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# Coordination of DNA Replication and Histone Modification by the Rik1-Dos2 Complex

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## Abstract

Histone modification marks play an important role in many chromatin processes[1,2]. During DNA replication, both heterochromatin and euchromatin are disrupted ahead of the replication fork and then reassembled into their original epigenetic states behind the fork[3,4]. How the histone marks are faithfully inherited during each generation is still poorly understood. In fission yeast RNA interference (RNAi)-mediated histone methylation is cell-cycle regulated. Centromere repeats are transiently transcribed at S phase and processed into small interference RNAs (siRNAs) by RITS and RDRC complexes[5-7]. The small RNAs, in concert with silencing factors, including Dos1/Clr8, Dos2/Clr7, Rik1 and Lid2, promote heterochromatic H3K9 methylation by a histone methyltransferase, Clr4[8-13]. H3K9 methylation serves as a binding site for Swi6, a structural and functional homolog of metazoan Heterochromatin Protein 1 (HP1)[14]. Here we characterize a silencing complex, which contains Dos2, Rik1, Mms19, and Cdc20 (DNA polymerase epsilon). The complex regulates RNA Pol II activity in heterochromatin, and is required for DNA replication and heterochromatin assembly. Our findings provide a molecular link between DNA replication and histone methylation, shedding light on how epigenetic marks are transmitted during each cell cycle.

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To further explore the role of Dos2 in heterochromatin assembly, we sought to identify Dos2-associated proteins by tandem affinity purification (TAP). Mass spectrometry analysis of TAP-tag purified Dos2 revealed, in addition to Rik1, two new interacting members: Cdc20, the DNA polymerase epsilon catalytic subunit[15], and a previous uncharacterized protein (SPAC1071.02) (Fig. 1a). SPAC1071.02 is highly conserved (Supplementary Fig. 1). Its homolog in budding yeast is *MMS19*, thus we named it Mms19[16]. The interactions of

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**Supplementary Information** is linked to the online version of the paper at www.nature.com/nature.

**Author Contributions** F.L. and W.Z.C. designed experiments and wrote the manuscript. F.L. performed the experiments. R.M. provided mass spectrometry expertise, equipment, and conceptual support.

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Dos2 with Mms19 and Cdc20 were confirmed by Co-immunoprecipitation (Co-IP) (Supplementary Fig. 2 and 3).

Cdc20 is a conserved DNA polymerase epsilon subunit, sharing extensive homology with its counterparts in human and budding yeast. Cdc20 regulates the elongation of leading strand DNA replication shortly after initiation and is essential for viability[15]. To test whether Cdc20 is required for silencing, we used a temperature sensitive mutant allele, *cdc20-p7*. At 37 °C, mutant cells arrest in early S phase. We crossed the mutant into the *otr*::*ura4*$^+$ background and performed a silencing assay. We found that, at the non-restrictive temperature, 34 °C, the mutant cells grew poorly on the control plate, compared to WT, likely resulting from the replication abnormality. However, on medium lacking uracil the mutants had more robust growth than WT, while on the 5′-FOA plate cells have little growth, demonstrating that centromere silencing was partially compromised (Fig. 1b). These results indicated that full heterochromatin silencing requires Cdc20.

In WT cells, heterochromatin transcripts are quickly processed by RNAi machinery, but in RNAi-processing defective mutants, such as *dcr1*Δ, these transcripts are readily detectable[17]. In *cdc20-p7* mutant cells incubated at 34 °C, similar to *dcr1*Δ, peri-centromere transcripts clearly accumulated (Fig. 1c). We then examined the level of small RNAs in the mutant by Northern blot. Since RNAi is temperature-sensitive, small RNAs in WT are considerably reduced at 34 °C but still detectable[6] (Fig. 1d). In the *cdc20-p7* mutant, however, siRNAs are completely abolished (Fig. 1d), showing that Cdc20 promotes siRNA generation.

