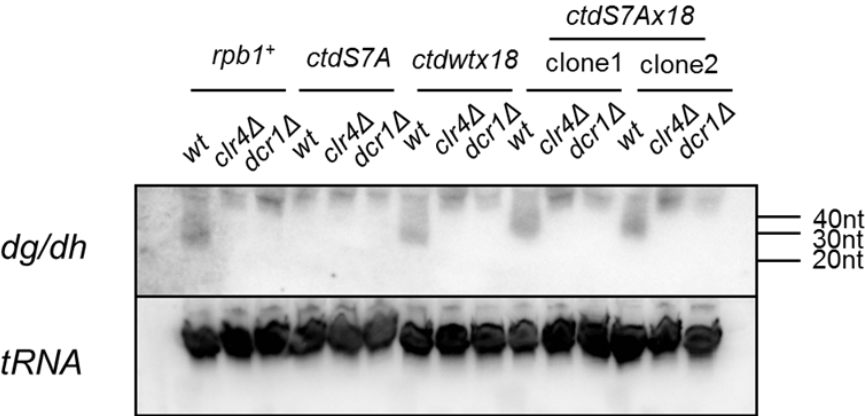


Fig. S4

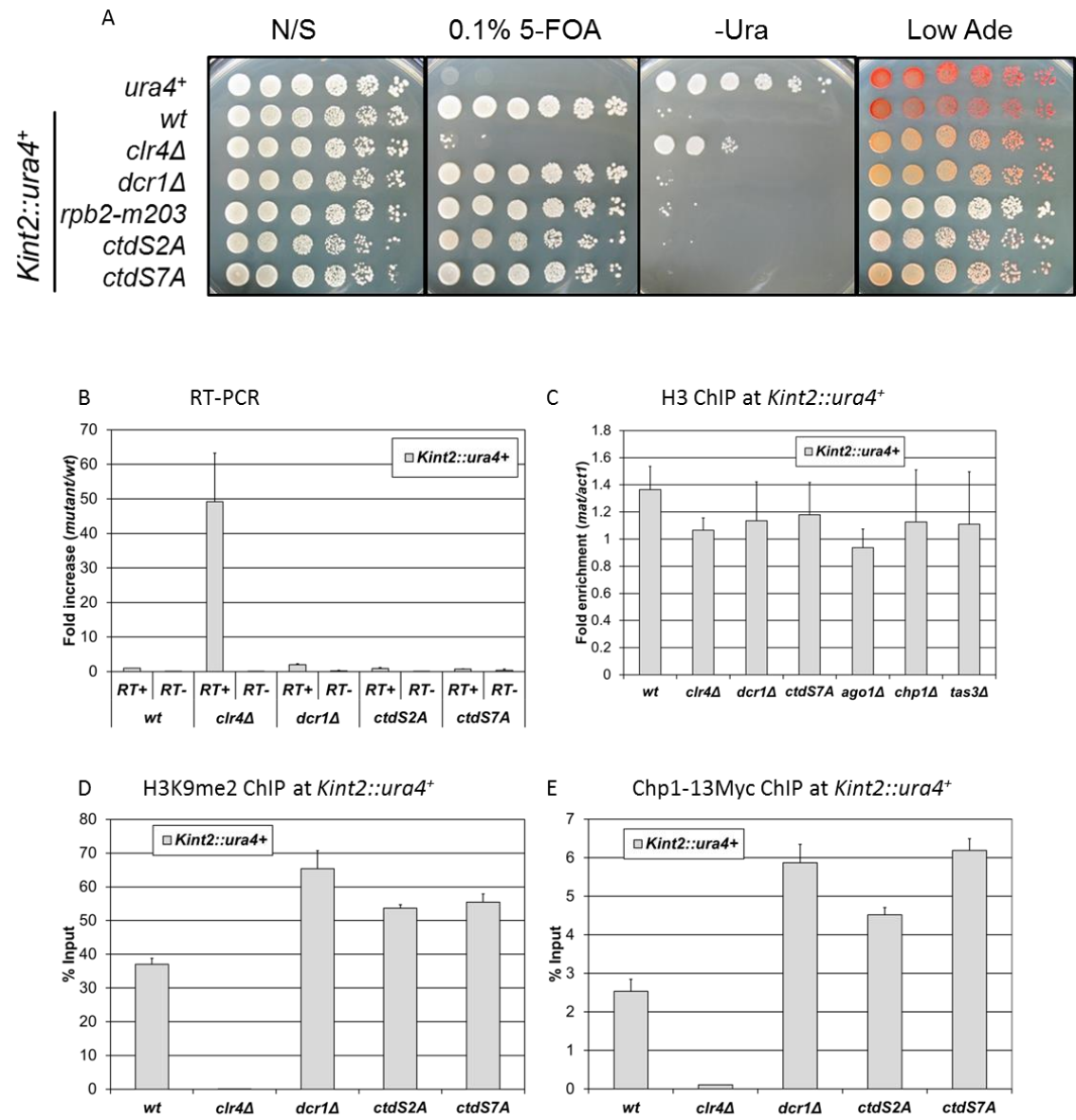


Fig. S5

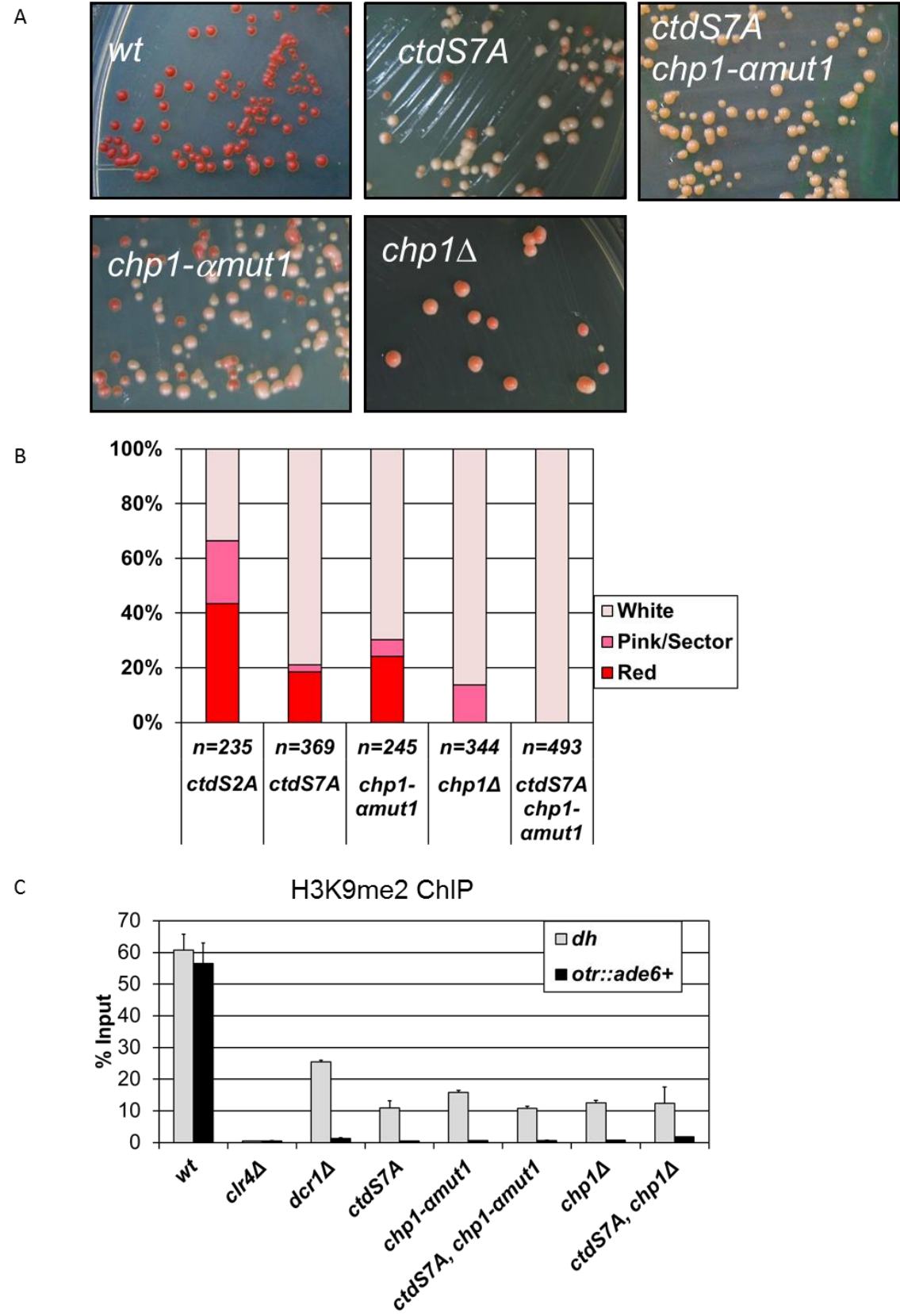
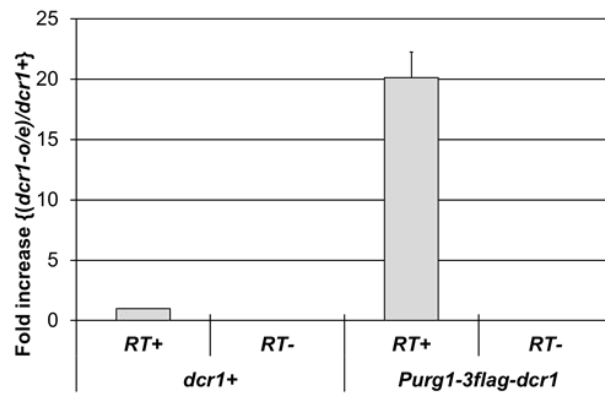


