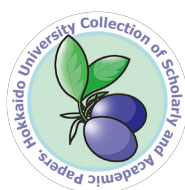




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DNA:RNA hybrid formation mediates RNAi-directed heterochromatin formation.

Mina Nakama^{1, #}, Kei Kawakami^{1, 2}, Takuya Kajitani², Takeshi Urano³ and Yota Murakami^{2,}

^{4, *}

¹ Laboratory of Cell Regulation and Molecular Network, Division of Systemic Life Science, Graduate School of Biostudies, Kyoto University, Kyoto, Kyoto 606-8501, Japan

² Division of Chemistry, Faculty of Science, Hokkaido University, Sapporo, Hokkaido 060-0810, Japan

³ Department of Biochemistry, Shimane University School of Medicine, Izumo, Shimane 693-8501, Japan,

⁴ Laboratory of Signal Transduction, Department of Cell Biology, Institute for Virus Research, Kyoto University, Kyoto, Kyoto 606-8501, Japan,

†Present address: Air Liquide Japan Ltd., Japan Air Gases, Granpark Tower 3-4-1, Shibaura, Minato-ku, Tokyo 108-8509

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*corresponding author: Yota Murakami, email: yota@sci.hokudai.ac.jp

Abstract

Certain non-coding RNAs (ncRNAs) implicated in the regulation of chromatin structure associate with chromatin. During the formation of RNAi-directed heterochromatin in fission yeast, ncRNAs transcribed from heterochromatin are thought to recruit the RNAi machinery to chromatin for the formation of heterochromatin; however, the molecular details of this association are not clear. Here, using RNA-immunoprecipitation assay, we showed that the heterochromatic ncRNA associated with chromatin via the formation of a DNA:RNA hybrid and bound to the RNA-induced transcriptional silencing (RITS) complex. The presence of DNA:RNA hybrid in the cell was also confirmed by immunofluorescence analysis using anti-DNA:RNA hybrid antibody. Overexpression and depletion of RNase H *in vivo* decreased and increased the amount of DNA:RNA hybrid formed, respectively, and both disturbed heterochromatin. Moreover, DNA:RNA hybrid was formed on, and overexpression of RNase H inhibited the formation of, artificial heterochromatin induced by tethering of RITS to mRNA. These results indicate that heterochromatic ncRNAs are retained on chromatin via the formation of DNA:RNA hybrids and provide a platform for the

RNAi-directed heterochromatin assembly and suggest that DNA:RNA hybrid formation plays a role in chromatic ncRNA function.

INTRODUCTION

The higher-order chromatin structure is an important determinant for epigenetic gene regulation and maintenance of genome integrity. A recent study revealed that a specific combination of histone modifications determines chromatin higher-order structures. One important issue of chromatin higher-order structure is its temporal and spatial regulation. One of the mechanisms involved in this regulation involves sequence-specific DNA binding proteins, which recruit histone-modifying enzymes. In addition to DNA-binding proteins, recent analyses revealed that many non-coding RNAs (ncRNAs) are transcribed from the eukaryotic genome and that some are involved in the regulation of chromatin higher-order structure (Bernstein & Allis 2005; Cam *et al.* 2009), such as dosage compensation in mammals (Chang *et al.* 2006) and fruit flies (Rea & Akhtar 2006). In both cases, relatively long ncRNAs (i.e., *xist* RNA in mammals and *rox* RNA in flies) are transcribed from the X chromosome and associate with chromatin to set up chromatin higher-order structure for transcriptional inactivation and activation, respectively. Similarly, the *Kcnqlot1* and *Air* RNAs associate with chromatin and are essential for the onset of silent chromatin at

imprinting regions (Nagano *et al.* 2008; Pandey *et al.* 2008). These ncRNAs are assumed to function as a scaffold for silent chromatin assembly.

RNAi-mediated transcriptional gene silencing has been reported in fission yeast and plants (Bühler & Moazed 2007; Grewal & Elgin 2007). In these systems, ncRNAs transcribed from silenced loci appear to be important for gene silencing. During RNAi-mediated heterochromatin formation in fission yeast, ncRNAs are transcribed from heterochromatic repeats by RNA polymerase II (Pol2) (Djupedal *et al.* 2005; Kato *et al.* 2005). ncRNAs bind to the RITS complex, which is an RNAi effector complex that comprises an argonaute family protein, Ago1, and a small interference RNA (siRNA) derived from the ncRNA (Noma *et al.* 2004; Verdel *et al.* 2004). siRNAs are thought to be utilized as guiding molecules for the binding of RITS to ncRNAs. RITS complex bound to ncRNA recruits the RNA-dependent RNA polymerase complex (RDRC) to produce double-stranded RNA (Motamedi *et al.* 2004), which is processed to siRNA by the Dicer RNase (Verdel *et al.* 2004; Colmenares *et al.* 2007). In parallel, RITS recruits Clr4 histone methyl transferase to methylate lysine 9 of histone H3 (H3K9), which attracts the heterochromatic protein Swi6

and Chp2, fission yeast homologues of HP1, and results in the formation of heterochromatin (Verdel *et al.* 2004; Zhang *et al.* 2008; Bayne *et al.* 2010). Binding of RITS to ncRNA is a key step for the RNAi-directed formation of heterochromatin. This is demonstrated by the fact that the artificial recruitment of the RITS complex using an RNA binding protein to *ura4* mRNA induces the generation of the *ura4*-siRNA and heterochromatin formation at the *ura4* locus (Bühler *et al.* 2006). As siRNA generation seems to be coupled with transcription (Kato *et al.* 2005) and with the localization of RITS at heterochromatin (Verdel *et al.* 2004), heterochromatic ncRNAs are assumed to remain on chromatin and function as a platform for the RNAi machinery (Cam *et al.* 2009). Similarly, during RNAi-mediated transcriptional gene silencing in plants, ncRNAs work on chromatin as a platform for the assembly of RNAi factors, which include argonaute family proteins (for example see (Wierzbicki *et al.* 2009)). However, there is no direct evidence of the association of ncRNAs to chromatin in these systems and the mechanisms that underlie the association are not understood.

In this report, we used an RNA precipitation assay that was targeted to histones to confirm that ncRNAs transcribed from heterochromatic repeats in fission yeast bound to

chromatin. Surprisingly, we found that the ncRNAs were retained on chromatin through the formation of a DNA:RNA hybrid. Immuno-fluorescence analysis using an antibody specific for the DNA:RNA hybrid detected nuclear foci that co-localized with the heterochromatic regions and the foci were diminished by the RNase H treatment, which confirmed that the formation of DNA:RNA hybrid *in vivo*. This hybrid formation did not depend on heterochromatin, while it required the RNAi machinery. Overexpression or depletion of RNase H caused a decrease and an increase in hybrid formation and chromatin-association of ncRNA, respectively. Both situations disrupted heterochromatin formation, which suggests a dosage effect of the DNA:RNA hybrid on heterochromatin formation. Similarly, a DNA:RNA hybrid was formed in artificial heterochromatin induced by tethering of RITS to the *ura4* RNA and overproduction of RNase H inhibited the establishment and maintenance of artificial heterochromatin. These data demonstrated the importance of the retention of ncRNAs on chromatin via the DNA:RNA hybrid to form the RNAi-directed heterochromatin.

RESULTS

Detection of an association between heterochromatic ncRNA and chromatin by the histone-RNA immunoprecipitation (RIP) assay

To investigate the association of ncRNAs with chromatin, we performed a RIP assay using an antibody against histone. In the RIP assay, association of specific RNAs with a protein of interest is detected by performing RT-PCR analysis on immunoprecipitates from *in vivo* formaldehyde-crosslinked cells (Gilbert *et al.* 2004). The RIP assay using antibodies against histone (the histone-RIP assay) detects chromatin-associated RNAs (Fig. S1 in supporting information) Note that, in the RIP assay performed previously (Gilbert *et al.* 2004; Motamedi *et al.* 2004), cell extracts prepared from crosslinked cells were treated with DNase I to degrade DNA before the immunoprecipitation of target proteins not to detect the indirect RNA-protein interaction mediated by DNA, while in this study the samples were treated with DNase I after immunoprecipitation of target proteins (Fig. S1 in supporting information), because we wanted to precipitate the intact chromatin-RNA complexes and DNase I treatment before precipitation would disrupt the complexes.

Heterochromatic ncRNA was transcribed from both strands of centromeric *dh* repeats (Fig. 1 A) (Volpe *et al.* 2002; Dutrow *et al.* 2008) and the ncRNA was shown to bind to the RITS complex via the RIP assay (Motamedi *et al.* 2004). To confirm the reliability of our RIP assay, we precipitated myc-tagged Chp1, which is a component of the RITS complex (Verdel *et al.* 2004) that was shown to bind ncRNA using the previous RIP assay (Motamedi *et al.* 2004) and an anti-myc antibody. We detected the association of Chp1 to heterochromatic ncRNA (Fig. 1 B) (Motamedi *et al.* 2004). Note that the high background signal obtained in the RT-PCR reactions performed without reverse transcriptase (–RT), which also observed in other RIP-assays in Fig. 1, were the result of DNA:RNA hybrid formation, as shown below.

The H3-RIP assay effectively detected both forward and reverse transcripts from *dh* repeats (Fig. 1 C), which demonstrated that both ncRNAs associate with heterochromatin. Under the same conditions, the *act1* and *cdc2* mRNAs used as control were poorly precipitated with histone H3 (Fig. 1 C), which indicated that mRNAs did not associate with chromatin. Since the *cdc2* mRNA, which is transcribed constitutively but is less abundant

than the *act1* mRNA (the relative amounts of *act1* mRNA, *cdc2* mRNA, and *dhfor* ncRNA in the input fraction were 1, 0.03, and 0.005, respectively), the poor association of mRNA with chromatin was not affected by the efficiency of transcription. A RIP assay using the anti-Pol2 antibody, which can recognize elongating polymerase (Das *et al.* 2007), indicated that a fraction of heterochromatic ncRNA, *act1* mRNA, and *cdc2* mRNA, which probably represent elongating transcripts, associated with RNA Pol2 (Fig. 1 D). Interestingly, a larger fraction of heterochromatic ncRNA than *act1* or *cdc2* mRNAs associates with Pol2, which suggests that heterochromatic ncRNAs tend to remain close to Pol2. Since *cdc2* mRNA that is far less abundant than *act1* mRNA more weakly associated with Pol2, the weak transcription of *dhfor* ncRNA may not cause the higher association of the transcripts with Pol2. Thus, heterochromatic ncRNA appeared to remain at transcription sites, where it associated with chromatin after transcription, whereas most mRNA was promptly released from chromatin after transcription.