To further determine how heterochromatin structure is affected, we examined H3K9 methylation and Swi6 distribution in the *cdc20-p7* cells grown at 34 °C. H3K9 methylation at the pericentromere was significantly reduced at the elevated temperature (Fig. 2a) and association of Swi6 also was decreased (Supplementary Fig. 4), consistent with the heterochromatin defect showed by the silencing assay. We also used delocalization of Swi6-GFP to assess loss of heterochromatin, as the GFP-Swi6 pattern is unchanged in RNAi mutants[18]. We found that 53% of *cdc20-p7* cells at 34 °C and more than 70% at 37 °C had a diffuse GFP-Swi6 pattern, a defect similar to the *dos2*Δ mutant (Supplementary Fig. 5; Fig. 2b). WT cells incubated at the elevated temperatures did not show severe Swi6 delocalization (Fig. 2b) demonstrating that heterochromatin formation requires Cdc20. Since heterochromatin formation is mediated by both RNAi-dependent and –independent pathways[19], the silencing abnormality in *cdc20-p7* suggested that Cdc20 acts at an early stage of heterochromatin assembly.

Mms19, another Dos2 interacting factor, is a conserved protein, containing the HEAT repeat domain (Supplementary Fig. 1). Studies of its homolog in budding yeast, *MMS19*, and in human reveal that they function as a TFIIH regulator, participating in the initiation of RNA polymerase II (RNA Pol II) transcription[16,20]. Interestingly, human *MMS19* is also required for chromosome segregation[21]. To study the role of Mms19 in fission yeast, we first examined its distribution using a GFP-tagged version of Mms19, and found that the GFP signal is predominantly nuclear, consistent with its potential role as a transcription regulator (Supplementary Fig. 6). To elucidate the Mms19 function, we created an *mms19* null

mutant. The *mms19*Δ mutant grew slower than the wild type strain (WT) but it was viable, indicating that *Mms19*$^{+}$ is not an essential gene. Similar to the budding yeast *mms19* mutant, the growth of *mms19*Δ requires methionine (Supplementary Fig. 7).

Since the Mms19 homolog in other organisms associates with the TFIIH complex, we speculated that Mms19 may be involved in RNA Pol II transcription in heterochromatin regions. To address this possibility, we directly examined centromeric transcription by RT-PCR. The transcripts are abundant in siRNA-processing mutants, such as *dcr1*Δ, but it is difficult to detect them in WT[17] (Supplementary Fig. 8). The centromere transcripts were not discernible in the *mms19*Δ mutant by RT-PCR, similar to WT (Supplementary Fig. 8), and were greatly reduced in a *dcr1*Δ *mms19*Δ double mutant (Fig. 3a). We reasoned that, as a result of reduction of primary siRNA transcript, centromeric siRNA levels may also decrease. To test this, RNA extracted from the *mms19*Δ mutant was probed for centromeric siRNA by Northern blot which showed that siRNAs were partially reduced in *mms19*Δ(Fig. 3b). These data further demonstrated that Mms19 regulates centromere transcription.

Coinciding with heterochromatin expression, RNA Pol II is preferentially restricted to heterochromatin at S phase[5]. To further elucidate the role of Mms19, we investigated how Mms19 associates with heterochromatin during the cell cycle. After release from synchronization, cells carrying Mms19-TAP at different stages were collected. We found that Mms19 preferentially associated with heterochromatin at S phase (Fig. 3c), in concurrence with the enrichment of RNA Pol II. We then investigated how Mms19 affects the RNA Pol II distribution in heterochromatin at this stage. ChIP with an RNA Pol II antibody showed that RNA Pol II accumulation at S phase was reduced considerably in the *mms19*Δ mutant (Fig. 3d). Furthermore, Mms19 physically associates with RNA Pol II (Supplementary Fig. 9). Together, our results suggest that Mms19 is a transcription activator, required for the heterochromatic RNA Pol II transcription.

To gain further insight into how the *cdc20-p7* mutant affects its interaction with Dos2 and Mms19, we created a HA tagged version of the mutant gene. Co-IP showed that at 23 °C Cdc20-p7-HA maintains its association with Dos2 and Mms19; however, these interactions were lost at 34 °C, indicating that the point mutation reduced the interaction at elevated temperature (Fig. 4a). We also investigated the association of heterochromatin of Mms19 by ChIP in synchronized *cdc20-p7* cells released from metaphase. At 23 °C the peak enrichment of Mms19 in the mutant at S phase, ~80 min after release, was obvious; however, the accumulation of Mms19 was not observed when temperature was elevated to 34 °C, indicating that Cdc20 is required for Mms19's association with heterochromatin (Fig. 4b). A previous report showed that Dos2, and Rik1 start to accumulate in heterochromatin at S phase[5]. We then determined whether Cdc20 affects the recruitment of the two silencing factors. Using ChIP assays with antibodies for TAP or Myc tag, we found that at 23 °C Dos2-TAP and Rik1-myc are enriched at S phase, consistent with the previous study; however, the association is diminished at 34 °C (Fig. 4c and Supplementary Fig. 10). The results indicate that Cdc20 is required for recruitment of Dos2 and Rik1 to heterochromatin. Interestingly, heterochromatic silencing is also partially compromised in mutants in two different DNA polymerase alpha subunits[22,23]. As DNA polymerase alpha-primase is