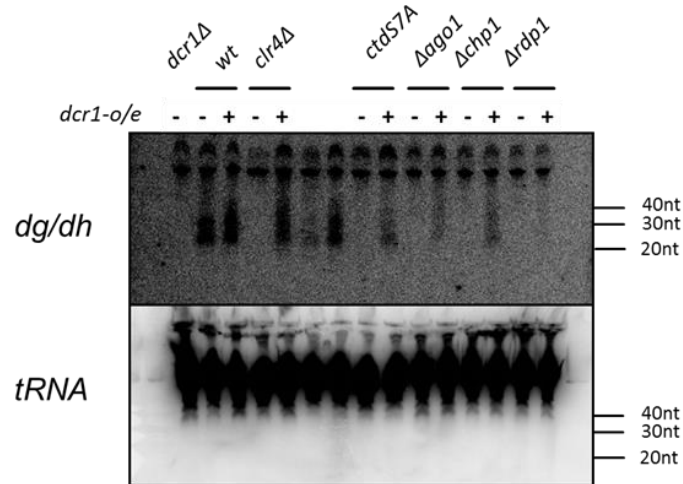
Fig. S6

A

RT-PCR of *dcr1*⁺ transcript

B

siRNA Northern blot



C

siRNA Northern blot

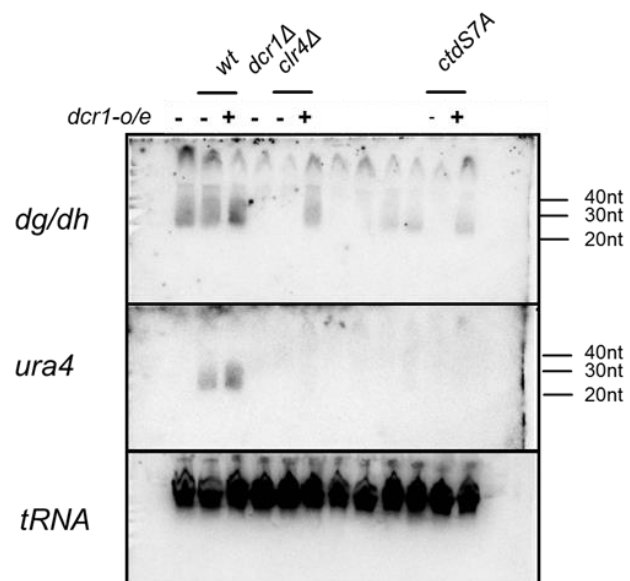


Fig. S7

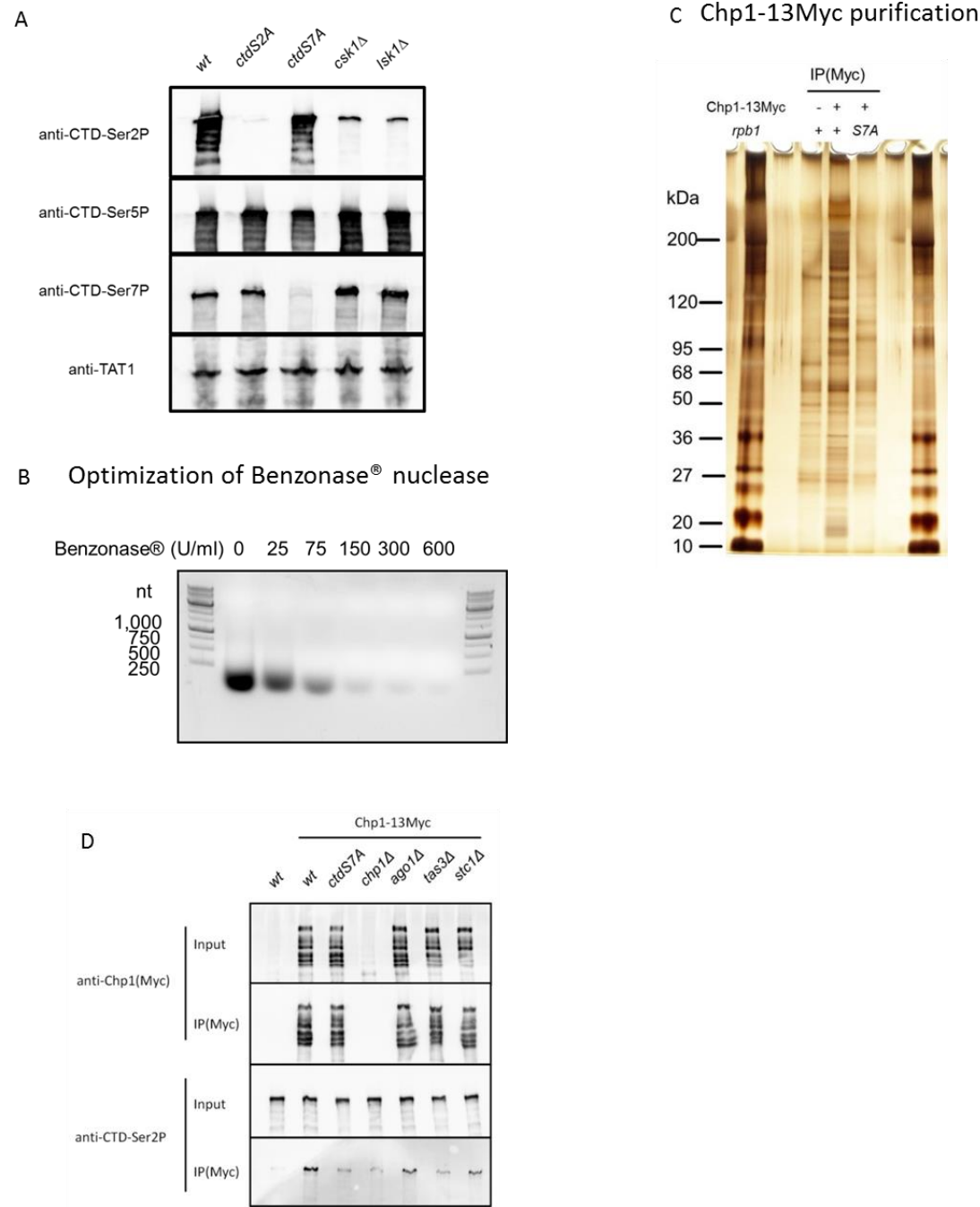


Table S1. List of genes up-regulated and down-regulated in *ctdS2A*, *ctdS7A*, and *dcr1Δ* cells ($\log_2(\text{ratio}) > 1$, $\log_2(\text{ratio}) < -1$)

Systematic Name	Gene Name	Description(ver6-2011-Dec)	log2(ctdS2A/wt)
SPBPB2B2.06c		phosphoprotein phosphatase (predicted)	4.54
SPNCRNA.554		antisense RNA (predicted)	4.49
SPBC56F2.03		actin-like protein Arp10 (predicted)	4.14
SPNCRNA.234		non-coding RNA, centromeric (predicted)	4.03
SPNCRNA.581		antisense RNA (predicted)	4.01
SPCC1223.02	nmt1	no message in thiamine Nmt1	3.81
SPNCRNA.569		antisense RNA (predicted)	3.60
SPBPB2B2.03c		not found	3.35
SPNCRNA.232		non-coding RNA, centromeric (predicted)	3.27
SPBPB2B2.08		conserved fungal protein	3.22
SPAC5H10.04		NADPH dehydrogenase (predicted)	3.15
SPBC1861.06c	mug131	S. pombe specific UPF0300 family protein 4	2.96
SPAPB18E9.04c		sequence orphan	2.95
SPAC22F3.04	mug62	AMP binding enzyme (predicted)	2.88
SPNCRNA.561		antisense RNA (predicted)	2.80
SPBC31F10.08	mde2	Mde2 protein	2.72
SPNCRNA.371		non-coding RNA, centromeric (predicted)	2.71
SPCC1259.14c	meu27	S. pombe specific UPF0300 family protein 5	2.70
SPCC1919.11	mug137	BAR adaptor protein	2.63
SPAC25G10.04c	rec10	meiotic recombination protein Rec10	2.57
SPNCRNA.133		non-coding RNA (predicted)	2.47
SPBC146.11c	mug97	meiotically upregulated gene Mug97	2.43
SPCC737.04		S. pombe specific UPF0300 family protein 6	2.41
SPNCRNA.560		antisense RNA (predicted)	2.40
SPAC23H3.15c		sequence orphan	2.35
SPAPB17E12.09		sequence orphan	2.32
SPNCRNA.563		antisense RNA (predicted)	2.29
SPNCRNA.373		non-coding RNA, centromeric (predicted)	2.28
SPNCRNA.314		not found	2.24
SPBC28E12.02		RNA-binding protein	2.24
SPBC428.07	meu6	meiotic chromosome segregation protein Meu6	2.21
SPBC1347.03	meu14	sporulation protein Meu14	2.19
SPNCRNA.536		antisense RNA (predicted)	2.19
SPNCRNA.11	prl11	non-coding RNA, poly(A)-bearing (predicted)	2.18
SPBP4G3.03		PI31 proteasome regulator related	2.14
SPNCRNA.564		antisense RNA (predicted)	2.12
SPAC977.06		S. pombe specific DUF999 family protein 3	2.11
SPAC139.05		succinate-semialdehyde dehydrogenase (predicted)	2.03
SPBC16E9.16c	lsd90	Lsd90 protein	2.03
SPNCRNA.402		non-coding RNA (predicted)	2.01