In addition to the centromere, other RNAi-mediated heterochromatin exists at the mating-type region (*mat* locus) and at the subtelomeres in fission yeast. The *mat* locus

contains sequences that are highly homologous to centromeric repeats (*cenH*) and the subtelomeric region of chromosomes 1 and 2 also contains *cenH*-like sequences (Fig. 1 E). Both sequences are transcribed and act as nucleation sites for RNAi-mediated heterochromatin assembly (Noma *et al.* 2004; Cam *et al.* 2005; Kanoh *et al.* 2005). The H3-RIP assay detected chromatin-associated ncRNAs at the *mat* locus and at the subtelomeric region (Fig. 1 F). Thus, the association of heterochromatic ncRNA with chromatin was generally observed in RNAi-mediated heterochromatin formation.

Formation of an DNA:RNA hybrid by heterochromatic ncRNA

Using H3-RIP assays of heterochromatic ncRNA, we observed a high background signal in the reaction lacking reverse transcriptase (–RT) (Fig. 1 C, F), which typically represents DNA contamination. Similar signals in –RT reactions were also observed in the Chp1- and Pol2-RIP assays of *dhc* for ncRNA, but not in the Pol2-RIP assay of *act1* mRNA (Fig. 1 B, D). Therefore, the high background detected in –RT reactions seemed to be specific for heterochromatic ncRNA. We suspected the presence of DNA:RNA hybrids that are resistant

to DNase I (Sutton *et al.* 1997). To test this possibility, we treated RNA-IP samples with RNase H, which specifically degrades the RNA in DNA:RNA hybrids, before DNase I treatment. If DNA:RNA hybrids existed in the precipitates of the RIP-assay, sequential treatment with RNase H and DNase I would decrease both +RT and –RT signals. RNase H treatment decreased the signal of the H3-RIP assay of *dhfor* RNA to 40–50% of the signal in the absence of RNase H treatment in both +RT and –RT reactions (Fig. 2 A), which suggests that a significant portion of the RNA detected by the H3-RIP assay formed DNA:RNA hybrids. A RIP assay using an anti-Swi6 antibody indicated that the *dhfor* ncRNA associated with Swi6 and that most of it was also RNase H-sensitive (Fig. 2 B), which confirmed the formation of a DNA:RNA hybrid at heterochromatin. Importantly, a fraction of the ncRNA bound to Chp1 or Pol2 was also RNase H-sensitive, while the *act1* mRNA associated with Pol2 was not (Fig. 2 C). This suggested that DNA:RNA hybrids are located close to Pol2 and RITS. The RIP assay against Rdp1 did not show such a high background signal (Fig. S2 in supporting information), suggesting that RDRC associates with ncRNA at a position away from the DNA:RNA hybrid. Similar DNA:RNA hybrid formation was observed at the mating

locus and at subtelomeric heterochromatin (Fig. 2 D), which suggests that hybrid formation is a common feature of ncRNAs transcribed at heterochromatic regions. Note that although a 8–9 bp-long DNA:RNA hybrid is formed at the RNA Pol2 complex during RNA synthesis (Gnatt *et al.* 2001), this hybrid was not detected by our RIP assay, even at the highly transcribed *act1* gene (Fig. 2 C). As we used primer sets that produced RT-PCR products that were ~150 bp-long, the length of the hybridized region is likely to be greater than 150 bp. These results suggest that the formation of a DNA:RNA hybrid by ncRNA is coupled with transcription and that the resulting ncRNA on chromatin becomes a target for the RITS complex.

We tested for the presence of DNA:RNA hybrids at four transcriptionally active loci (Dutrow *et al.* 2008) on centromeric *dh* repeats (Fig. S3 in supporting information) using an antibody against methylated histone H3K9 that represents heterochromatin. At all four sites examined, 40-60% of the chromatin-associated ncRNAs were sensitive to RNase H, which suggests that the DNA:RNA hybrids are formed entire transcribed-region in the centromeric *dh* repeats, though it is not clear whether the results represents the formation of small

DNA:RNA formed at each region or the formation of long DNA:RNA hybrid encompassing entire region.

When the insertion of euchromatic genes such as *ade6* or *ura4* into centromeric heterochromatin, the heterochromatin structure (H3K9me and Swi6/Chp2) spreads into the inserted genes and suppresses gene expression. However, a small amount of RNA is transcribed from the inserted genes and is converted to siRNA (Bühler *et al.* 2007), which indicates that the RNAi system is operative at the inserted transcription units. Therefore, we analyzed the association of the *ade6* and *ura4* mRNAs inserted into centromeric repeats (*otr::ade6* and *imr::ura4*; Fig. 2 F) using the H3-RIP assay. The *ade6* and *ura4* mRNAs did not associate with chromatin at native loci, such as the *act1* or *cdc2* mRNAs (Fig. 1 and Fig. 2), while the insertion of the genes into centromeric repeats led to the coprecipitation of a portion of the *ade6* or *ura4* mRNAs with histone H3 (Fig. 2 F). In addition, as observed with centromeric ncRNA, this fraction was sensitive to RNase H treatment (Fig. 2 E), which suggests that the local environment of centromeric repeats induces the formation of DNA:RNA hybrids.

To confirm the formation of the DNA:RNA hybrid *in vivo*, immunofluorescence analysis was performed using an antibody specific for the DNA:RNA hybrid (S9.6) (Boguslawski *et al.* 1986) (Fig. 3). Chp1-GFP was used as a control, which formed a few (1–3) nuclear foci that represent heterochromatin (Sadaie *et al.* 2004). The anti-DNA:RNA hybrid antibody detected signals in the cytoplasm of almost all cells, and the number of signals varied from cell to cell. The cytoplasmic signals may arise from mitochondrial DNA since mitochondrial DNA is thought to harbor an R-loop at its origin of replication (Brown *et al.* 2008) and DAPI-staining signals in the cytoplasm colocalized with the foci (Fig. S4 in supporting information). Nuclear foci that mainly localized to the periphery of the nucleus were also observed, and some of these foci co-localized with one of the Chp1-GFP signals (Fig. 3, arrows) in about 5% of the cells, showing the existence of DNA:RNA hybrids in some of the heterochromatic regions. One of the reasons for the low percentage of co-localization would be the S-phase specific transcription of heterochromatic ncRNA (Chen *et al.* 2008). The nuclear foci that did not colocalize with heterochromatin (Fig. 3, arrow heads) may indicate DNA:RNA hybrids formed at euchromatic regions. Importantly, both

nuclear and cytoplasmic foci detected by the DNA:RNA hybrid antibody were diminished by the treatment of the cells with RNase H before immuno-staining (Fig. 3, lower panels), which confirmed that the antibody detected the DNA:RNA hybrid. These data showed that the DNA:RNA hybrid is formed in the heterochromatic region *in vivo*.

Requirements for chromatin association and DNA:RNA hybrid formation of heterochromatic ncRNA

To examine whether heterochromatin is required for chromatin association or DNA:RNA hybrid formation, we performed the H3-RIP assay on yeast strains that carry defects in heterochromatin formation (Fig. 4 A). Compared with the wild-type strain, the increased level of ncRNA was retained on chromatin in deletion strains of *swi6* or *chp2* (both of which encode major structural components of heterochromatin). Similarly, ncRNA bound to chromatin in *clr4Δ* cells, in which heterochromatin was completely erased by the loss of the H3K9-specific methyltransferase Clr4. Hence, heterochromatin per se was not required for DNA:RNA hybrid formation and chromatin binding and hybrid formation are intrinsic

properties of heterochromatic ncRNA. Interestingly, the RNase H-sensitive fraction of chromatin-associated ncRNA was significantly increased in *swi6Δ*, *chp2Δ*, and *clr4Δ* cells., suggesting that heterochromatin decreased DNA:RNA hybrid formation. Notably, *clr4* deletion did not influence chromatin association of *otr::ade6* or *imr::ura4* RNAs; rather, it increased the RNase H-sensitive fraction of the RNAs (Fig. 2 F).

Next, we analyzed the effect of mutation of the RNAi machinery (Fig. 4 A). The deletion of genes encoding a subunit of the RITS complex (*chp1* or *ago1*), RDRC (*rdp1* or *hrr1*) or an siRNA-generating RNase (*dcr1*) resulted in a significant decrease in the association of ncRNA with chromatin. Similarly, the *rpb2-m203* mutation, which is a point mutation in Pol2 that disturbs siRNA generation but does not affect ncRNA transcription (Kato *et al.* 2005) also resulted in a decrease in the association of *dhf* ncRNA with chromatin. These results suggest that the RNAi machinery is required for the efficient association of heterochromatic ncRNA to chromatin via DNA:RNA hybrid.

Binding of the RITS complex to ncRNA is a key step for the RNAi-directed formation of heterochromatin; it induces H3K9 methylation (for heterochromatin formation)

and processing of ncRNA (for siRNA generation). This was demonstrated by the fact that the tethering of RITS to the *ura4* RNA induces RNAi- and heterochromatin-dependent gene silencing of the *ura4* gene (Bühler *et al.* 2008). Tethering of RITS is achieved by fusion of Tas3, which is a subunit of RITS, with the λ N protein, which binds to the 5BoxB sequence inserted at the 3' UTR region of the *ura4* RNA (Fig. 4 B). In this system, the *ura4* RNA should remain on chromatin to recruit RITS close to chromatin. Thus, we analyzed the chromatin binding and DNA:RNA hybrid formation of the *ura4* RNA in the RITS-tethering system using the H3-RIP assay. The expression of the Tas3- λ N protein in cells harboring the *ura4-5BoxB* gene rendered only a small portion (~0.1–0.5 %) of the cells resistant to 5-fluoro-orotic acid (FOA), which indicates silencing of *ura4* gene expression. Once the silent state was established, it was stably maintained for generations under non-selective conditions; about 90% of cells were FOA resistant after 10 generations (Fig. 4 C). We compared the chromatin association of *ura4-5BoxB* RNA in Tas3- λ N-expressing cells that were not selected or selected for FOA resistance. In non-selected cells, only a small portion of the *ura4-5boxB* RNA bound to histone H3, which is similar to what was observed for

native *ura4* RNA (less than 0.1% of the input RNA) (Fig. 2 F). In contrast, FOA-resistant cells exhibited a significant amount of *ura4-5boxB* RNA bound to chromatin and most of the RNA was RNase H sensitive (Fig. 4 D). These results suggest that simple association of RITS complex to RNA did not induce DNA:RNA hybrid formation; rather DNA:RNA hybrid formation correlated with heterochromatin formation induced by RITS tethering.