required before elongation by polymerase epsilon, it is possible that interaction of the Rik1 complex with Cdc20 underlies this silencing defect.

We reasoned that the loss of silencing in the mutant may be linked to the impairment of DNA replication. In fact, *cdc20-p7*cells at 34 °C grew much more slowly than at 23 °C, and had an extended S phase (Fig. 1b). Efficiency of replication recovery from UV-induced damage demonstrates the replication state of the tested strain. We found that *cdc20-p7* was highly sensitive to UV at 34 °C but not at 23 °C (Fig. 2c). Furthermore, heterochromatic fragments that contain ARS elements cannot replicate efficiently in the mutant at 34 °C (Supplementary Fig. 11). Thus, the loss of heterochromatin silencing in *cdc20-p7* appears coupled to a defect in DNA replication.

To gain further insight into the role of Cdc20 in the heterochromatin pathway, we analyzed an N-terminal deletion of Cdc20, $cdc20^{\Delta N\text{-}term}$. The N-terminus of Cdc20, which contains the catalytic domain, is not essential for survival[24]. To test how the mutant affects heterochromatin silencing, $cdc20^{\Delta N\text{-}term}$ in *otr*::$ade6^{+}$ background was analyzed on rich medium supplemented with no extra adenine at room temperature. WT cells form red colonies due to transcriptional silencing, but $cdc20^{\Delta N\text{-}term}$ colonies have a white color (Fig. 2d), indicating that heterochromatin silencing is alleviated in the mutant. Consistent with this, H3K9 methylation in peri-centromeric repeats is significantly reduced (Fig. 2e). Thus, DNA replication and heterochromatin function were decoupled in this mutant, further showing that Cdc20 is directly involved in heterochromatin silencing.

We demonstrate that the Dos2 complex, which contains Dos2, Mms19, Rik1 and Cdc20, is critical for DNA replication, siRNA production and heterochromatin assembly. Our findings establish the first physical and functional link between DNA replication, small RNA generation and H3K9 methylation, and provide a novel mechanism to explain how they are coordinated (Fig. 4d).

Our results provide insight into how the epigenetic states of heterochromatin are faithfully duplicated in each cell cycle (Fig. 4d). In budding yeast, heterochromatin assembly requires S phase progression but not origin firing[25,26]. Our findings suggest that DNA replication is required for heterochromatin assembly in *S. pombe*. In plants and mammals, DNA replication and DNA polymerase epsilon also have been implicated in silencing heterochromatin[27-29]. This suggests that a molecular mechanism linking DNA replication to heterochromatin formation, similar to the one elucidated in this study, is likely conserved in multicellular eukaryotes.

## METHODS SUMMARY

*Schizosaccharomyces pombe* strains used in this study are listed in Supplementary Table 1. Cells synchronization was performed by hydroxyurea method. For mass spectrometry, TAP-tagged Dos2 was purified from a total of $9 \times 10^{10}$ cells as described previously[11]. Fluorescent immunofluorescence images were taken by a Delta Vision System (Applied Precision, Issaquah, WA). SoftWoRX2.50 (Applied Precision) was used for processing the final projections. For UV Survival Assay, exponential growing cells were collected, and

plated with appropriate dilutions onto YES media. The plates were then irradiated by various UV doses. After incubation at 23 °C for 5 day, the colonies were counted. Detailed descriptions of Immunoprecipitation assays, ChIP, RT-PCR and Northern blot can be found in the Supplementary Information.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Appendix

## Methods

### Fission Yeast Strains, Media and Genetic Procedures

*Schizosaccharomyces pombe* strains used in this study are listed in Supplementary Table 1. Yeast extract with supplements (YES) was used as a complete culture medium, EMM as a minimum media, and SPAS used for conjugation and sporulation. Cells synchronization was performed by hydroxyurea method. Briefly, cells were treated with 12mM hydroxyurea for 4 h, then released into 100 μg of TBZ/ml for 1 h to block mitotic entry. Standard genetic protocols for fission yeast were used[30].