Systematic Name	Gene Name	Description(ver6-2011-Dec)	log2(ctdS7A/wt)
SPNCRNA.234		non-coding RNA, centromeric (predicted)	8.41
SPNCRNA.371		non-coding RNA, centromeric (predicted)	8.18
SPNCRNA.360		non-coding RNA, centromeric (predicted)	7.92
SPNCRNA.373		non-coding RNA, centromeric (predicted)	7.70
SPNCRNA.231		non-coding RNA, centromeric (predicted)	6.92
SPNCRNA.232		non-coding RNA, centromeric (predicted)	5.79
SPNCRNA.372		non-coding RNA, centromeric (predicted)	5.72
SPBC23G7.09	matmc_2	mating-type m-specific polypeptide mc 2	5.35
SPNCRNA.359		non-coding RNA, centromeric (predicted)	5.10
SPBC3E7.02c	hsp16	heat shock protein Hsp16	4.34
SPBC839.06	cta3	P-type ATPase, calcium transporting Cta3	4.03
SPCC737.04		S. pombe specific UPF0300 family protein 6	3.79
SPNCRNA.388		non-coding RNA (predicted)	3.73
SPNCRNA.574		antisense RNA (predicted)	3.55
SPCC70.12c	ec11	extender of the chronological lifespan protein Ecl1	3.49
SPBC1271.09		glycerophosphodiester transporter (predicted)	3.44
SPCC1020.01c	pma2	P-type proton ATPase, P3-type Pma2	3.29
SPCC1840.12		OPT oligopeptide transporter family protein (predicted)	3.17
SPAC212.11	tlh1	RecQ type DNA helicase	3.15
SPCC191.11	inv1	beta-fructofuranosidase	3.09
SPNCRNA.314		not found	3.04
SPBC19C7.04c		conserved fungal protein	2.99
SPBCPT2R1.08c	tlh2	RecQ type DNA helicase Tlh1	2.97
SPAC513.03	mfm2	M-factor precursor Mfm2	2.94
SPAC23H3.15c		sequence orphan	2.69
SPBC16E9.16c	lsd90	Lsd90 protein	2.69
SPNCRNA.230		non-coding RNA, centromeric (predicted)	2.59
SPBC56F2.06	mug147	sequence orphan	2.58
SPAC13G7.02c	ssa1	heat shock protein Ssa1 (predicted)	2.58
SPCC794.04c		membrane transporter (predicted)	2.49
SPAC22H10.13	zym1	metallothionein Zym1	2.48
SPNCRNA.365		non-coding RNA, centromeric (predicted)	2.43
SPBPB21E7.04c		human COMT ortholog 2	2.37
SPNCRNA.554		antisense RNA (predicted)	2.18
SPBC23G7.10c		NADH-dependent flavin oxidoreductase (predicted)	2.15
SPBC8E4.12c	ec13	extender of the chronological lifespan protein Ecl3	2.13
SPAC17A2.07c		sequence orphan	2.09
SPNCRNA.98	srp7	7SL signal recognition particle component	2.08
SPAC3G9.11c		pyruvate decarboxylase (predicted)	2.06
SPAC3C7.03c	rhp55	RecA family ATPase Rhp55	2.03
SPAC637.03		conserved fungal protein	2.03
SPCC1259.14c	meu27	S. pombe specific UPF0300 family protein 5	2.02

Systematic Name	Gene Name	Description(ver6-2011-Dec)	log2(<i>dcr1Δ</i> /wt)
SPNCRNA.234		non-coding RNA, centromeric (predicted)	7.82
SPNCRNA.371		non-coding RNA, centromeric (predicted)	7.47
SPNCRNA.360		non-coding RNA, centromeric (predicted)	7.45
SPNCRNA.373		non-coding RNA, centromeric (predicted)	7.30
SPNCRNA.231		non-coding RNA, centromeric (predicted)	6.71
SPNCRNA.232		non-coding RNA, centromeric (predicted)	5.65
SPNCRNA.372		non-coding RNA, centromeric (predicted)	5.15
SPBC3E7.02c	hsp16	heat shock protein Hsp16	5.00
SPNCRNA.359		non-coding RNA, centromeric (predicted)	4.98
SPBC839.06	cta3	P-type ATPase, calcium transporting Cta3	4.94
SPCC70.12c	ecl1	extender of the chronological lifespan protein Ecl1	4.09
SPCC1840.12		OPT oligopeptide transporter family protein (predicted)	3.89
SPNCRNA.574		antisense RNA (predicted)	3.87
SPCC191.11	inv1	beta-fructofuranosidase	3.78
SPCC338.18		sequence orphan	3.45
SPNCRNA.288		not found	3.39
SPNCRNA.314		not found	2.99
SPBC1683.06c		uridine ribohydrolase (predicted)	2.91
SPCC737.04		S. pombe specific UPF0300 family protein 6	2.90
SPNCRNA.583		antisense RNA (predicted)	2.86
SPBPB21E7.04c		human COMT ortholog 2	2.72
SPAC23H3.15c		sequence orphan	2.65
SPNCRNA.540		non-coding RNA (predicted)	2.63
SPBC947.05c		ferric-chelate reductase Frp2 (predicted)	2.58
SPBC19C7.04c		conserved fungal protein	2.58
SPBC56F2.06	mug147	sequence orphan	2.55
SPAC13G7.02c	ssa1	heat shock protein Ssa1 (predicted)	2.53
SPNCRNA.230		non-coding RNA, centromeric (predicted)	2.53
SPAC22H10.13	zym1	metallothionein Zym1	2.47
SPBPB21E7.08		pseudogene	2.39
SPBC1773.03c		aminotransferase class-III, unknown specificity	2.30
SPAC22F3.02	atf31	transcription factor Atf31	2.27
SPBC21C3.19		SBDS family protein Rtc3 (predicted)	2.25
SPNCRNA.67	prl67	antisense RNA (predicted)	2.23
SPBC1685.05		serine protease (predicted)	2.17
SPCPB16A4.06c		sequence orphan	2.11
SPBC16E9.16c	lsd90	Lsd90 protein	2.11
SPAC139.05		succinate-semialdehyde dehydrogenase (predicted)	2.11
SPBC1683.12		nicotinic acid plasma membrane transporter (predicted)	2.07
SPCC663.06c		short chain dehydrogenase (predicted)	2.06
SPCC965.11c		amino acid transporter (predicted)	2.06

Systematic Name	Gene Name	Description(ver6-2011-Dec)	log2(ctdS2A/wt)
SPBPB10D8.02c		arylsulfatase (predicted)	-3.86
SPBC359.02	alr2	alanine racemase Alr2 (predicted)	-3.83
SPBC1271.07c		N-acetyltransferase (predicted)	-3.60
SPNCRNA.347		not found	-3.41
SPCC576.19c		dubious	-3.23
SPBPB21E7.07	aes1	enhancer of RNA-mediated gene silencing	-2.95
SPCC622.05		dubious	-2.93
SPBPB10D8.03		pseudogene transporter	-2.85
SPBC15D4.13c		human ASCC1 ortholog (predicted)	-2.78
SPCC330.03c		NADPH-hemoprotein reductase (predicted)	-2.78
SPBC359.04c		cell surface glycoprotein (predicted), DIPSY family	-2.73
SPCC965.14c		cytosine deaminase (predicted)	-2.69
SPBC887.19	rft1	Man5GlcNac2-PP-Dol translocation protein Rft1 (predicted)	-2.61
SPAP27G11.15	slx1	structure-specific endonuclease catalytic subunit	-2.57
SPBC1703.08c		5-formyltetrahydrofolate cyclo-ligase (predicted)	-2.56
SPCC330.19c		sequence orphan	-2.52
SPNCRNA.379		non-coding RNA (predicted)	-2.50
SPAC25G10.02	cce1	mitochondrial cruciform cutting endonuclease Cce1	-2.41
SPBC1709.14		peptide N-glycanase (predicted)	-2.39
SPAPB15E9.02c		dubious	-2.36
SPNCRNA.277		non-coding RNA (predicted)	-2.34
SPBC713.05		WD repeat protein, human MAPK organizer 1 (MORG1) family (predicted)	-2.32
SPAC227.16c	psf3	GIN5 complex subunit Psf3	-2.29
SPAC11D3.02c		ELLA family acetyltransferase (predicted)	-2.29
SPAC56F8.15		sequence orphan	-2.26
SPAC1F8.01	ght3	hexose transporter Ght3	-2.26
SPCC330.10	pcm1	P-TEFb-cap methyltransferase Pcm1	-2.24
SPNCRNA.301		non-coding RNA (predicted)	-2.24
SPAC1B3.16c	vht1	vitamin H transporter Vht1	-2.24
SPCC1393.14	ten1	nuclear telomere cap complex subunit Ten1	-2.22
SPNCRNA.42	prl42	non-coding RNA, poly(A)-bearing (predicted)	-2.19
SPBC1271.08c		sequence orphan	-2.14
SPBC29A10.09c		CAF1 family ribonuclease (predicted)	-2.14
SPAC3G6.05		Mpv17/PMP22 family protein 1 (predicted)	-2.12
SPNCRNA.440		non-coding RNA (predicted)	-2.11
SPAC23C11.04c	pnk1	DNA kinase/phosphatase Pnk1	-2.06