Requirement for a specific amount of DNA:RNA for hybrid heterochromatin formation

To test the importance of the DNA:RNA hybrid in the formation of heterochromatin, we first tried overexpressing RNase H in cells, as the overexpression of RNase H reduces the amount of DNA:RNA hybrids in human cells and budding yeast (Huertas & Aguilera 2003; Li & Manley 2005). Overexpression of *rnh201*, which encodes the catalytic subunit of RNase H2, neither affected the growth rate (data not shown, Fig. S6 in supporting information, N/S) nor the DNA content of cells (Fig. S5B in supporting information), while it caused a decrease in the level of RNase H-sensitive H3-associated ncRNA at heterochromatin (Fig. 5 A). This showed a reduction in the amount of DNA:RNA hybrids. Under the same conditions, the

level of H3K9me was decreased to that observed in *dcrl1Δ* cells (Fig. 5B), in which the RNAi-mediated heterochromatin formation system is defective. Swi6, as measured using the chromatin immunoprecipitation (ChIP) assay, was also significantly reduced (Fig. 5 B), though the decrease was less than that of H3K9me. These results indicate that the reduction in the amount of DNA:RNA hybrids caused a disturbance in heterochromatin structure.

The decrease in the quantity of DNA:RNA hybrids after overexpression of RNase H raised the possibility that RNase H is involved in the regulation of DNA:RNA hybrid formation at heterochromatin. Thus, we analyzed the effect of deletion of two genes that encode RNase H, *rnh201* and *rnh1* (the latter encodes the catalytic subunit of RNase H1). As expected, the amounts of RNase H-sensitive *dh* for ncRNA increased in cells harboring a deletion of *rnh201* or *rnh1*, by about two- and three-fold, respectively (Fig. 5 C). This demonstrated that both gene products contributed to the decrease in the levels of DNA:RNA hybrids. Interestingly, the levels of H3K9me and Swi6 were decreased in these mutants (Fig. 5 D). Moreover, the greater the increase in DNA:RNA hybrid levels in *rnh1Δ* cells, the greater the decrease in H3K9me. Note that the reduction in Swi6 was more obvious than that

of H3K9me in *rnh201Δ* cells. Together with the result showing that Swi6 tended to remain on chromatin in *rnh201*-overexpressing cells (Fig. 5 B), these findings led us to suspect that the DNA:RNA hybrids may inhibit the association of Swi6 with heterochromatin, independently of H3K9me.

We next analyzed effect of overproduction or depletion of RNase H on the silencing of the *ura4* gene inserted into heterochromatin (*imr::ura4*) (Fig. 4D). Consistent with the reduction of H3K9me and Swi6, we observed that both overexpression of *rnh201* and deletion of *rnh201* or *rnh1* caused the decrease of the silencing of *imr::ura4*, which was shown by the enhanced growth on the plates lacking uracil (-URA, Fig. 5 D). The results suggest that the proper turnover of the DNA:RNA hybrid is important for heterochromatin formation and that RNase H is involved in the regulation of this process.

To test the importance of DNA:RNA hybrid formation in RITS-tethering-induced heterochromatin formation, we analyzed the effect of overproduction of RNase H on the establishment and maintenance of the silent state (Fig. 6 A). Overexpression of Rnh201 in RITS-tethering cells led to a significant decrease in the number of FOA-resistant cells (Fig.

6A, upper panel); the conversion rate from the FOA-sensitive to the FOA-resistant state per generation, which represents the efficiency of heterochromatin establishment, was reduced from 1.4 to 0.05% (Fig. 6 B). Therefore, overexpression of RNase H inhibited the formation of RITS-tethering-mediated heterochromatin. We picked FOA-resistant colonies from control and Rnh201-overexpressing cells, cultured them in nonselective medium for more than 10 generations, and then analyzed the number of FOA-resistant cells. For control cells, the number and size of the colonies on FOA plates were similar to those grown on nonselective plates (Fig. 6 B, lower panel, and Fig. S6 in supporting information), which suggests the stable maintenance of the silent state. For Rnh210-overexpressing cells, the number of colonies was decreased significantly and their size was variable on FOA plates, which demonstrated the instability of the silent state (Fig. 6 B and Fig. S6 in supporting information). The conversion rates from the FOA-resistant to the FOA-sensitive state in control cells and in Rnh201-overexpressing cells were 1.0 % and 12 %, respectively (Fig. 6 B, right panel). This indicates that overexpression of Rnh201 destabilized RITS-tethering-mediated silencing. These results suggest that the association of *ura4-5BoxB*

RNA to chromatin via DNA:RNA hybrid formation is a necessary step for the establishment and maintenance of RITS-tethering-induced heterochromatin.

DISCUSSION

Here, we showed that ncRNA transcribed from heterochromatin associated with chromatin via the formation of a DNA:RNA hybrid. Importantly, hybrid formation was involved in heterochromatin formation. We propose a model for the association of heterochromatic ncRNA to chromatin and its function in heterochromatin formation (see Fig. 7, (I)-(III)).

As shown in Fig. 7 (I), the ncRNAs transcribed by RNA Pol2 from centromeric repeats form DNA:RNA hybrids, probably via a transcription-coupled mechanism. In budding yeast transcription-coupled DNA:RNA hybrid formation at normal gene loci is induced by depletion of HPR1, which functions at the interface of transcription and mRNA metabolism as a component of a component of the THO/TREX complex, or Sen1, which functions in transcription termination as a component of NRD complex (Huertas & Aguilera 2003; Mischo *et al.* 2011). Similarly, depletion of the ASF/SF2 (alternative splicing factor 2)

in avian lymphoid cells or senataxin (homologue of Sen1) in human cells leads to DNA:RNA hybrid formation (Li & Manley 2005; Skourti-Stathaki *et al.* 2011). These results suggest that a mechanism that includes mRNA export/processing factors is involved in the prevention of DNA:RNA hybrid formation at normal protein-coding genes. Therefore, we speculate that an active mechanism induces DNA:RNA hybrid formation. Recently, several splicing factors were shown to be involved in RNAi-directed heterochromatin formation (Bayne *et al.* 2008; Chinen *et al.* 2010). Considering the requirements of RNAi machinery for DNA:RNA hybrid formation, we suggest that hybrid formation involves the cooperation of the RNAi machinery and the RNA processing machinery (Fig. 7, (I)a).

Our data suggests that there are two distinct mechanisms of DNA:RNA hybrid formation, heterochromatin-dependent and –independent mechanisms. As heterochromatin ncRNA transcription exhibited an intrinsic property to form DNA:RNA hybrid, independent of heterochromatin (Fig. 4), cis-element(s) may confer this property to the transcription apparatus. This might be a similar mechanism to DNA:RNA hybrid formation at the DNA replication origin of the colE1 plasmid in *E. coli*, in which a specific secondary structure of

the transcribed primer RNA triggers DNA:RNA hybrid formation via an interaction with RNA polymerase (Itoh & Tomizawa 1979; Masukata & Tomizawa 1986). Alternatively, convergent transcription may be a signal for DNA:RNA hybrid formation, as a couple of recent reports suggested that the convergent transcription triggers RNAi-directed heterochromatin formation (Gullerova & Proudfoot 2008; Iida *et al.* 2008). In both situations, transcribed RNA should be retained on chromatin and become a target for RITS for the formation of heterochromatin. We found that transcripts derived from the euchromatic genes inserted into heterochromatic region (*otr::ade6* and *imr::ura4*) formed DNA:RNA hybrids in a heterochromatin-independent manner (Fig. 2 F). We assumed that the observed DNA:RNA hybrid was derived from read-through transcripts of heterochromatic ncRNA. This type of read-through product was reported previously and was suggested to be necessary for the spreading of heterochromatin onto inserted genes (Irvine *et al.* 2006). Alternatively, read-through transcripts from the downstream of the marker genes may generate the convergent transcription, which could induce the DNA:RNA hybrid formation.

In addition to the ncRNA-specific hybrid formation, there might be another mode

of DNA:RNA hybrid formation, a heterochromatin-dependent system (Fig. 7 (I)b), because the hybrid was formed only when heterochromatin was formed in an RITS-tethering system (Fig. 4 B-D). In this system, the *ura4* gene was transcribed from its native promoter, and there was no heterochromatic DNA/RNA element except 5BoxB sequence. Importantly, simple tethering of RITS to *ura4* RNA did not induce DNA:RNA hybrid formation; we could detect DNA:RNA hybrid after cells established heterochromatin. Therefore, heterochromatin formation on the *ura4* gene itself seemed to induce DNA:RNA hybrid formation. We propose, hence, that there are two modes of DNA:RNA hybrid formation: the heterochromatin-independent mode, which requires the cis element present in heterochromatic repeats, and the heterochromatin-dependent mode, which does not require the cis element. The latter mode of hybrid formation might contribute to the spreading and maintenance of heterochromatin by inducing siRNA generation from transcription units that are newly embedded in the heterochromatin.

We assumed that the RITS complex is targeted to the ncRNA that is tethered on the chromatin via partial DNA:RNA hybrid formation (Fig. 7 (II)c). Alternatively,

single-stranded DNA generated in a R-loop, which might be covered by single-stranded DNA proteins, such as RP-A, may become a target of the RITS complex (Fig. 7 (II)d), as it is by the recombination protein AID during immunoglobulin class switch recombination (Reaban & Griffin 1990; Yu *et al.* 2003). In both cases, the chromatin-associated RITS complex promotes siRNA production and heterochromatin formation. It is noteworthy that *Drosophila* Piwi, which is an argonaute family protein implicated in heterochromatin formation (Pal-Bhadra *et al.* 2004; Huisinga & Elgin 2008), was shown to associate with the heterochromatic region and to be released from heterochromatin by RNase H treatment (Brower-Toland *et al.* 2007). This finding suggests that the Piwi complex may thus target DNA:RNA hybrids formed at heterochromatin, as is the case in fission yeast.