### Mass Spectrometry

TAP-tagged Dos2 was purified from a total of $9 \times 10^{10}$ cells as described previously[31]. Briefly, cell lycates in 1x lysis buffer (50 mM bis-Tris propane, pH 7.0, 0.1 M KCl, 5 mM EDTA, 5 mM EGTA, 10% glycerol) were incubated with IgG sepharose (Amersham Pharmacia Biotech) for two hrs. After washing with lysis buffer, the IgG sepharose was incubated overnight with TEV protease (Invitrogen). Supernatant was removed from the IgG sepharose and added to S protein agarose slurry (Novagen) for 3 hrs. The S protein agarose was washed by lysis buffer. The elute from S protein agarose was analyzed by silver staining and subjected to mass spectrometry (Cold Spring Harbor Laboratory).

### Immunoprecipitation Assays

Cells were lysed by glass bead method in HB buffer[30]. Lysates were pre-cleared with protein A agarose beads, followed by 2-hour incubation with anti-HA or GFP antibody (Sigma), or IgG Sepharose 6 Fastflow beads (Amersham Bioscience) at 4°C. After washing, eluted proteins and input extracts were analyzed by Western blotting using anti-S tag (ABR, MA1-981) or anti-PolII (ab5408, Abcam).

### ChIP Analysis

CHIP assays were carried out as previously described[32]. Cells growing at log phase were crosslinked by 1% formaldehyde. Immunoprecipitation was performed with S protein agarose (Novagen) or the following antibodies: dimethylated H3-K9 (Upstate, 07-441) or Swi6 (Abcam, ab14898). Precipitated DNA was analyzed by competitive PCR using oligonucleotides specific to centromeric *dh* region or to the control gene, *act1*$^+$. PCR products were separated on a 1.7% agarose gel and post-stained with ethidium bromide. Primers used are listed in Supplementary Table 2.

### RT-PCR

Total RNA was isolated from cells growing at log phase by RNeasy mini kit (Qiagen). After treated with DNAase I (Promega), 50 ng of purified RNA was analyzed by RT-PCR in a 25 μl reaction volume using a one-step RT-PCR kit (Qiagen). Equal loading of RNA samples were assessed by amplification of the control gene, *act1*$^+$. For strand-specific RT-PCR, RNA samples were incubated with primers complimentary to either forward or reverse centromeric transcripts for synthesis of the first cDNA strand. After heat inactivation of the reverse transcriptase at 95°C for 15 min, a second primer was added for the subsequent cycles of PCR amplification. Primers used for RT-PCR are listed in Supplementary Table 2.

### Small RNA Northern Blot

Small RNA Northern blot was performed as described previously[10]. Briefly, siRNAs were extracted from exponentially growing cells in YES media using a mirVana miRNA isolation kit (Ambion). 25 μg of small total RNA was resolved by a 15% denaturing acrylamide gel, and blotted to a charged nylon membrane (Hybond-N+, Amersham). RNA blots were cross-linked and hybridized with DNA probes specific for centromere *otr* region or snoRNA69 as a loading control. DNA oligonucleotides used are listed in Supplementary Table 2.

### Microscopy

Fluorescent images were taken by a Delta Vision System (Applied Precision, Issaquah, WA). SoftWoRX2.50 (Applied Precision) was used for processing the final projections. DAPI staining was followed the standard procedure.

### UV Survival Assay

Exponential growing cells were collected, and plated with appropriate dilutions onto YES media. The plates were irradiated by various UV doses. After incubation at 23°C for 5 days, the colonies were counted.

### DNA Replication Assay

A pBluescript plasmid carrying a 3.0-kb fragment from heterochromatic *cen3* repeats (3.0-K) [33] and also an *ura4*$^+$ selective marker was transformed into the *cdc20-p7* mutant strain. The centromeric fragment contains efficient ARS elements[33]. After incubation at 23°C or 34°C for 4 days, total DNA was isolated following standard procedure[33]. 50 ng of purified DNA was analyzed by PCR (BioRad) using primers specific for *ura4*$^+$. Equal loading were assessed by amplification of the control gene, *act1*$^+$.