Systematic Name	Gene Name	Description(ver6-2011-Dec)	log2(<i>ctdS7A/wt</i>)
SPBPB2B2.09c		2-dehydropantoate 2-reductase (predicted)	-7.58
SPBPB2B2.13		galactokinase Gal1	-5.65
SPBPB10D8.02c		arylsulfatase (predicted)	-4.77
SPBC359.04c		cell surface glycoprotein (predicted), DIPSY family	-4.64
SPNCRNA.532		non-coding RNA (predicted)	-4.61
SPBPB10D8.01		cysteine transporter (predicted)	-4.44
SPNCRNA.293		non-coding RNA (predicted)	-4.24
SPBPB2B2.10c		galactose-1-phosphate uridylyltransferase Gal7	-4.02
SPBPB2B2.01		amino acid permease (predicted)	-3.92
SPBPB2B2.05		peptidase family C26 protein	-3.67
SPBC359.02	alr2	alanine racemase Alr2 (predicted)	-3.49
SPBPB10D8.03		pseudogene transporter	-3.43
SPAC186.03		L-asparaginase (predicted)	-3.37
SPBPB21E7.09		L-asparaginase (predicted)	-3.29
SPBPB2B2.12c		UDP-glucose 4-epimerase/aldose 1-epimerase Gal10	-3.20
SPNCRNA.347		not found	-3.11
SPAC212.04c		S. pombe specific DUF999 family protein 1	-3.10
SPAC1039.08		serine acetyltransferase (predicted)	-3.07
SPCC622.05		dubious	-2.93
SPCP20C8.02c		S. pombe specific UPF0321 family protein 1	-2.75
SPAC186.06		ER unfolded protein response protein (predicted)	-2.66
SPAC1039.10	mmf2	homologous Pmf1 factor 1, implicated in isoleucine biosynthesis (predicted)	-2.60
SPBPB2B2.02	mug180	esterase/lipase (predicted)	-2.54
SPAC11D3.11c		zinc finger protein, truncated	-2.53
SPNCRNA.189		non-coding RNA (predicted)	-2.31
SPNCRNA.277		non-coding RNA (predicted)	-2.27
SPBC1773.12		transcription factor (predicted)	-2.26
SPNCRNA.178		non-coding RNA (predicted)	-2.24
SPAC11D3.10		nifs homolog, possible cysteine desulfurase	-2.24
SPAC11D3.18c		nicotinic acid plasma membrane transporter (predicted)	-2.18
SPNCRNA.508		non-coding RNA (predicted)	-2.17
SPBPB2B2.06c		phosphoprotein phosphatase (predicted)	-2.11
SPBC1271.07c		N-acetyltransferase (predicted)	-2.10
SPNCRNA.181		non-coding RNA (predicted)	-2.09
SPAC186.05c		human TMEM165 homolog	-2.08
SPBC557.05		arrestin	-2.07
SPAC5H10.11	gmh1	alpha-1,2-galactosyltransferase Gmh1 (predicted)	-2.07
SPNCRNA.253		non-coding RNA (predicted)	-2.06
SPNCRNA.14	pr14	antisense RNA (predicted)	-2.03
SPAC2E1P3.05c		fungus cellulose binding domain protein	-2.02
SPNCRNA.115		non-coding RNA (predicted)	-2.02
SPAC6C3.03c		sequence orphan	-2.02

Systematic Name	Gene Name	Description(ver6-2011-Dec)	log2(<i>dcr1Δ/wt</i>)
SPAC2E1P3.05c		fungus cellulose binding domain protein	-4.62
SPAC186.05c		human TMEM165 homolog	-4.30
SPAC186.06		ER unfolded protein response protein (predicted)	-3.43
SPAC2E1P3.04	cao1	copper amine oxidase Cao1	-3.29
SPNCRNA.347		not found	-2.99
SPNCRNA.194		non-coding RNA (predicted)	-2.41
SPNCRNA.364		non-coding RNA, centromeric (predicted)	-2.38
SPBCPT2R1.08c	tlh2	RecQ type DNA helicase Tlh1	-2.18
SPNCRNA.178		non-coding RNA (predicted)	-2.16
SPNCRNA.253		non-coding RNA (predicted)	-2.11
SPNCRNA.115		non-coding RNA (predicted)	-2.08
SPNCRNA.580		antisense RNA (predicted)	-2.05
SPBC32H8.06	mug93	TPR repeat protein, meiotically spliced	-2.03

Table S2. Changes in silencing-related gene expression in *ctdS2A* and *ctdS7A* mutants.

Complex or Function	gene name	log2(<i>ctdS2A</i> /wt)	log2(<i>ctdS7A</i> /wt)
CLRC	<i>clr4</i>	-0.174	1.037
	<i>rik1</i>	0.522	-0.352
	<i>raf1</i>	0.209	0.235
	<i>raf2</i>	0.111	-0.148
	<i>pcu4</i>	-0.622	0.353
	<i>stc1</i>	-1.088	-0.232
	<i>ned8</i>	0.223	0.110
RITS	<i>ago1</i>	-0.225	0.066
	<i>chp1</i>	-0.36	0.145
	<i>tas3</i>	0.292	-0.191
	<i>tri1</i>	-2.178	-0.722
ARC	<i>arb1</i>	0.094	-0.408
	<i>arb2</i>	-0.759	-0.687
RDRC	<i>rdp1</i>	0.455	-0.262
	<i>hrr1</i>	0.563	-0.037
	<i>cid12</i>	-1.234	1.105
other RNAi	<i>dcr1</i>	-0.186	1.391
	<i>dsh1</i>	0.488	-0.282
	<i>ers1</i>	0.083	-0.768
	<i>swi6</i>	-0.172	-0.084
SHREC	<i>chp2</i>	0.199	-0.009
	<i>clr1</i>	-0.068	-0.329
	<i>clr2</i>	-0.337	-0.605
	<i>clr3</i>	0.138	-0.049
	<i>mit1</i>	-0.01	0.046
	<i>ckb1</i>	0.088	0.462
Splicing	<i>prp10</i>	0.103	0
	<i>cwf10</i>	-0.129	-0.022
	<i>prp5</i>	-0.131	-0.079
	<i>prp8</i>	0.186	-0.056
	<i>prp12</i>	0.096	-0.102
	<i>prp39</i>	-0.014	-0.033

Complex or Function	gene name	log2(ctdS2A/wt)	log2(ctdS7A/wt)
transcription	<i>med20</i>	-0.34	-0.038
	<i>med18</i>	-0.11	0.479
	<i>rpb2</i>	-0.058	-0.055
	<i>rpb7</i>	0.09	0.192
	<i>epe1</i>	-0.16	0.368
	<i>spt6</i>	-0.087	-0.037
	<i>iws1</i>	-0.2	0.087
	<i>mms19</i>	-0.393	-0.362
Epe1	<i>epe1</i>	-0.16	0.368
	<i>bdf2</i>	0.024	0.246
	<i>cdt2</i>	0.255	0.253
CENP-B	<i>abp1</i>	0.142	-0.316
	<i>cbh1</i>	-0.24	-0.205
	<i>cbh2</i>	1.014	-0.174
Lid2	<i>sdc1</i>	0.527	0.076
	<i>snt2</i>	0.375	-0.021
	<i>lid2</i>	0.008	-0.327
	<i>ash2</i>	-0.036	-0.139
	<i>jmj3</i>	-0.207	-0.266
	<i>lid2</i>	0.008	-0.327
Lsd1/2	<i>lsd1</i>	0.045	-0.02
	<i>lsd2</i>	0.278	-0.331
	<i>phf1</i>	-0.077	0.075
	<i>phf2</i>	0.108	-0.028
	<i>clr5</i>	-0.293	0.172
Clr6(Rpd3L)	<i>sds3</i>	-0.289	-0.077
	<i>png2</i>	0.647	0.378
	<i>pst1</i>	-0.096	0.187
	<i>clr6</i>	-0.371	0.142
	<i>prw1</i>	-0.116	0.126
Clr6(Rpd3S)	<i>laf2</i>	-0.247	0.141
	<i>cph1</i>	0.325	0.144
	<i>laf1</i>	-0.349	-0.184
	<i>pst3</i>	-0.51	0.22
	<i>cph2</i>	0.136	-0.283
	<i>alp13</i>	0.09	-0.086