The produced DNA:RNA hybrid must be removed quickly because the R-loop would inhibit subsequent transcription, which would prevent the supply of ncRNA for heterochromatin formation. This conclusion is supported by the observation that the deletion of RNase H genes resulted in an increase in DNA:RNA hybrid levels and in the disruption of

heterochromatin (Fig. 5 C and D). In addition to the S-phase specific transcription of the heterochromatic ncRNA, the dynamic turnover of the DNA:RNA hybrid could explain why only a small fraction of cells showed the DNA:RNA hybrid signal during the immunofluorescence analysis (Fig. 3). Our data suggested that RNase H is involved in such a dynamic regulation of DNA:RNA hybrid. It is possible that DNA/RNA helicases like Sen1 (Senataxin) helicase, which is recently suggested to resolve DNA:RNA hybrid in the cell (Mischo *et al.* 2011; Skourti-Stathaki *et al.* 2011), is also involved in the regulation of DNA:RNA hybrid formed in heterochromatin. Since RDRC binding to ncRNA at a position away from DNA:RNA hybrid (Fig. S2 in supporting information), it is attractive to speculate that a putative helicase, Hrr1, in RDRC unwinds DNA:RNA hybrid and the released RNA is used as a substrate for dsRNA synthesis by RDRC.

Our immunofluorescence analysis using an antibody against the DNA:RNA hybrid sometimes detected nuclear signals that did not colocalize with Chp1 (Fig. 2 E arrowheads), suggesting that the DNA:RNA hybrids exist in the euchromatic region. Considering the recent reports that show that some ncRNA transcribed at regulatory regions is involved in the

regulation of transcription (Hirota *et al.* 2008; Wang *et al.* 2008; Kim *et al.* 2010; Bertani *et al.* 2011), we speculate that the DNA:RNA hybrids also play regulatory roles in the euchromatin. Indeed, ncRNA transcribed in the promoter region of the cyclin D1 gene in human cells and the intergenic region of Hoxa6 and Hoxa7 genes in mouse cells thought to form DNA:RNA hybrids and recruits the RNA-binding transcriptional regulatory proteins TLS and MLL1, respectively to regulate the gene expression (Wang *et al.* 2008; Bertani *et al.* 2011). Since DNA:RNA hybrids inhibit nucleosome formation (Dunn & Griffith 1980), it is also possible that hybrid formation can be utilized to remove or reorganize nucleosomes around regulatory regions. Judging from the nuclear signals in the immunofluorescence analysis, euchromatic DNA/RNA hybrid regions might form clusters like heterochromatin. Since R-loop provides ssDNA region that can be used for pairing of homologous sequence, it is possible that the hybrids are formed at repeated sequences and contribute to cluster formation. In this sense, it is noteworthy that *Tf2* retroposons make clusters into “Tf bodies” in fission yeast (Cam *et al.* 2007).

The R-loop is hyper-recombinogenic, as has been shown for the induction of

DNA:RNA hybrids by the mutation/depletion of the RNA processing/transport factors mentioned above (Huertas & Aguilera 2003; Li & Manley 2005; Mischo *et al.* 2011). It is noteworthy that deletion of *swi6* or *clr4*, which exhibited a significant increase in hybrid formation (Fig. 4 A), show synthetic growth defects with deletion of several genes involved in DNA repair/recombination, while deletion mutants of *dcr1* or *chp1*, which exhibited a decrease in hybrid formation (Fig. 4 A), do not (Roguev *et al.* 2008). This may reflect the fact that an increase of the R-loop in heterochromatin in *clr4Δ* or *swi6Δ* cells in the cells deficient in DNA repair/recombination causes an increase in hyper-recombination at the R-loop to a level that is toxic for cell growth. Similar genetic interaction between SEN1 and DNA repair/recombination genes has been reported recently in budding yeast (Mischo *et al.* 2011). We want to point out that the hyper-recombinogenic property of the R-loop raised the possibility that DNA:RNA hybrid formation provides a hot spot for recombination or homologous chromosome pairing during meiosis. Therefore, DNA:RNA hybrid formation of ncRNA may regulate chromatin and/or DNA metabolism in the eukaryotic genome.

EXPERIMENTAL PROCEDURES

***Schizosaccharomyces pombe* strains and culture media**

All strains used in this study are listed in Table S1 in supporting information. The media and genetic methods used in *S. pombe* experiments were as described (Moreno *et al.* 1991). Yeast cells were cultured in YES, EMMS at 30°C. For deletion or epitope-tagging of the target genes, the PCR-based module method (Krawchuk & Wahls 1999) was used.

Antibodies and oligonucleotids

The following antibodies were used in this study: anti-c-myc (9E11, Santa Cruz), anti-Pol2 (8WG16, Abcam), anti-H3K9me monoclonal antibodies (Kato *et al.* 2005), anti-Swi6 polyclonal antibodies (Sadaie *et al.* 2004) and anti-DNA:RNA hybrid monoclonal antibodies (Boguslawski *et al.* 1986).

Oligonucleotides used in this study are listed in Table S2 in supporting information.

Overexpression and depletion of RNase H

For expression of RNase H in *S. pombe* cells, the *rnh201* (SPAC4G9.02) cDNA was amplified by PCR from genomic DNA and ligated between the BamHI and SalI sites of pREP1, which contains the thiamine-repressible *nmt1* promoter (Maundrell 1993). For deletion of the *rnh1* (SPBC336.06c) and *rnh201* genes, the PCR-based module method (Krawchuk & Wahls 1999) was used.

Chromatin immunoprecipitation (ChIP)

ChIP analysis was performed as described previously (Nakagawa *et al.* 2002), using the anti-Swi6 rabbit polyclonal antibody and the anti-H3K9me monoclonal antibody.

RNA immunoprecipitation (RNA-IP)

RNA immunoprecipitation was performed as described (Motamedi *et al.* 2004), with the following modifications: to prepare whole-cell extracts, cells were crosslinked with 1% formaldehyde, suspended in chilled lysis buffer (50 mM HEPES-KOH [pH 7.5] containing

140 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 0.1% Na-deoxycholate) with proteinase inhibitors (Nacalai), RNase inhibitors (Promega), and 10 mM phenylmethanesulfonyl fluoride (**PMSF**) and disrupted with glass beads. Nucleic acids in whole-cell extracts were fragmented by sonication to an average length of 0.5 kb. The sample was immunoprecipitated using antibodies against target proteins and Dynabeads M-280 anti-mouse or anti-rabbit IgGs (Invitrogen). Precipitated protein–nucleic acid complexes were washed twice with lysis buffer, twice with lysis/NaCl buffer (50 mM HEPES-KOH [pH 7.5], 500 mM NaCl, 1 mM EGTA, 1% Triton X-100, 0.1% Na-deoxycholate, proteinase inhibitors, RNase inhibitors, and 10 mM PMSF), and twice with wash buffer (10 mM Tris [pH 8.0], 250 mM LiCl, 1 mM EGTA, 0.5% NP-40, 0.5% Na-deoxycholate, RNase inhibitors, and 10 mM PMSF). After washing, the beads were resuspended in diethylpyrocarbonate (DEPC) -treated distilled water. Samples were adjusted to 0.05% SDS and 100 µg/ml proteinase K and incubated for 45 min at 45°C and then at 65°C for reversal of crosslinking. Samples were then extracted once with phenol–chloroform and once with chloroform–isoamyl alcohol. After ethanol precipitation, samples were resuspended in a

suitable volume of DEPC-treated distilled water.

Samples were treated with DNase I (150 U per 100 μ L; Invitrogen) at 37°C for 60 min before RT–PCR analysis. When necessary, nuclease treatment with RNase H and/or RNase T1 was performed before DNase I treatment: RNase T1 (1 U per 50 μ L; Ambion) at 25°C for 30 min and/or RNase H (1 U per 50 μ L; Takara) at 37°C for 60 min. RNA samples were reverse-transcribed into cDNA using PrimeScript Reverse Transcriptase (Takara) and strand-specific primers. We performed quantitative PCR on an ABI7500 real-time PCR machine (Applied Biosystems) using SYBR Premix ExTaq (Takara). Centromeric ncRNA, mating-type locus-specific ncRNA, subtelomeric ncRNA, and *act1* mRNA were detected using the primers listed in Table S2, each of which generated 150–180 bp-long PCR products. The ratio of precipitated RNA to input RNA in the samples treated with reverse transcriptase was calculated from quantitative RT–PCR data.

Note that the difference of immunoprecipitation efficiency between experiments using the same antibody caused by the difference of the lots of the antibodies.

Immuno-fluorescence analysis

Immuno-fluorescence was performed as described in(Dohke *et al.* 2008).

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FIGURE LEGENDS

Figure 1. The histone-RIP assay detected the association of heterochromatic ncRNAs with chromatin. (A) Schematic diagram showing the structure of the centromere region of chromosome 2 (*cen2*), which includes the outer repeats (*dg*, *dhI*, *dhII*), the innermost repeats (*imr2*), and the center region (*cnt2*). The positions of the forward and reverse ncRNAs (*dhfor* and *dhrev*) transcribed from the *dh* repeats are indicated. The box indicates the position of a primer set used for detection of the *dh* ncRNA. (B–E) RIP assays in the presence (+) or absence (–) of RT were performed to detect ncRNA associated with Chp1 tagged with myc tag (B), histone H3 (C), and Pol2 (D). The association of *act1* and *cdc2* mRNAs with H3 and Pol2 was also analyzed in (C) and (D). Note that the relative amounts of *act1* mRNA, *cdc2* mRNA, and *dhfor* ncRNA in the input fraction were 1, 0.03, and 0.005, respectively. (E) Schematic indication of the mating-type locus and the subtelomeric region of the left arm of

chromosome 1. The *mat2* and *mat3* genes, the *cenH* region (orange box), which is highly homologous to the centromeric repeats, IR-L and IR-R repeats, which form the boundary of heterochromatin, and a subtelomeric gene (SPAC212.11, white boxes) containing *cenH*-like sequences (orange boxes) are indicated. The boxes and arrows indicate the positions of the primer sets used and the direction of transcripts detected by qRT-PCR in (F), respectively.