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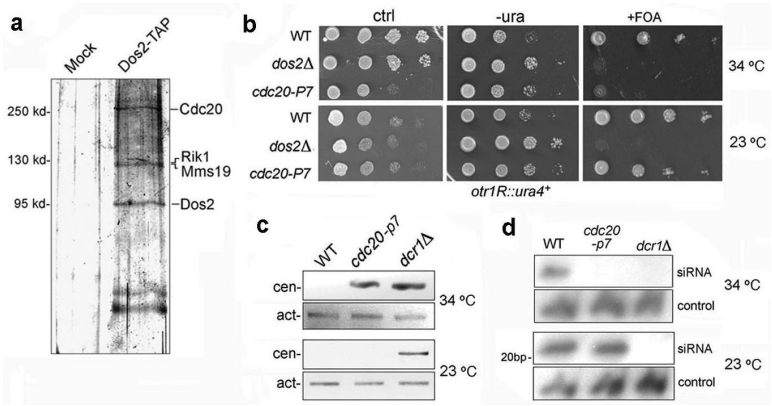


**Figure 1. Cdc20 is essential for gene silencing and siRNA generation**

**(a)** Protein extracts made from a Dos2-TAP strain or untagged control strain were purified by the TAP method. Purified products were separated by SDS-page gel and visualized by silver staining. **(b)** Growth assay of serial dilutions of strains carrying *ura4*$^{+}$ inserted at the pericentromeric *otr* region on –ura or counter-selective 5′-FOA media incubated at 23°C or 34°C. **(c)** Accumulation of centromeric transcripts was analyzed by RT-PCR from strains incubated at indicated temperatures. **(d)** Analysis of small RNA corresponding centromeirc repeats by Northern blot. Control, snoRNA69 as a loading control.

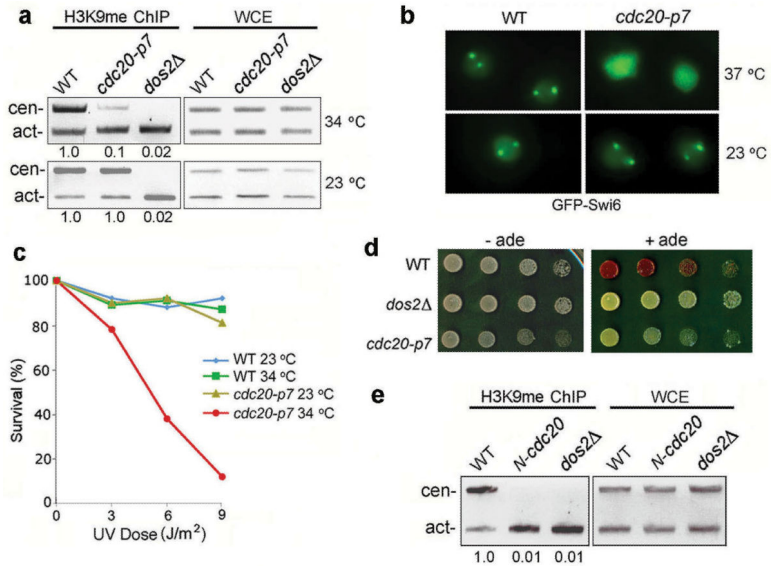


**Figure 2. Heterochromatin abnormality is coupled to the DNA replication defects**

**(a)** H3K9 methylation at the peri-centromere in *cdc20-p7* was significantly lost at 34°C but not at 23°C. **(b)** Fluorescent images of GFP-Swi6 in the strains incubated at 23°C or 37°C. **(c)** UV sensitivity of *cdc20-p7* and WT at 23°C or 34°C. **(d)** Colony color silencing assay of strains carrying *ade6*$^{+}$ inserted at the pericentromeric *otr* region on YES rich media without further supplementing with adenine at room temperature. **(e)** Analysis of H3K9 methylation at the peri-centromere in the indicated strains. *N-cdc20*, N-terminal deleted *cdc20* mutant.