Complex or Function	gene name	log2(<i>ctdS2A</i> /wt)	log2(<i>ctdS7A</i> /wt)
Clr6(Rpd3S)	<i>sds3</i>	-0.289	-0.077
	<i>png2</i>	0.647	0.378
	<i>rxt3</i>	0.111	0.513
	<i>cti6</i>	0.386	0.287
	<i>pst2</i>	0.495	-0.109
	<i>dep1</i>	-0.364	0
	<i>rxt2</i>	-0.405	0.277
	<i>pst1</i>	-0.096	0.187
	<i>clr6</i>	-0.371	0.142
	<i>prw1</i>	-0.116	0.126
Sir2 family	<i>sir2</i>	-0.012	-0.694
	<i>hst2</i>	-0.129	0.054
	<i>hst4</i>	0.27	0.082
HDAC non sir2	<i>hos2</i>	-0.017	-0.067
TRAMP	<i>cid14</i>	0.15	-0.295
	<i>air1</i>	-0.066	-0.061
	<i>mtr4</i>	-0.136	-0.079
	<i>SPAC17H9.02</i>	0.234	-0.171
	<i>mlo3</i>	-0.222	-0.411
	<i>tfs1</i>	-0.103	-0.142
exosome	<i>rrp6</i>	0.074	-0.143
	<i>pla1</i>	-0.231	-0.028
	<i>red1</i>	0.227	0.145
	<i>pab2</i>	0.02	0.314
	<i>mmi1</i>	0.174	0.009
	<i>dis3</i>	-0.176	-0.106
Chromatin remodeling	<i>hrp1</i>	-0.25	-0.131
	<i>hrp3</i>	-0.196	-0.092
Histone chaperone	<i>spt16</i>	-0.208	0.23
	<i>pob3</i>	0.136	0.334
	<i>cia1</i>	-1.042	0.171
HIRA	<i>slm9</i>	0.17	-0.201
	<i>hip1</i>	-0.014	-0.007
	<i>hip3</i>	0.362	-0.08
	<i>seb1</i>	0.147	-0.343
	<i>swc3</i>	0.31	0.004

Complex or Function	gene name	log2(<i>ctdS2A</i> /wt)	log2(<i>ctdS7A</i> /wt)
HAT	<i>mst1</i>	-0.02	-0.349
	<i>mst2</i>	-0.835	-0.073
	<i>rtt109</i>	-0.722	-0.731
HULC	<i>rhp6</i>	0.073	-0.003
	<i>brl1</i>	-0.373	0.053
	<i>brl2</i>	-0.078	0.241
	<i>shf1</i>	-1.278	0.006
SUMO	<i>ulp1</i>	0.083	-0.038
	<i>smt3</i>	-0.673	0.007
rRNA	<i>grc3</i>	-0.316	1.084
	<i>rix1</i>	-0.295	-0.037
	<i>las1</i>	-0.379	0.01

Table S3. List of strains used in this study

Strain	mating type	Genotype	Source
TKY570	<i>h</i> ⁺	<i>ade6-DN/N, leu1-32, ura4-DS/E, imr1L(Ncol)::ura4⁺, otr1R(SphI)::ade6⁺</i>	R. Allshire
TKY311	<i>h</i> ⁺	<i>ade6-DN/N, leu1-32, ura4-DS/E, imr1L(Ncol)::ura4⁺, otr1R(SphI)::ade6⁺, clr4Δ::nat</i>	This study
TKY318	<i>h</i> ⁺	<i>ade6-DN/N, leu1-32, ura4-DS/E, imr1L(Ncol)::ura4⁺, otr1R(SphI)::ade6⁺, dcr1Δ::nat</i>	This study
TKY572	<i>h</i> ⁺	<i>ade6-DN/N, ura4-DS/E, imr1L(Ncol)::ura4⁺, otr1R(SphI)::ade6⁺, rpb2-m203</i>	This study
TKY1191	<i>h</i> ⁺	<i>ade6-DN/N, leu1-32?, ura4-DS/E, imr1L(Ncol)::ura4⁺, otr1R(SphI)::ade6⁺, rpb1-11-kan</i>	derivative of gift of R. Allshire
TKY547	<i>h</i> ⁺	<i>ade6-DN/N, leu1-32?, ura4-DS/E, imr1L(Ncol)::ura4⁺, otr1R(SphI)::ade6⁺, rpb1-ctdS2A-kan</i>	This study
TKY1195	<i>h</i> ⁺	<i>ade6-DN/N, ura4-DS/E, leu1-32, imr1L(Ncol)::ura4⁺, otr1R(SphI)::ade6⁺, rpb1-ctdS7A-kan</i>	This study
TKY846	?	<i>ade6-DN/N, leu1-32?, ura4-DS/E, imr1L(Ncol)::ura4⁺, otr1R(SphI)::ade6⁺, nat-Pago1-3FLAG-ago1</i>	This study
TKY856	?	<i>ade6-DN/N, leu1-32?, ura4-DS/E, imr1L(Ncol)::ura4⁺, otr1R(SphI)::ade6⁺, nat-Pago1-3FLAG-ago1, clr4Δ::kan</i>	This study
TKY859	?	<i>ade6-DN/N, leu1-32?, ura4-DS/E, imr1L(Ncol)::ura4⁺, otr1R(SphI)::ade6⁺, nat-Pago1-3FLAG-ago1, dcr1Δ::kan</i>	This study
TKY861	?	<i>ade6-DN/N, leu1-32?, ura4-DS/E, imr1L(Ncol)::ura4⁺, otr1R(SphI)::ade6⁺, nat-Pago1-3FLAG-ago1, rpb1-11-kan</i>	derivative of gift of R. Allshire
TKY865	<i>h</i> ⁹⁰	<i>ade6-DN/N, leu1-32?, ura4-DS/E, imr1L(Ncol)::ura4⁺, otr1R(SphI)::ade6⁺, nat-Pago1-3FLAG-ago1, rpb1-ctdS2A-kan</i>	This study
TKY866	?	<i>ade6-DN/N, leu1-32?, ura4-DS/E, imr1L(Ncol)::ura4⁺, otr1R(SphI)::ade6⁺, nat-Pago1-3FLAG-ago1, rpb1-ctdS7A-kan</i>	This study
TKY328	<i>h</i> ⁺	<i>ade6-DN/N, leu1-32?, ura4-DS/E, imr1L(Ncol)::ura4⁺, otr1R(SphI)::ade6⁺, chp1-13myc-kan</i>	This study
TKY407	<i>h</i> ⁺	<i>ade6-DN/N, leu1-32?, ura4-DS/E, imr1L(Ncol)::ura4⁺, otr1R(SphI)::ade6⁺, chp1-13myc-kan, clr4Δ::nat</i>	This study
TKY410	<i>h</i> ⁺	<i>ade6-DN/N, leu1-32?, ura4-DS/E, imr1L(Ncol)::ura4⁺, otr1R(SphI)::ade6⁺, chp1-13myc-kan, dcr1Δ::nat</i>	This study
TKY870	<i>h</i> ⁺	<i>ade6-DN/N, leu1-32?, ura4-DS/E, imr1L(Ncol)::ura4⁺, otr1R(SphI)::ade6⁺, chp1-13myc-nat, rpb1-11-kan</i>	derivative of gift of R. Allshire
TKY880	<i>h</i> ⁹⁰	<i>ade6-DN/N, leu1-32?, ura4-DS/E, imr1L(Ncol)::ura4⁺, otr1R(SphI)::ade6⁺, chp1-13myc-kan, rpb1-ctdS2A-hph</i>	This study
TKY884	?	<i>ade6-DN/N, leu1-32?, ura4-DS/E, imr1L(Ncol)::ura4⁺, otr1R(SphI)::ade6⁺, chp1-13myc-kan, rpb1-ctdS7A-hph</i>	This study
TKY919	<i>h</i> ⁺	<i>ade6-DN/N, leu1-32?, ura4-DS/E, imr1L(Ncol)::ura4⁺, otr1R(SphI)::ade6⁺, rdp1-13myc-nat</i>	This study
TKY922	<i>h</i> ⁺	<i>ade6-DN/N, leu1-32?, ura4-DS/E, imr1L(Ncol)::ura4⁺, otr1R(SphI)::ade6⁺, rdp1-13myc-nat, clr4Δ::kan</i>	This study
TKY925	<i>h</i> ⁺	<i>ade6-DN/N, leu1-32?, ura4-DS/E, imr1L(Ncol)::ura4⁺, otr1R(SphI)::ade6⁺, rdp1-13myc-nat, dcr1Δ::kan</i>	This study
TKY1015	?	<i>ade6-DN/N, leu1-32?, ura4-DS/E, imr1L(Ncol)::ura4⁺, otr1R(SphI)::ade6⁺, rdp1-13myc-nat, rpb1-11-kan</i>	derivative of gift of R. Allshire
TKY985	?	<i>ade6-DN/N, leu1-32?, ura4-DS/E, imr1L(Ncol)::ura4⁺, otr1R(SphI)::ade6⁺, rdp1-13myc-nat, rpb1-ctdS2A-kan</i>	This study