(F) Results of histone-RIP assays using antibodies against H3K9me (H3K9me-RIP) and H3 (H3-RIP) in the presence (+RT) or absence (–RT) of RT. The proportion of precipitated RNA to input RNA in +RT samples calculated from quantitative RT-PCR data was plotted, with error bars showing the standard error of the mean (n = 3).

Figure 2. Heterochromatic ncRNA formed a DNA:RNA hybrid. (A–E) Heterochromatic ncRNA formed a DNA:RNA hybrid. A RIP assay was performed with (+) or without (–) RNase H treatment and in the presence (+RT) or absence (–RT) of RT to detect centromeric *dh* forward ncRNA associated with H3 (A), Swi6 (B), and Chp1 and Pol2 (C). In the Pol2-RIP assay (C), the association of *act1* mRNA with Pol2 was also examined. (D)

ncRNAs transcribed from the mating-type locus and subtelomere associated with chromatin.

A RIP assay was performed with (+) or without (–) RNase H treatment. (E, F) DNA:RNA hybrid formation of RNAs transcribed from the marker genes inserted into centromeric heterochromatin. (E) Schematic diagram showing the structure of the centromere region of chromosome 1 (*cen1*), which includes the outer repeats (*dg*, *dhI*, *dhII*), the innermost repeats (*imr1*), the center region (*cnt1*), and the inserted marker genes (*ura4* and *ade6*). The positions of forward and reverse ncRNAs (*dgfor* and *dgrev*) transcribed from the *dg* and *imr1* repeats are indicated by arrows. The box indicates the position of the primer set used for the detection of the *ura4* and *ade6* ncRNAs. (E) Results of histone-RIP assays for the inserted marker genes using antibodies against H3 (H3-RIP) with (+) or without (–) RNase H treatment. The proportion of precipitated RNA to input RNA calculated from quantitative RT–PCR data was plotted, with error bars showing the standard error of the mean (n = 3). The proportion of precipitated RNA to input RNA calculated from strand-specific qRT–PCR was plotted, with error bars showing the standard error of the mean (n = 3). *P* values were determined in (A–D) using a two-sided Student’s *t* test: ** *P* < 0.01, n = 3; *** *P* < 0.001, n =

3.

Figure 3. Detection of DNA:RNA hybrids by immunofluorescence using a monoclonal antibody against DNA:RNA hybrid. (Upper two panels) The localization of DNA:RNA hybrid and heterochromatin was visualized by immunofluorescence with anti-DNA:RNA hybrid (S9.6#) and anti-GFP antibodies, respectively using cells expressing Chp1 tagged with GFP (Chp1-GFP). DAPI staining indicates nuclear DNA. Cells observed by Normarski optics are indicated in the right panels, Arrows and arrowheads indicate the nuclear DNA foci overlapped with Chp1 foci and not overlapped with Chp1 foci respectively. (Bottom panels) Cells were treated with RNase H before immuno-detection (Materials and Methods). Both cytoplasmic and nuclear signals detected by the anti-DNA:RNA hybrid antibody are diminished by RNase H treatment.

Figure 4. Requirements for the formation of DNA:RNA hybrids. (A) H3-RIP assays for DNA:RNA hybrid formation of *dh* forward ncRNA in the indicated mutants. The relative

amount of signal obtained from each mutant to that of the wild type was plotted. Error bars represent s.e.m. (n = 2). (B) Schematic indication of the artificial heterochromatin formation by RITS-tethering to the *ura4* mRNA. In this system, RITS was tethered artificially to the *ura4* RNA via binding of the λ N protein fused to Tas3 (which is a subunit of RITS) to its recognition sequence, boxB, five copies of which are inserted in to 3' UTR of the *ura4* mRNA. This induced siRNA generation and heterochromatin at the *ura4* locus in an RNAi-dependent manner. (C) Gene silencing of *ura4* via tethering of RITS. Strains harboring the RITS-tethering system (*ura4-5boxB, tas3 λ N*) or *ura4-5boxB* alone were grown to a density of 1.0×10^7 cells/ml and serial dilutions (1:5) of the cultures were spotted onto non-selective (N/S), counter selective (FOA), and selective (-URA) plates for silencing of the *ura4* gene. A strain harboring *clr4 Δ* was also included, as a control. A FOA-resistant colony was picked, grown in non-selective medium for 10 generations, and respotted on the plates to analyze the stability of the silent state (box with an arrow and lower panel). (D) H3-RIP assays in the presence or absence of RNase H treatment were performed to detect DNA:RNA hybrid formation in the RITS-tethering system, using cells grown in

non-selective condition (in which only a small fraction of cells formed heterochromatin) and cells selected for FOA resistance (in which heterochromatin was stably formed) (see panel (C)).

Figure 5. Effect of overexpression and deletion of RNase H *in vivo*. (A) The catalytic subunit of RNase H2, which is encoded by *rnh201*, was overexpressed from a strong *nmt1* promoter and its effects on chromatin association and DNA:RNA hybrid formation of *dhfor* RNA were analyzed using the H3-RIP assay. (B) Heterochromatin status was also analyzed by measuring the level of H3K9me and Swi6 using the ChIP assay. (C and D) Effects of deletion of *rnh1* and *rnh201*, which encode the catalytic subunit of RNase H1 and H2, respectively, on the chromatin association and DNA:RNA hybrid formation of *dhfor* ncRNA (C) and on heterochromatin status. (D). The proportion of precipitated RNA to input RNA calculated from the quantitative PCR data was plotted, with error bars showing the standard error of the mean (n = 3). *P* values were determined using a two-sided Student's *t* test: * *P* < 0.05, n = 3; ** *P* < 0.01, n = 3; *** *P* < 0.001, n = 3. (E) Strains harboring *imr::ura4* and a plasmid for the

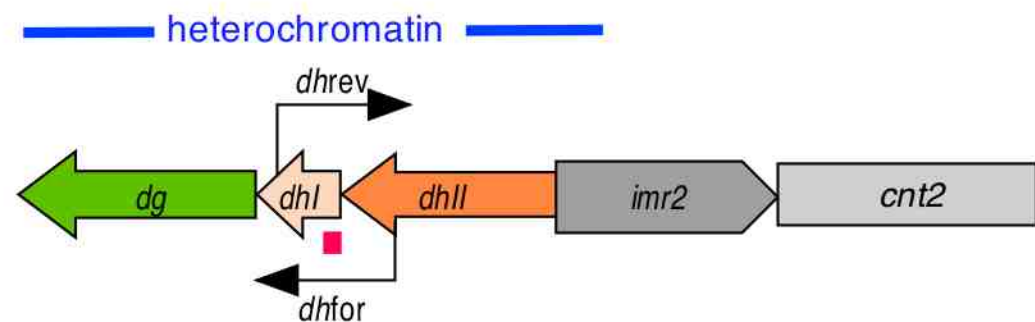
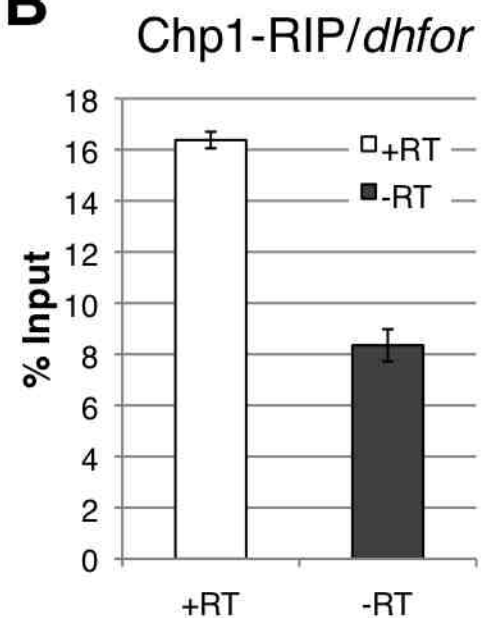
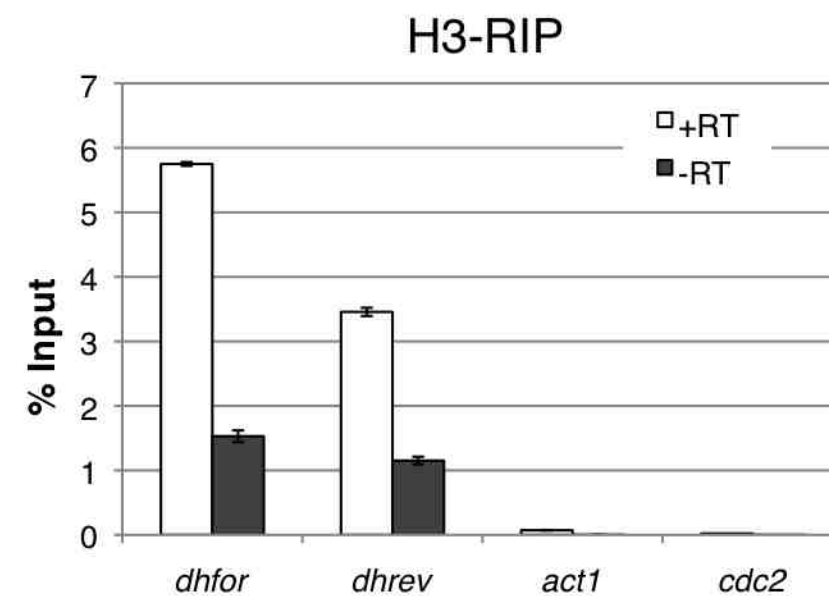
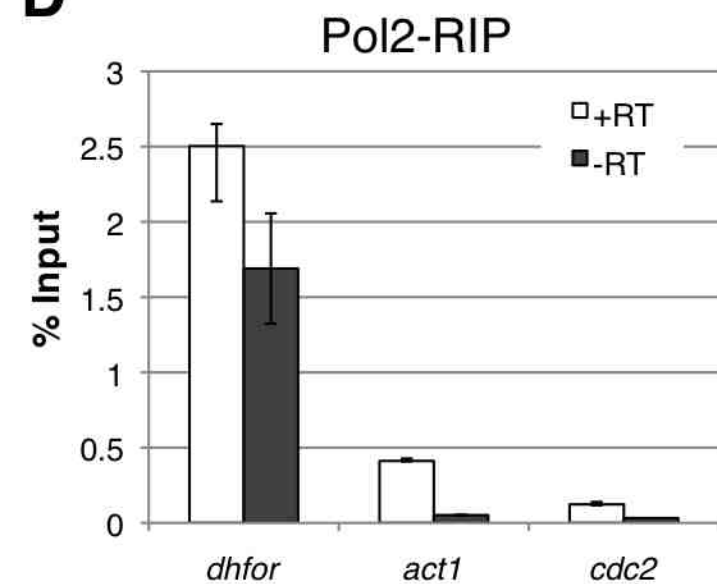
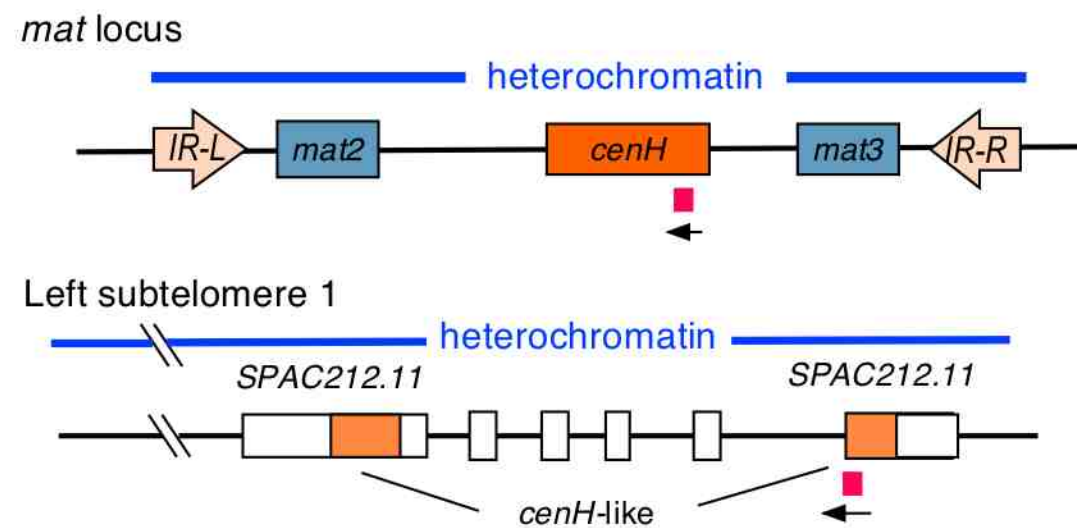
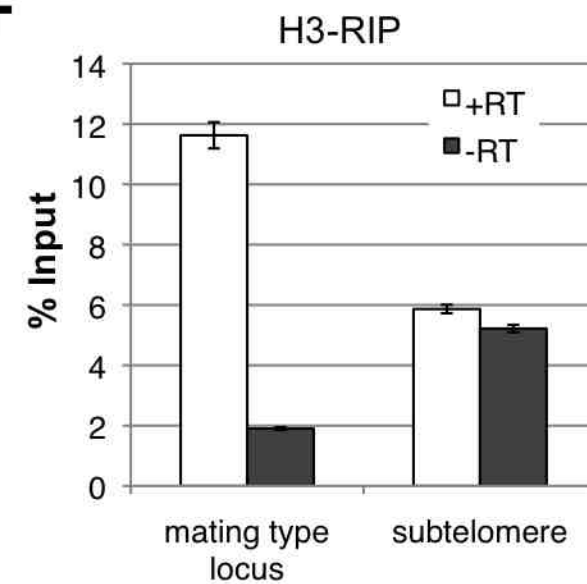
overexpression of RNase H or the indicated deletions were grown to a density of 1.0×10^7 cells/ml and serial dilutions (1:5) of the cultures were spotted onto non-selective (N/S), counter selective (FOA), and selective (–URA) plates for silencing of the *ura4* gene.