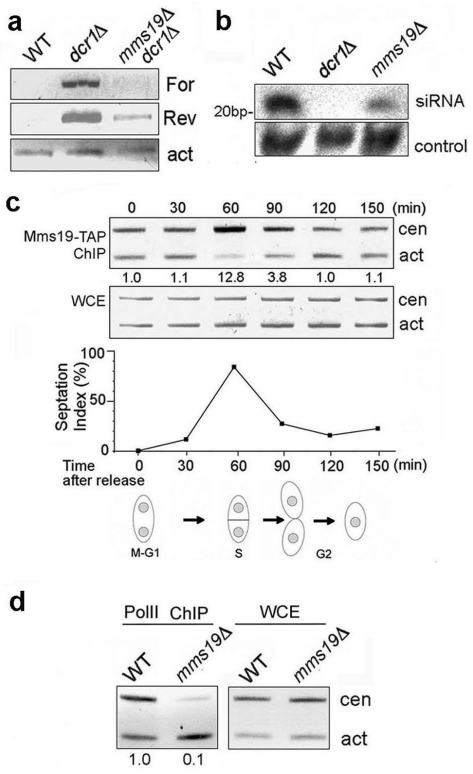


**Figure 3. Mms19 is required for heterochromatin RNA Pol II transcription**

**(a)** strand-specific RT-PCR analysis of accumulation of the transcripts from centromeric *dh* repeats. For, forward strand; Rev, reverse strand. **(b)** Analysis of small RNA corresponding centromeirc *dg-dh* repeats by Northern blot. Control, snoRNA69 as a loading control. **(c)** Mms19 preferentially associates with heterochromatin during S phase. Protein extract were prepared from synchronized cell carrying Mms19-TAP, and analyzed by ChIP with an antibody for TAP. **(d)** ChIP analysis of RNA Pol II accumulation in cells synchronized in S phase. Cell cycle progression was monitored by a Septum Index.

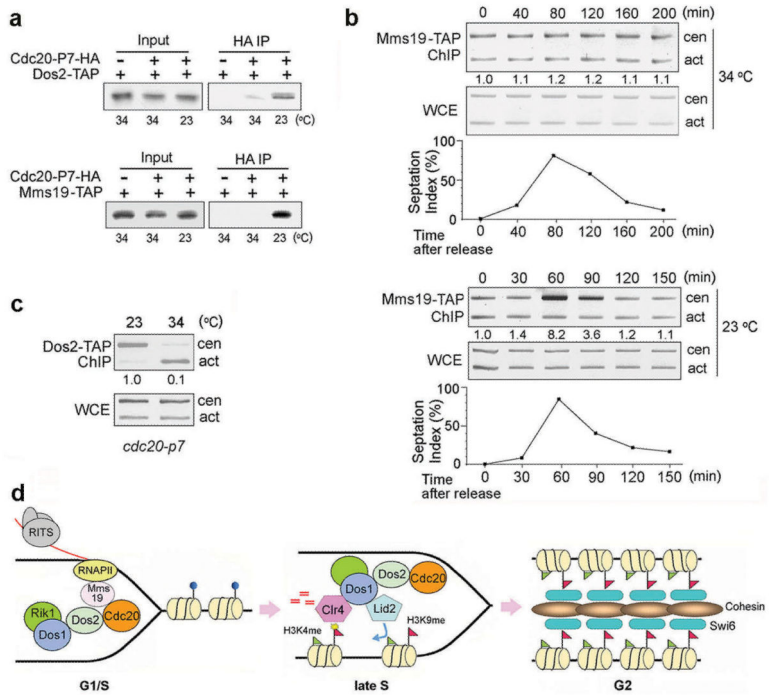


**Figure 4. Functional interactions between components of Dos2 complex**

**(a)** The interaction of *cdc20-p7* mutant with Dos2 or Mms19 was abolished at 34°C but not at 23°C. **(b-c)** Mms19-TAP and Dos2-TAP accumulation in heterochromatin at S phase were lost in *cdc20-p7* cells at 34°C. **(d) Model:** (1) during G1/S phase, while synthesizing the leading heterochromatin strands, Cdc20 regulates heterochromatin transcription by interacting with Mms19, and also recruits Dos2 and Rik1. Another DNA polymerase subunit may be responsible for similar process in the lagging strand. Heterochromatin transcripts are subsequently processed into siRNAs by RITS complex. (2) Dos1-Dos2-Rik1 complex together with siRNAs promotes H3K9 methylation by Clr4. (3) Swi6 binds to H3K9 methylation to reassemble chromatin into a repressed state.