Strain	mating type	Genotype	Source
TKY937	?	<i>ade6-DN/N, leu1-32?, ura4-DS/E, imr1L(Ncol)::ura4⁺, otr1R(SphI)::ade6⁺, rdp1-13myc-nat, rpb1-ctdS7A-nat</i>	This study
TKY887	<i>h⁻</i>	<i>ade6-DN/N, leu1-32?, ura4-DS/E, imr1L(Ncol)::ura4⁺, otr1R(SphI)::ade6⁺, rik1-13myc-nat</i>	This study
TKY893	<i>h⁻</i>	<i>ade6-DN/N, leu1-32?, ura4-DS/E, imr1L(Ncol)::ura4⁺, otr1R(SphI)::ade6⁺, rik1-13myc-nat, clr4Δ::kan</i>	This study
TKY896	<i>h⁻</i>	<i>ade6-DN/N, leu1-32?, ura4-DS/E, imr1L(Ncol)::ura4⁺, otr1R(SphI)::ade6⁺, rik1-13myc-nat, dcr1Δ::kan</i>	This study
TKY899	<i>h⁺</i>	<i>ade6-DN/N, leu1-32?, ura4-DS/E, imr1L(Ncol)::ura4⁺, otr1R(SphI)::ade6⁺, rik1-13myc-nat, rpb1-11-kan</i>	derivative of gift of R. Allshire
TKY899	<i>h⁺</i>	<i>ade6-DN/N, leu1-32?, ura4-DS/E, imr1L(Ncol)::ura4⁺, otr1R(SphI)::ade6⁺, rik1-13myc-nat, rpb1-11-kan</i>	This study
TKY911	<i>h⁻</i>	<i>ade6-DN/N, leu1-32?, ura4-DS/E, imr1L(Ncol)::ura4⁺, otr1R(SphI)::ade6⁺, rik1-13myc-nat, rpb1-ctdS2A-kan</i>	This study
TKY915	<i>h⁻</i>	<i>ade6-DN/N, leu1-32?, ura4-DS/E, imr1L(Ncol)::ura4⁺, otr1R(SphI)::ade6⁺, rik1-13myc-nat, rpb1-ctdS7A-kan</i>	This study
TKY1778	<i>h⁺</i>	<i>ade6-DN/N, leu1-32, ura4-DS/E, imr1L(Ncol)::ura4⁺, otr1R(SphI)::ade6⁺, ago1Δ::kan</i>	This study
TKY1800	<i>h⁺</i>	<i>ade6-DN/N, leu1-32, ura4-DS/E, imr1L(Ncol)::ura4⁺, otr1R(SphI)::ade6⁺, chp1Δ::hph</i>	This study
TKY694	<i>h⁺</i>	<i>ade6-DN/N, leu1-32, his2-?, ura4-DS/E, mr1L(Ncol)::ura4⁺, otr1R(SphI)::ade6⁺, rdp1Δ::kan</i>	This study
TKY2365	<i>h⁺</i>	<i>ade6-DN/N, leu1-32, ura4-DS/E, otr1R(SphI)::ade6⁺, imr1L(Ncol)::ura4⁺, rpb1-ctdS7A-kan, chp1Δ::hph</i>	This study
TKY2206	<i>h⁺</i>	<i>ade6-DN/N, leu1-32, ura4-DS/E, imr1L(Ncol)::ura4⁺, otr1R(SphI)::ade6⁺, hph-Pchp1-3flag-chp1</i>	derivative of gift of J. Nakayama
TKY2218	<i>h⁺</i>	<i>ade6-DN/N, leu1-32, ura4-DS/E, imr1L(Ncol)::ura4⁺, otr1R(SphI)::ade6⁺, hph-Pchp1-3flag-chp1-amut1</i>	derivative of gift of J. Nakayama
TKY2225	<i>h⁺</i>	<i>ade6-DN/N, ura4-DS/E, leu1-32, otr1R(SphI)::ade6⁺, imr1L(Ncol)::ura4⁺, rpb1-ctdS7A-kan, hph-Pchp1-3flag-chp1</i>	derivative of gift of J. Nakayama
TKY2236	<i>h⁺</i>	<i>ade6-DN/N, ura4-DS/E, leu1-32, otr1R(SphI)::ade6⁺, imr1L(Ncol)::ura4⁺, rpb1-ctdS7A-kan, hph-Pchp1-3flag-chp1-amut1</i>	derivative of gift of J. Nakayama
TKY1744	<i>h⁺</i>	<i>ade6-DN/N, leu1-32, ura4-DS/E, imr1L(Ncol)::ura4⁺, otr1R(SphI)::ade6⁺, hph-Purg1-3flag-dcr1</i>	This study
TKY1748	<i>h⁺</i>	<i>ade6-DN/N, leu1-32, ura4-DS/E, imr1L(Ncol)::ura4⁺, otr1R(SphI)::ade6⁺, hph-Purg1-3flag-dcr1, clr4Δ::nat</i>	This study
TKY1754	<i>h⁺</i>	<i>ade6-DN/N, leu1-32, ura4-DS/E, imr1L(Ncol)::ura4⁺, otr1R(SphI)::ade6⁺, hph-Purg1-3flag-dcr1, chp1Δ::kan</i>	This study
TKY1757	<i>h⁺</i>	<i>ade6-DN/N, leu1-32, ura4-DS/E, imr1L(Ncol)::ura4⁺, otr1R(SphI)::ade6⁺, hph-Purg1-3flag-dcr1, rdp1Δ::kan</i>	This study
TKY1771	<i>h⁺</i>	<i>ade6-DN/N, leu1-32, ura4-DS/E, imr1L(Ncol)::ura4⁺, otr1R(SphI)::ade6⁺, hph-Purg1-3flag-dcr1, ago1Δ::kan</i>	This study
TKY3742	<i>h⁺</i>	<i>ade6-DN/N, leu1-32, ura4-DS/E, imr1L(Ncol)::ura4⁺, otr1R(SphI)::ade6⁺, hph-Purg1-3flag-dcr1, rpb1-ctdS7A-kan, chp1Δ::nat</i>	This study
TKY2345	<i>h⁺</i>	<i>ade6-DN/N, leu1-32, ura4-DS/E, imr1L(Ncol)::ura4⁺, otr1R(SphI)::ade6⁺, hph-Purg1-3flag-dcr1, kan-Pchp1-3flag-chp1</i>	derivative of gift of J. Nakayama
TKY2349	<i>h⁺</i>	<i>ade6-DN/N, leu1-32, ura4-DS/E, imr1L(Ncol)::ura4⁺, otr1R(SphI)::ade6⁺, hph-Purg1-3flag-dcr1, kan-Pchp1-3flag-chp1-amut1</i>	derivative of gift of J. Nakayama
TKY2353	<i>h⁺</i>	<i>ade6-DN/N, leu1-32, ura4-DS/E, imr1L(Ncol)::ura4⁺, otr1R(SphI)::ade6⁺, hph-Purg1-3flag-dcr1, kan-Pchp1-3flag-chp1, rpb1-ctdS7A-nat</i>	derivative of gift of J. Nakayama