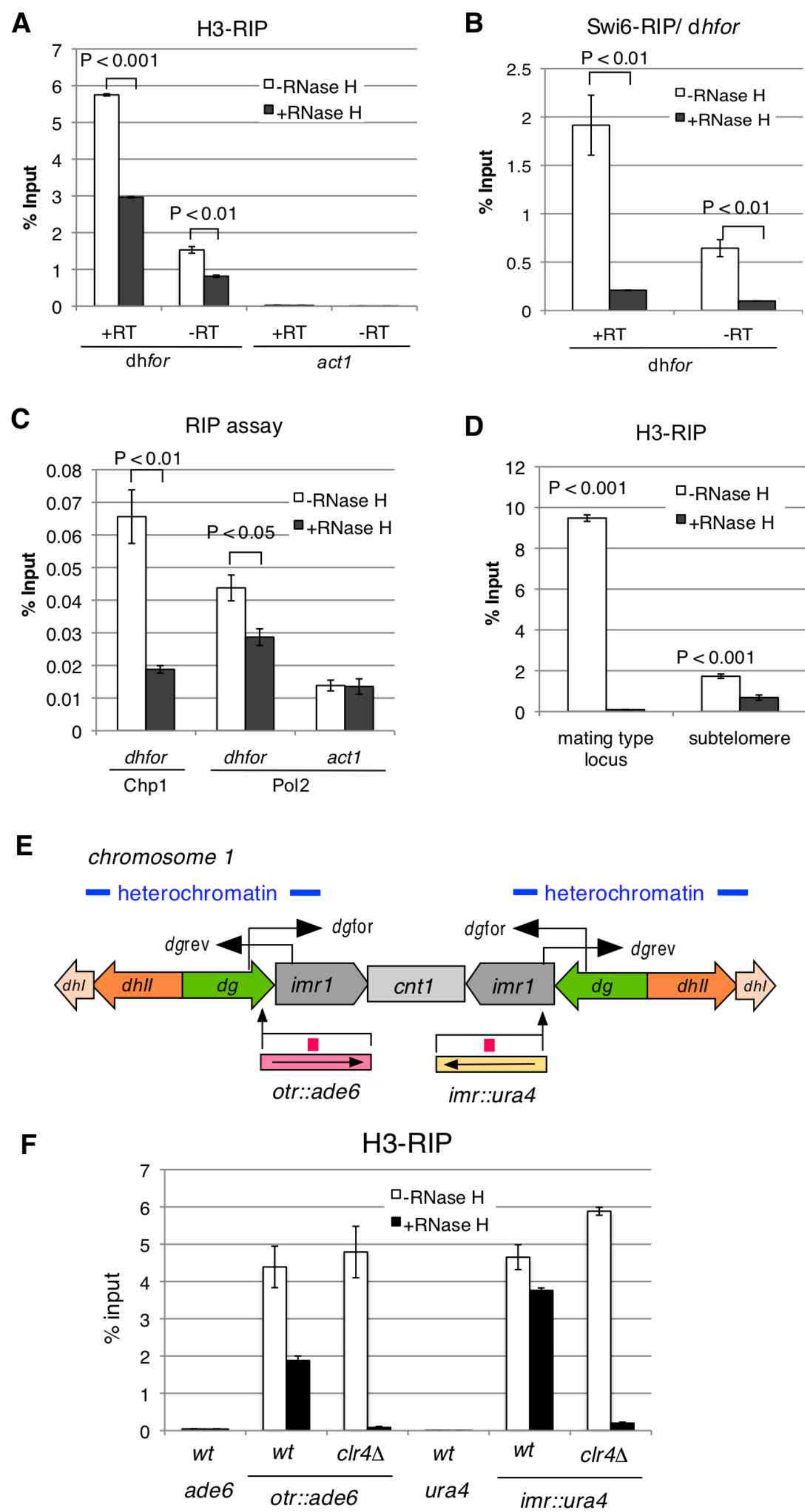
Figure 6. Effect of the overexpression of RNase H on gene silencing by artificial heterochromatin induced by RITS-tethering was analyzed using a spot assay. (A) Spot assays were performed using cells harboring RITS-tethering system, a plasmid for the overexpression of RNase H, or vector alone. Indicated strains that did not harbor the plasmid were also included as a control. Two FOA-resistant colonies from each strain were picked and stability of the FOA resistance was analyzed after growth for 10 generations in non-selective medium (arrow and lower panel). (B) Conversion rates from FOA-sensitive (FOA^S) to FOA-resistant (FOA^R) or FOA^R to FOA^S were measured after 10 generations of growth in non-selective medium. The former represents the efficiency of heterochromatin establishment, while the latter represents the stability of heterochromatin. The conversion rates were calculated in at least three independent experiments and the standard error was indicated.