Strain	mating type	Genotype	Source
TKY2359	<i>h</i> ⁺	<i>ade6-DN/N, leu1-32, ura4-DS/E, imr1L(NcoI)::ura4⁺, otr1R(SphI)::ade6⁺, hph-Purg1-3flag-dcr1, chp1::kan-Pchp1-3flag-chp1-amut1, rpb1-ctdS7A-nat</i>	derivative of gift of J. Nakayama
TKY1326	<i>h</i> ⁻	<i>ura4⁺, leu1-32, ade6-DN/N, chp1-mycx6-his3, his3-D1?, otr1R(SphI)::ade6⁺</i>	derivative of gift of R. Allshire
TKY1455	<i>h</i> ⁹⁰	<i>ura4-DS/E, leu1-32, ade6-DN/N, his3-D1?, Kint2::ura4⁺, his2, otr1R(SphI)::ade6⁺, chp1-13myc-nat</i>	derivative of gift of S. Grewal
TKY1460	<i>h</i> ⁹⁰	<i>ura4-DS/E, leu1-32, ade6-DN/N, his3-D1?, Kint2::ura4⁺, his2 otr1R(SphI)::ade6⁺, chp1-13myc-nat, clr4Δ::kan</i>	This study
TKY1463	<i>h</i> ⁹⁰	<i>ura4-DS/E, leu1-32, ade6-DN/N, his3-D1?, Kint2::ura4⁺, his2 otr1R(SphI)::ade6⁺, chp1-13myc-nat, dcr1Δ::kan</i>	This study
TKY1729	<i>h</i> ⁹⁰	<i>ura4-DS/E, leu1-32, ade6-DN/N, his3-D1?, Kint2::ura4⁺, his2, otr1R(SphI)::ade6⁺, chp1-13myc-nat, ago1Δ::kan</i>	This study
TKY1046	<i>h</i> ⁹⁰	<i>ura4-DS/E, leu1-32, ade6-DN/N, his3-D1?, Kint2::ura4⁺, his2, otr1R(SphI)::ade6⁺, chp1Δ::kan</i>	This study
TKY1741	<i>h</i> ⁹⁰	<i>ura4-DS/E, leu1-32, ade6-DN/N, his3-D1?, Kint2::ura4⁺, his2, otr1R(SphI)::ade6⁺, chp1-13myc-nat, tas3Δ::kan</i>	This study
TKY1732	<i>h</i> ⁹⁰	<i>ura4-DS/E, leu1-32, ade6-DN/N, his3-D1?, Kint2::ura4⁺, his2, otr1R(SphI)::ade6⁺, chp1-13myc-nat, stc1Δ::kan</i>	This study
TKY1472	<i>h</i> ⁹⁰	<i>ura4-DS/E, leu1-32, ade6-DN/N, his3-D1?, Kint2::ura4⁺, his2, otr1R(SphI)::ade6⁺, chp1-13myc-nat, rpb2-m203</i>	This study
TKY1475	<i>h</i> ⁹⁰	<i>ura4-DS/E, leu1-32, ade6-DN/N, his3-D1?, Kint2::ura4⁺, his2, otr1R(SphI)::ade6⁺, chp1-13myc-nat, rpb1-ctdS2A-kan</i>	This study
TKY2441	<i>h</i> ⁹⁰	<i>ura4-DS/E, leu1-32, ade6-DN/N, his3-D1?, Kint2::ura4⁺, his2, otr1R(SphI)::ade6⁺, hph-Pchp1-3flag-chp1-13myc-nat</i>	derivative of gift of J. Nakayama
TKY3786	<i>h</i> ⁹⁰	<i>ura4-DS/E, leu1-32, ade6-DN/N, his3-D1?, Kint2::ura4⁺, his2, otr1R(SphI)::ade6⁺, hph-Pchp1-3flag-chp1-amut1-13myc-nat,</i>	derivative of gift of J. Nakayama
TKY2250	<i>h</i> ⁹⁰	<i>ura4-DS/E, leu1-32, ade6-DN/N, Kint2::ura4⁺, his2, otr1R(SphI)::ade6⁺, hph-Pchp1-3flag-chp1-13myc-nat, rpb1-ctdS7A-kan</i>	derivative of gift of J. Nakayama
TKY4086	<i>h</i> ⁹⁰	<i>ura4-DS/E, leu1-32, ade6-DN/N, his3-D1?, Kint2::ura4⁺, otr1R(SphI)::ade6⁺, hph-Pchp1-3flag-chp1-amut1-13myc-nat, rpb1-ctdS7A-kan</i>	derivative of gift of J. Nakayama
TKY1031	<i>h</i> ⁹⁰	<i>ura4-DS/E, leu1-32, ade6-DN/N, his3-D1?, Kint2::ura4⁺, his2, otr1R(SphI)::ade6⁺, chp1-6myc-his3⁺</i>	derivative of gift of S. Grewal and R. Allshire
TKY1174	<i>h</i> ⁹⁰	<i>ura4-DS/E, leu1-32, ade6-DN/N, his3-D1?, Kint2::ura4⁺, his2, otr1R(SphI)::ade6⁺, chp1-6myc-his3⁺, clr4Δ::nat</i>	This study
TKY4336	<i>h</i> ⁺	<i>ade6-DN/N, leu1-32, ura4-DS/E, imr1L(NcoI)::ura4⁺, otr1R(SphI)::ade6⁺, rpb1-(ctdwt)x18-nat</i>	derivative of gift of S. Shuman
TKY4437	<i>h</i> ⁺	<i>ade6-DN/N, leu1-32, ura4-DS/E, imr1L(NcoI)::ura4⁺, otr1R(SphI)::ade6⁺, rpb1-(ctdwt)x18-nat, clr4Δ::kan</i>	derivative of gift of S. Shuman
TKY4441	<i>h</i> ⁺	<i>ade6-DN/N, leu1-32, ura4-DS/E, imr1L(NcoI)::ura4⁺, otr1R(SphI)::ade6⁺, rpb1-(ctdwt)x18-nat, dcr1Δ::kan</i>	derivative of gift of S. Shuman
TKY4352	<i>h</i> ⁺	<i>ade6-DN/N, leu1-32, ura4-DS/E, imr1L(NcoI)::ura4⁺, otr1R(SphI)::ade6⁺, rpb1-(ctdS7A)x18-nat</i>	derivative of gift of S. Shuman
TKY4445	<i>h</i> ⁺	<i>ade6-DN/N, leu1-32, ura4-DS/E, imr1L(NcoI)::ura4⁺, otr1R(SphI)::ade6⁺, rpb1-(ctdS7A)x18-nat, clr4Δ::kan</i>	derivative of gift of S. Shuman
TKY4449	<i>h</i> ⁺	<i>ade6-DN/N, leu1-32, ura4-DS/E, imr1L(NcoI)::ura4⁺, otr1R(SphI)::ade6⁺, rpb1-(ctdS7A)x18-nat, dcr1Δ::kan</i>	derivative of gift of S. Shuman
TKY1097	<i>h</i> ⁻	<i>ura4-3ERIS-hph, leu1-32 ?, ade6-DN/N, chp1-mycx6-his3, his3-D1? otr1R(SphI)::ade6⁺</i>	This study
TKY2829	<i>h</i> ⁻	<i>ura4-3ERIS-hph, leu1-32 ?, ade6-DN/N, chp1-mycx6-his3, his3-D1? otr1R(SphI)::ade6⁺, natMX6-Purg1-3flag-dcr1</i>	This study

Strain	mating type	Genotype	Source
TKY1131	<i>h⁻</i>	<i>ura4-3ERIS-hph, leu1-32[?], ade6-DN/N, chp1-mycx6-his3, his3-D1[?] otr1R(Sph1)::ade6+, dcr1Δ::nat</i>	This study
TKY1127	<i>h⁻</i>	<i>ura4-3ERIS-hph, leu1-32[?], ade6-DN/N, chp1-mycx6-his3, his3-D1[?], otr1R(Sph1)::ade6+, clr4Δ::kan</i>	This study
TKY2837	<i>h⁻</i>	<i>ura4-3ERIS-hph, leu1-32[?], ade6-DN/N, chp1-mycx6-his3, his3-D1[?] otr1R(Sph1)::ade6+, natMX6-Purg1-3flag-dcr1, clr4Δ::nat</i>	This study
TKY5307	<i>h⁻</i>	<i>ura4-3ERIS-hph, leu1-32[?], ade6-DN/N, chp1-mycx6-his3, his3-D1[?] otr1R(Sph1)::ade6+, rdp1Δ::kan</i>	This study
TKY2834	<i>h⁻</i>	<i>ura4-3ERIS-hph, leu1-32[?], ade6-DN/N, chp1-mycx6-his3, his3-D1[?] otr1R(Sph1)::ade6+, natMX6-Purg1-3flag-dcr1, rdp1Δ::kan</i>	This study
TKY1143	<i>?</i>	<i>ura4-3ERIS-hph, leu1-32[?], ade6-DN/N, chp1-mycx6-his3, his3-D1[?], otr1R(Sph1)::ade6+, rpb1-ctdS7A-kan</i>	This study
TKY2858	<i>?</i>	<i>ura4-3ERIS-hph, leu1-32[?], ade6-DN/N, chp1-mycx6-his3, his3-D1[?] otr1R(Sph1)::ade6+, rpb1-ctdS7A-kan, natMX6-Purg1-3flag-dcr1</i>	This study
TKY5314	<i>h⁻</i>	<i>ura4-3ERIS-hph, leu1-32[?], ade6-DN/N, kan-Pchp1-3flag-chp1-amut1-6myc-his3+, his3-D1[?] otr1R(Sph1)::ade6+</i>	This study
TKY5316	<i>h⁻</i>	<i>ura4-3ERIS-hph, leu1-32[?], ade6-DN/N, kan-Pchp1-3flag-chp1-amut1-6myc-his3+, his3-D1[?] otr1R(Sph1)::ade6+, natMX6-Purg1-3flag-dcr1,</i>	This study

Table S4. List of oligonucleotides used in this study

Oligo name	Sequences	Experiment
act1RT-FW	TGCCGATCGTATGCAAAAGG	RT-PCR, ChIP, RIP
act1RT-RV	CCGCTCTCATCATACTCTTG	RT-PCR, ChIP, RIP
ade6- DN/N124 FW	GTAGTACGCAGTTTAGACGG	RT-PCR, ChIP, RIP
ade6- DN/N124 RV	GAGCACGCTGTTGAATTGAG	RT-PCR, ChIP, RIP
cen hotspot siRNA-F1	GTTTTATTGTGTATCATCTTCCTGGATATCCTTTGTAATAG TT	Northern blot
cen hotspot siRNA-F2	TAATCGTAGTAGATAAACAAGCAATGAGTAAGAAAAAAAT GTA	Northern blot
cen hotspot siRNA-F3	GTACGACGATGATGTGTTTTCTCAAGATGATAACGGATCTA GCT	Northern blot
cen hotspot siRNA-F4	TCGCCATCAATAAGTATGAGGCAAAGGAGTCCATCTTTGA TGAA	Northern blot
cen hotspot siRNA-F5	ACATCCATTTGCCTGTTGTACATTTTTGCAGGACAACCAG CCTG	Northern blot
cen hotspot siRNA-F6	AGCACAAGAGACATGGTGTACTAGACTTTGTGCGGAATGT CTAC	Northern blot
cen hotspot siRNA-F7	TTCAAACTCGCATCTTTATATCTGATATATAGAGATATAGA GATATAGAGAGAGGCTGA	Northern blot
cen hotspot siRNA-F8	TGATTTATAAACACAAACGATAAGACTTGTAACATGAAGTT TGTCGAAAA	Northern blot
cen hotspot siRNA-F9	AGTCAATCTTTAAATCCCTTTCTGGATTTCTTTCTATGTTG AG	Northern blot
cen hotspot siRNA-F10	CTTTGGTACCATGCTTTTAGTGCGGTCATTTAAAGGCATAG CGATGA	Northern blot
cen hotspot siRNA-R1	AATATTACAAAGGATATCCAGGAAGATGATACACAATGAA AAC	Northern blot
cen hotspot siRNA-R2	TACATTTTTTTCTTACTCATTGCTTGTTTATCTACTACGATT A	Northern blot
cen hotspot siRNA-R3	AGCTAGATCCGTTATCATCTTGAGAAAACACATCATCGTC GTAC	Northern blot
cen hotspot siRNA-R4	TTCATCAAAGATGGACTCCTTGCCTCATACTTATTGATGG CGA	Northern blot
cen hotspot siRNA-R5	CAGGCTGGTTGTCCTGCAAAAATGTACAACAGGCAAATG GATGT	Northern blot
cen hotspot siRNA-R6	GTAGACATTCCGCACAAAGTCTAGTACACCATGTCTCTTG TGCT	Northern blot
cen hotspot siRNA-R7	TCAGCCTCTCTCTATATCTCTATATCTCTATATATCAGATATA AAGATGCGAGTTTTGAA	Northern blot
cen hotspot siRNA-R8	TTTCGACAACTTCATGTTACAAGTCTTATCGTTTGTGTT TATAAATCA	Northern blot

Oligo name	Sequences	Experiment
cen hotspot siRNA-R9	CTCGAACATAGAAAGAAATCCAGAAAGGGATTAAAGATTG ACT	Northern blot
cen hotspot siRNA-R10	TCATCGCTATGCCTTTAAATGACCGCACTAAAAGCATGGTA CCAAAG	Northern blot
cen siRNA-1	CCTTAATTAAAAACGACCAATATG	Northern blot
cen siRNA-2	CTGCGGTTCAACCCTTAACATC	Northern blot
cen siRNA-3	CAACTGCGGATGGAAAAAGT	Northern blot
cen siRNA-4	CTCTATATATCAGATATAAAGATGCG	Northern blot
cen siRNA-5	GTTTTGAAGTAGACATTCCGCACAA	Northern blot
cen siRNA-6	CTGAGCACAAGAGACATGGTGTACTAGA	Northern blot
cen siRNA-7	GTACATTTTTGCAGGACAACCAG	Northern blot
cen siRNA-8	CAACAACAGTCTTGGATTATTTAG	Northern blot
dhRT-3	CTCTCATCTCGACTCGTTTG	RT-PCR, ChIP, RIP
dhRT-4	GGCATTACGAAACATAGCG	RT-PCR, ChIP, RIP
SPCTRNAS N.05 Rv	CGACCTCACGATTAACAGTCG	Northern blot
ura4-DS/E145 FW	GAATGGTTTGAGAAGCATACC	RT-PCR, ChIP, RIP
ura4-DS/E145 RV	GAGTACGATATTGCTGTCCC	RT-PCR, ChIP, RIP
ura4-siRNA- F1	GATGCTAGAGTATTTCAAAGCTATTCAGCTAGAGCTGAGG GGAT	Northern blot
ura4-siRNA- R1	CTTTGCTTTTCTTCCATCAAAGCCAACAATTCCTTGGCAAT GGG	Northern blot
ura4-siRNA- F2	TCGCGGTCGATTTGACGAAGAAATCCGAAATCTTAGAATTG GTAG	Northern blot
ura4-siRNA- R2	TCGACAACGTCAATATGTGTCTTGATAACACAGACATAGGG TCC	Northern blot
ura4-siRNA- F3	GAGGATCGCAAATTCGCAGACATTGGAAATACCGTCAAGC TACA	Northern blot
ura4-siRNA- R3	CCCTCGCCTGGCACTGTATGGCAATTTGTGATATGAGCCC AAGA	Northern blot
ura4-siRNA- F4	GGGACGTGGTCTCTTGCTTTTGGCTGAAATGTCTTCCAAA GGCT	Northern blot

Oligo name	Sequences	Experiment
cen hotspot siRNA-R9	CTCGAACATAGAAAGAAATCCAGAAAGGGATTAAAGATTG ACT	Northern blot
cen hotspot siRNA-R10	TCATCGCTATGCCTTTAAATGACCGCACTAAAAGCATGGTAC CAAAG	Northern blot
cen siRNA-1	CCTTAATTAAAAACGACCAATATG	Northern blot
cen siRNA-2	CTGCGGTTCCACCCTTAACATC	Northern blot
cen siRNA-3	CAACTGCGGATGGAAAAAGT	Northern blot
cen siRNA-4	CTCTATATATCAGATATAAAGATGCG	Northern blot
cen siRNA-5	GTTTTGAAGTAGACATTCCGCACAA	Northern blot
cen siRNA-6	CTGAGCACAAGAGACATGGTGTACTAGA	Northern blot
cen siRNA-7	GTACATTTTTGCAGGACAACCAG	Northern blot
cen siRNA-8	CAACAACAGTCTTGGATTATTTAG	Northern blot
dhRT-3	CTCTCATCTCGACTCGTTTG	RT-PCR, ChIP, RIP
dhRT-4	GGCATTACGAAACATAGCG	RT-PCR, ChIP, RIP
SPCTRNAS N.05 Rv	CGACCTCACGATTAAACAGTCG	Northern blot
ura4-DS/E145 FW	GAATGGTTTGAGAAGCATACC	RT-PCR, ChIP, RIP
ura4-DS/E145 RV	GAGTACGATATTGCTGTCCC	RT-PCR, ChIP, RIP
ura4-siRNA- F1	GATGCTAGAGTATTTCAAAGCTATTCAGCTAGAGCTGAGGG GAT	Northern blot
ura4-siRNA- R1	CTTTGCTTTTCTTCCATCAAAGCCAACAATTCCTTGGCAATG GG	Northern blot
ura4-siRNA- F2	TCGCGGTCGATTTGACGAAGAAATCCGAAATCTTAGAATTG GTAG	Northern blot
ura4-siRNA- R2	TCGACAACGTCAATATGTGTCTTGATAACACAGACATAGGG TCC	Northern blot
ura4-siRNA- F3	GAGGATCGCAAATTCGCAGACATTGGAAATACCGTCAAGCT ACA	Northern blot
ura4-siRNA- R3	CCCTCGCCTGGCACTGTATGGCAATTTGTGATATGAGCCCA AGA	Northern blot
ura4-siRNA- F4	GGGACGTGGTCTCTTGCTTTTGGCTGAAATGTCTTCCAAAG GCT	Northern blot

Oligo name	Sequences	Experiment
ura4-siRNA-R4	ATAGTTATGTAGTCGCTTTGAAGGTTAGGAAATCGACGAC CAGC	Northern blot
ura4-siRNA-F5	CCCCTGGTATCGGCTTGGATGTTAAAGGAGACGGGCTG GGACAG	Northern blot
ura4-siRNA-R5	CTCCATAGACTCCACGACCAACAATGATGATATCGCTACC GCAG	Northern blot
ura4-siRNA-F6	GGTCGTAATCCTGTTGTCGAAGCCAAGAGATATAGAGAA GCTGG	Northern blot
ura4-siRNA-R6	AGTCTTTTTTTAATGCTGAGAAAGTCTTTGCTGATATGCCT TCC	Northern blot