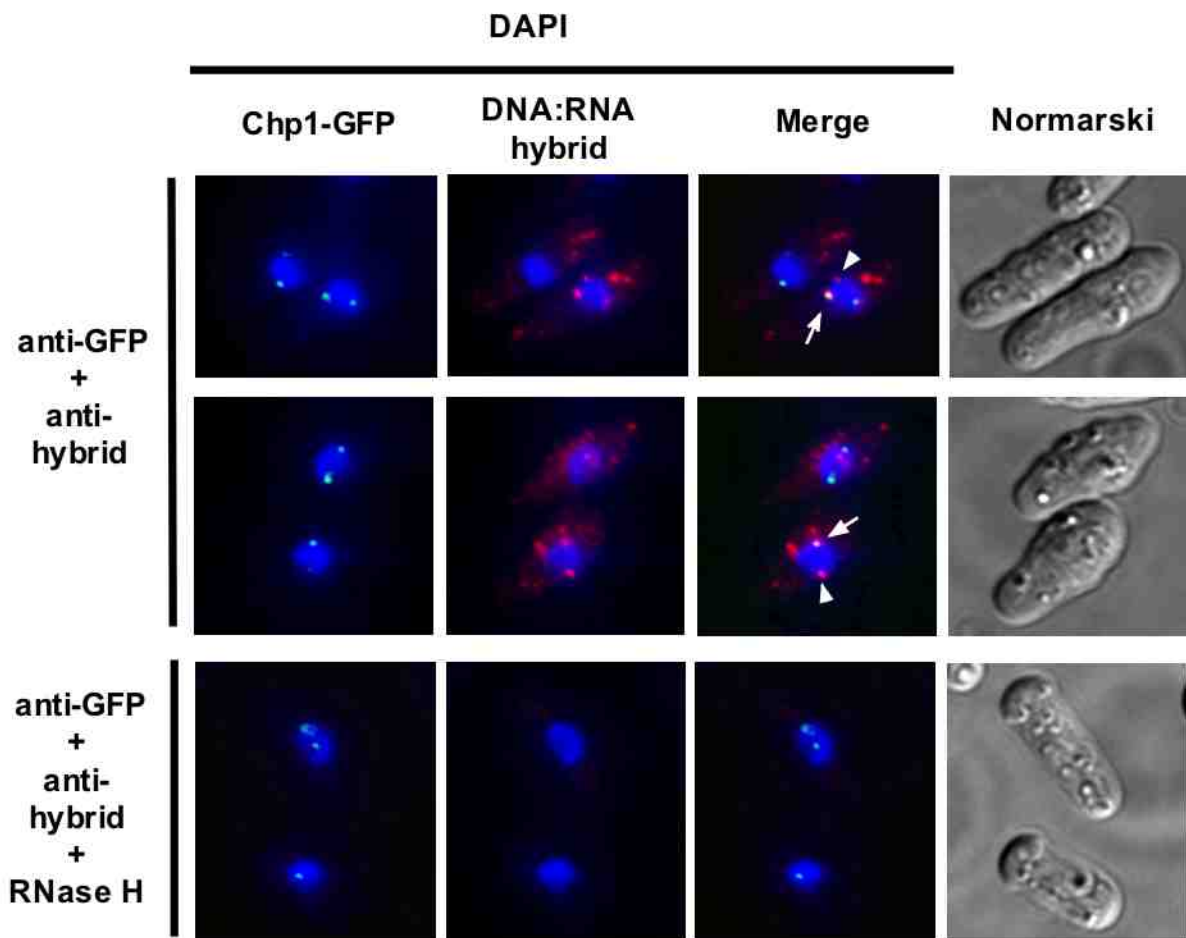
Figure 7. Model for the association of heterochromatic ncRNA with chromatin (I)

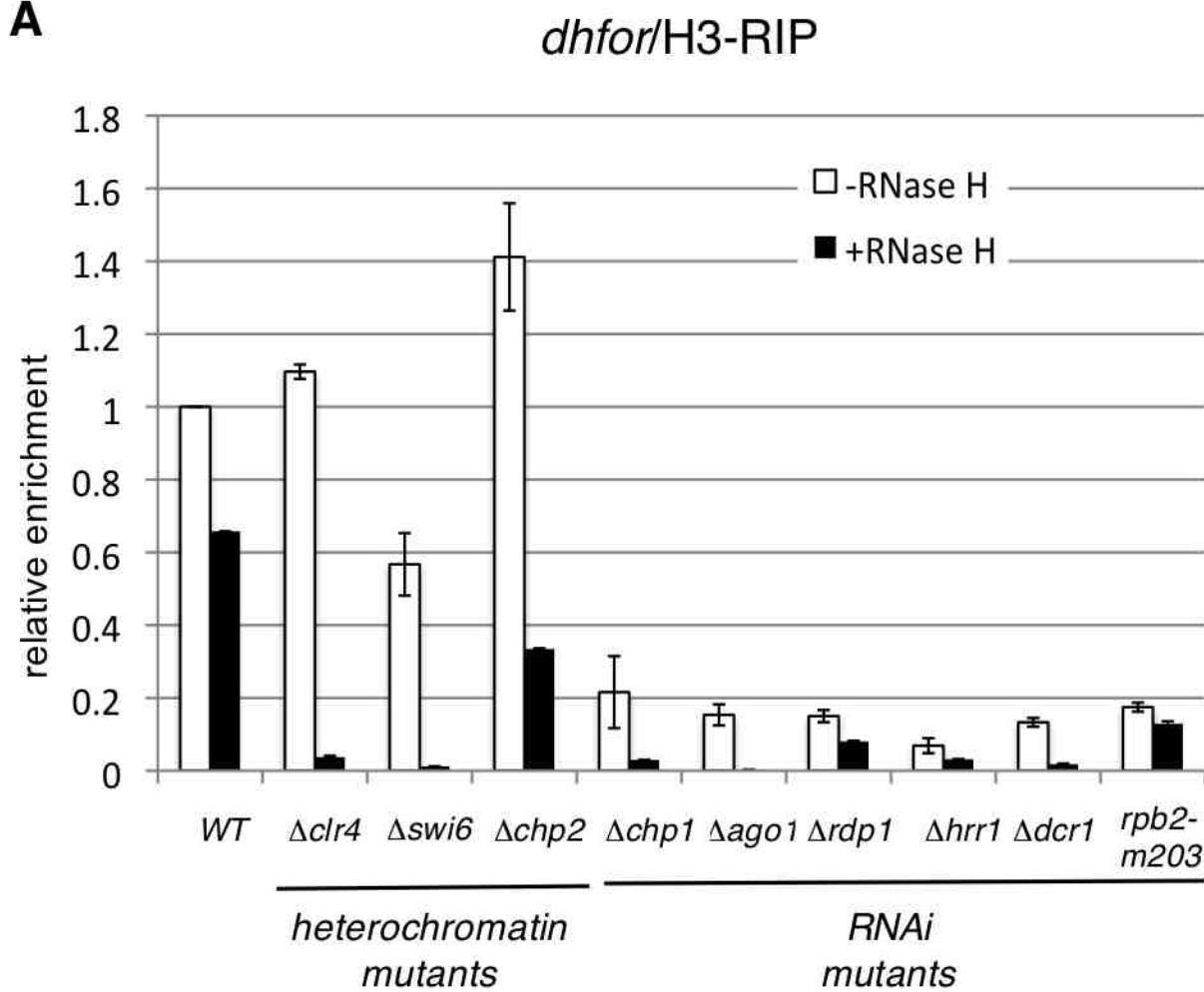
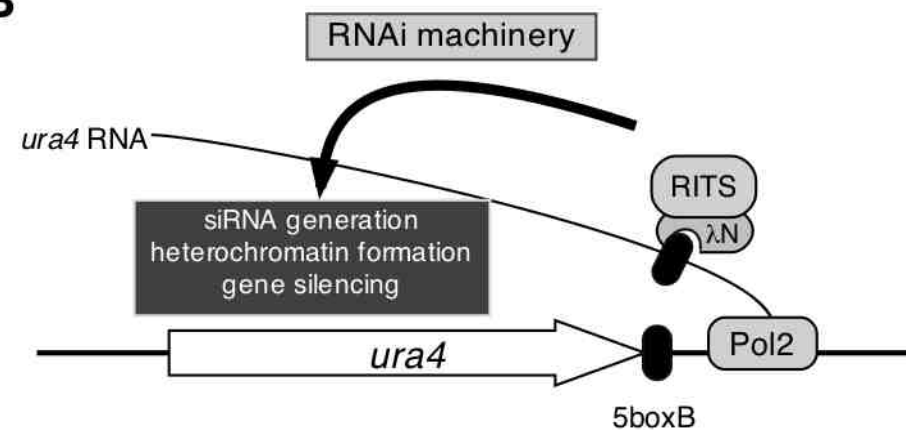
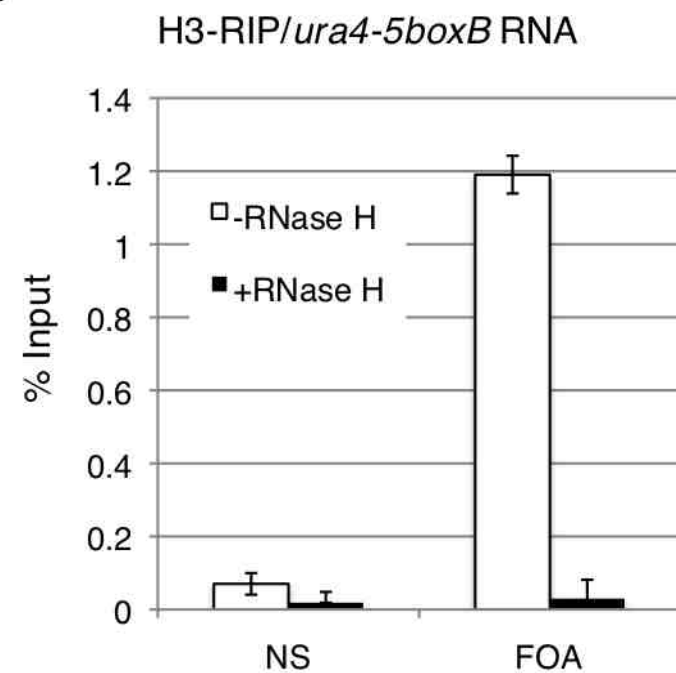
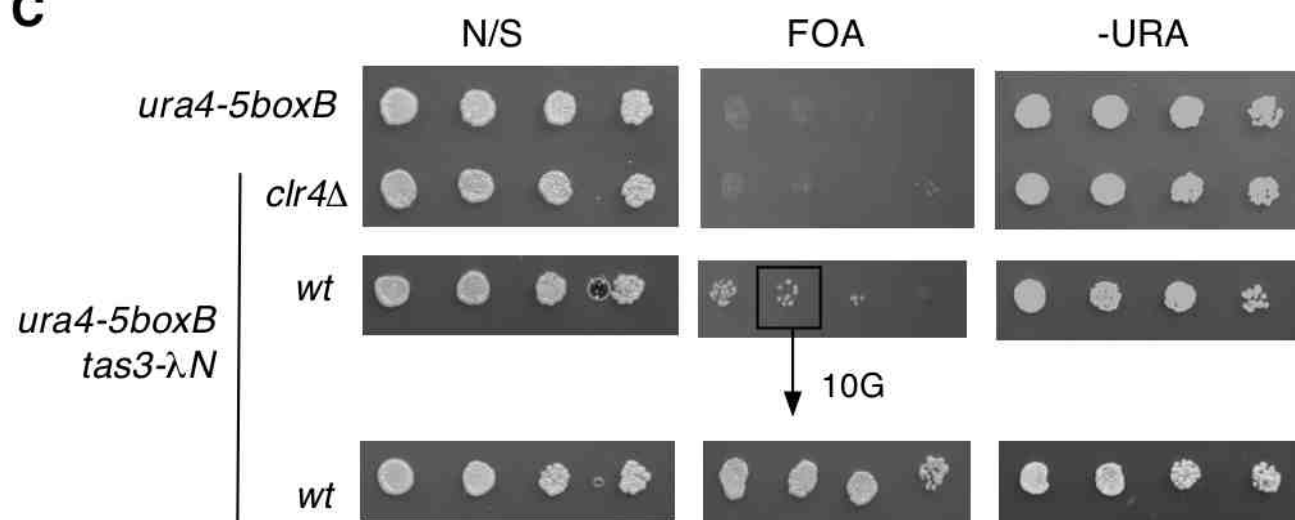
DNA:RNA hybrid is formed at the heterochromatic repeats by transcription-coupled mechanisms. (II) RITS complex targets to the chromatin-associated RNA (or the single stranded DNA) produced by DNA:RNA hybrid to generate siRNA and heterochromatin. (III)

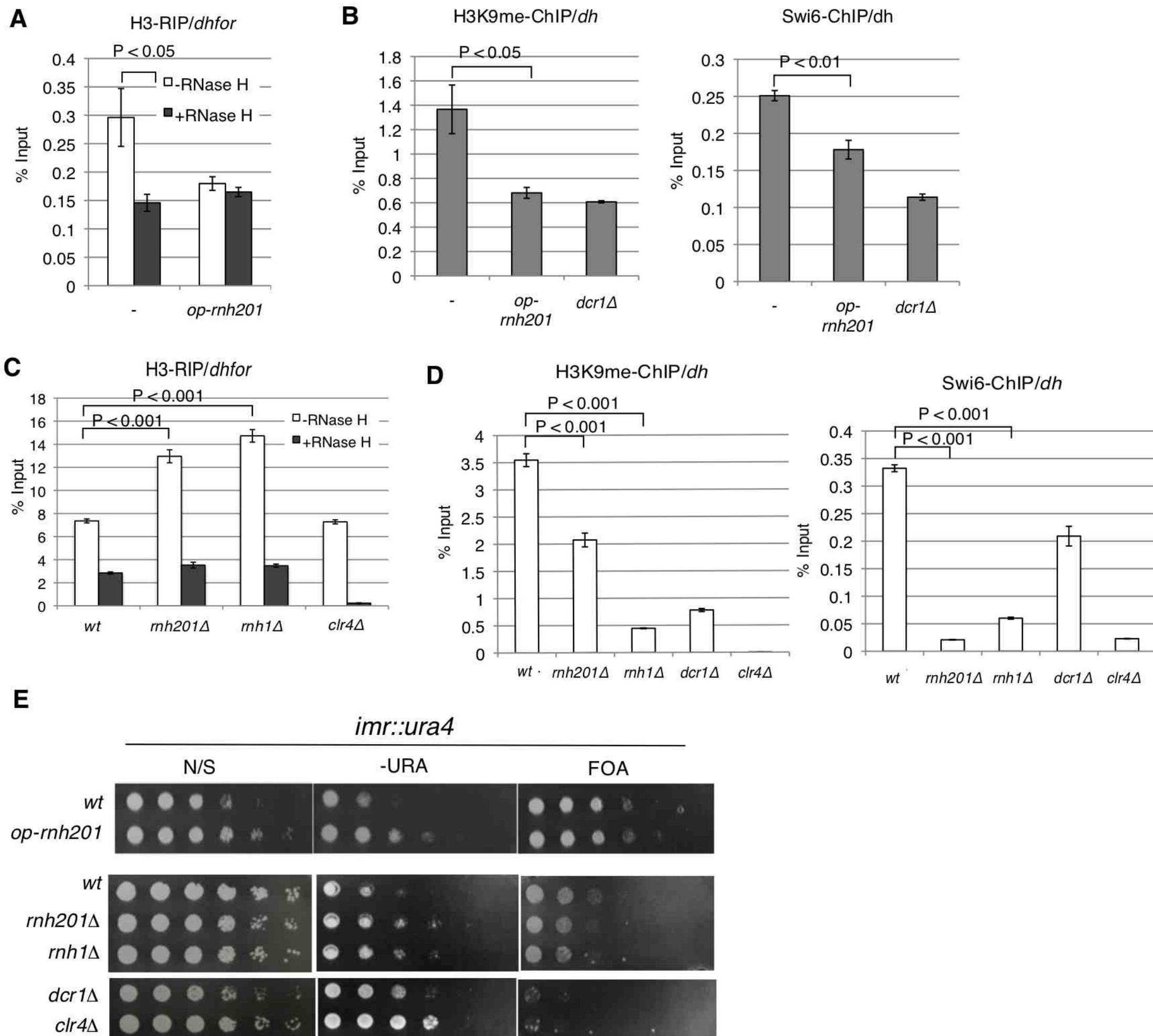
The amount of DNA:RNA hybrid is regulated by RNase H and possibly by DNA/RNA helicases.

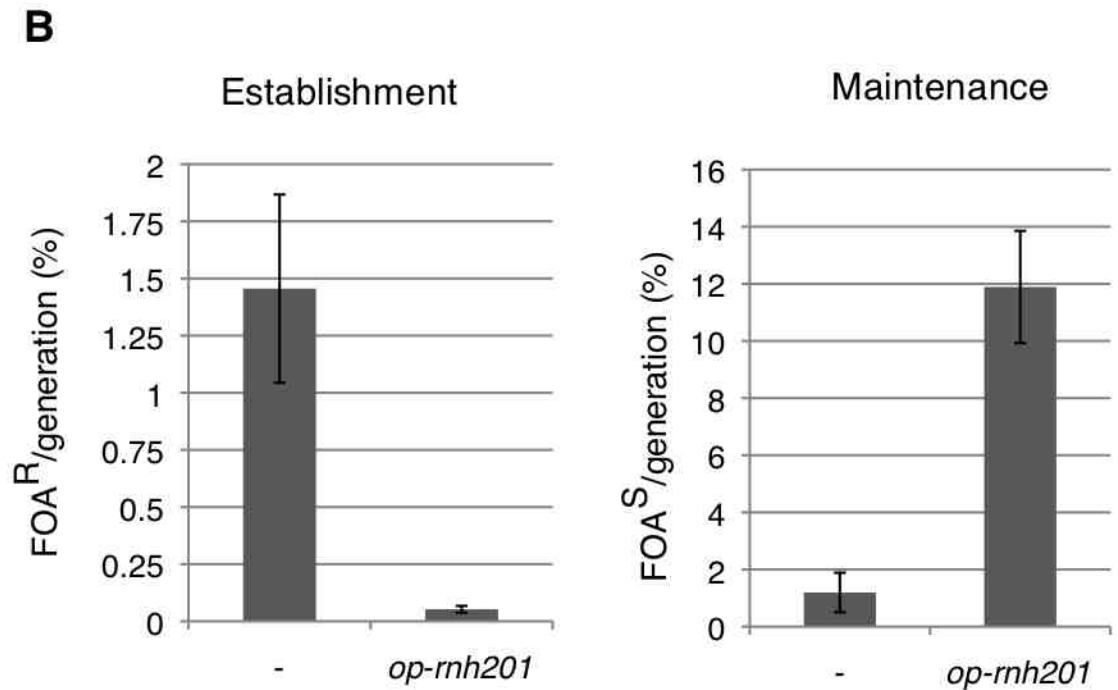
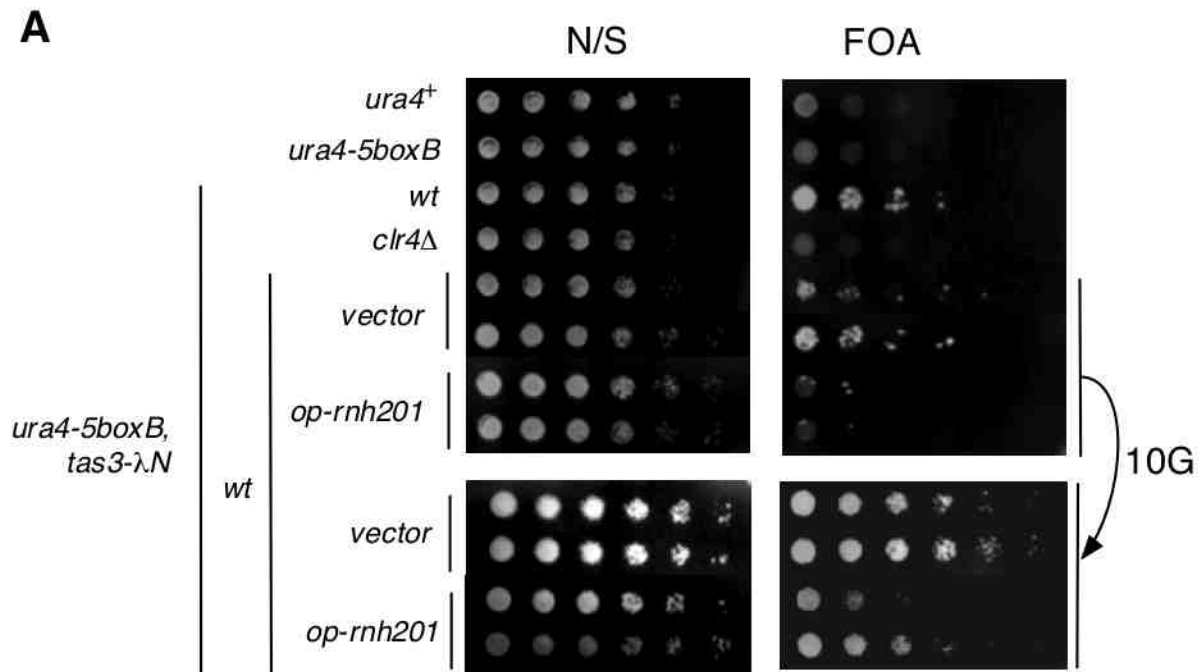
A**B****C****D****E****F**





A**B****D****C**





(II) Targeting of RITS complex