Current Biology, Vol. 15, 1448–1457, August 23, 2005, ©2005 Elsevier Ltd All rights reserved. DOI 10.1016/j.cub.2005.07.021

# Two Novel Proteins, Dos1 and Dos2, Interact with Rik1 to Regulate Heterochromatic RNA Interference and Histone Modification

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## Summary

**Background:** Chromosomal behavior during mitosis and meiosis depends in part on heterochromatic modifications such as histone H3 lysine-9 methylation (H3K9me). In fission yeast, the Heterochromatin Protein 1 homolog Swi6 recognizes H3K9me, silences transcription, and retains cohesin at pericentromeric repeats. Heterochromatin formation also depends on processing of transcripts derived from centromeric repeats by the RNAi machinery. The DDB1 homolog, Rik1, and histone methyltransferase, Clr4, act in a complex to promote H3K9me. However, the mechanism underlying this interaction is poorly understood.
**Results:** Using a cytological screen, we have identified two novel genes, *dos1*$^+$ and *dos2*$^+$, which are required for localization of Swi6. Deletion of either of these genes results in mitotic and meiotic chromosome missegregation, defects in mitotic centromeric cohesion and meiotic telomere clustering, and loss of heterochromatic silencing. Dos1 is predominantly located in the nucleus in a Dos2-dependent manner and directly interacts with Rik1. Each of these genes is required for the association of H3K9me with centromeric repeats, as well as for the production of small interfering RNAs.
**Conclusions:** Dos1 and Dos2 are required for the formation of heterochromatin in fission yeast. We hypothesize that the physical interaction between Dos1 and Rik1 represents a role in regulating activity of the Rik1/Clr4 complex. Dos2 contributes to this role by regulating Dos1 localization. Our findings suggest a mechanism for recruitment of Clr4 in the RNAi-dependent heterochromatin pathway, in which Dos1 and Dos2 are essential.

## Introduction

In eukaryotes, chromosomes are organized into higher-order structures known as euchromatin and heterochromatin. In interphase nuclei, heterochromatin is condensed and appears densely stained, whereas euchromatin is unraveled to allow gene expression. Heterochromatin has a relatively low gene density and is rich in highly repetitive sequences, but heterochromatin is not inert and plays important roles in gene silencing, genome stability, and chromosome segregation [1, 2]. In the fission yeast *Schizosaccharomyces pombe,* heterochromatin is relatively simple, comprising the pericentromeric, telomeric, and mating-type regions. Nonetheless, heterochromatin is required for silencing, switching, and suppression of recombination at the mating-type locus, for cohesion at centromeres, and for structural transitions at telomeres associated with the cell cycle [3–6].

Genes located within heterochromatic regions are silenced by position effect, and the assembly of heterochromatin has been studied genetically by isolating silencing mutants. Genes involved in mating-type silencing are almost always required for silencing of reporter genes at the centromere [3], but centromere-specific mutants (*csp*) have been recovered as well [7]. These studies have revealed that silencing requires histone deacetlyases Clr3, Clr6 [8], and Sir2 [9], as well as methylation of H3K9 by the SET domain methyltransferase, Clr4 [10]. Additional silencing factors include Rik1 [11, 12], a WD-propeller-repeat protein related to both the DNA damage binding protein, DDB1, and the large subunit of the mRNA-cleavage and polyadenylation-specificity factor, CPSF, in animals and plants [13]. Rik1 and Clr4 act in a complex to promote H3K9 methylation [14], although the mechanism underlying this interaction remains elusive. H3K9me recruits the chromodomain protein Swi6, a homolog of animal heterochromatin protein HP1, which is also required for silencing via position-effect variegation [15].

The RNA interference (RNAi) machinery, including Argonaute (Ago1), Dicer (Dcr1), and RNA-dependent RNA polymerase (Rdr1), is conserved in animals and plants, and is required for centromeric but not for mating-type silencing in fission yeast [16]. This was unexpected because silencing was known to occur at the transcriptional rather than the posttranscriptional level. The RNAi machinery processes noncoding RNA transcribed from both strands of the centromeric repeats into small interfering RNA (siRNA) [17], and this results in silencing of nearby reporter genes as well as "forward" strand transcripts from the repeats. Similar repeats are found at the mating-type locus, and a role for RNAi in initiating, though not maintaining, mating-type silencing has been proposed [18]. However, an RNAi-independent heterochromatin pathway involving the CREB family proteins, Atf1 and Pcr1, plays a redundant role in mating-type silencing, accounting for its maintenance in RNAi mutants [19–21].

Systematic purification of chromodomain-containing complexes revealed that Chp1 (a chromodomain protein), Ago1, and the novel protein Tas3 are associated in the RITS (RNA-induced initiation of transcriptional gene silencing) complex [22]. siRNA is also found in the complex, presumably bound to Ago1 via its PAZ domain [23]. A role in initiating silencing at the mating-type locus has been proposed [14, 22]. However, association of RITS with heterochromatin depends strongly on Clr4 but only weakly on RNAi, whereas association with het-

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erochromatic reporter genes depends on both factors, suggesting a role for RITS in spreading, as well as (or instead of) initiation [14, 22]. It is possible that RITS has a more important role in recruiting the RNAi apparatus to modified chromatin, rather than the other way around.

How, then, is it that Clr4 is guided to methylate H3K9 and thereby recruits Swi6? Although the mechanism is still unclear, it may involve Rdr1, which is bound to centromeric repeats [16]. Recently, the RdRP complex (RDRC) has been found to include Hrr1 (an RNA helicase) and Cid12, a member of the polyA polymerase family [24]. The RITS complex weakly interacts with RDRC, and this interaction requires both Dcr1 and Clr4 [24]. The presence of a polyA polymerase was provocative because it could be associated in some way with Rik1, which has homology to polyadenylation recognition factors [24].

Centromere function and faithful segregation of chromosomes requires both Swi6 and Clr4 [25], and for this reason it also requires RNAi [26, 27]. Swi6 directly interacts with cohesin, retaining it at pericentromeric heterochromatin after it has been lost from euchromatic arms [28, 29]. Heterochromatin is also important for telomere function. For example, during early stages of meiosis, telomeres cluster at the nuclear periphery and attach to the nuclear envelope beneath the spindle pole body (SPB), the fission-yeast equivalent of the centrosome. The telomere bouquet, which is found in most eukaryotes, aligns the ends of homologous chromosomes as the nucleus is pulled back and forth in the cells by microtubules attached to the SPB. This movement, referred to as the "horsetail movement," is believed to facilitate homologous-chromosome pairing and recombination. Maintenance of the bouquet during the horsetail stage requires Rik1 and Clr4 along with telomere protein Taz1, but not Swi6 [12]. In RNAi mutants, the telomere-clustering defects are mild compared with centromere-segregation defects, consistent with the absence of telomeric-silencing defects [27].

To further study the mechanisms that regulate heterochromatin, we performed a novel genetic screen based on cytological defects rather than silencing. In order to find mutants in which heterochromatin organization is disrupted, we visually screened mutants carrying a sensitive marker, GFP-Swi6. This led to identification of two novel heterochromatin factors, Dos1 (*D*elocalization *o*f *S*wi6) and Dos2, which are required for silencing of all heterochromatin regions. Deletions of these two genes resulted in diffuse GFP-Swi6 and defects in chromosome segregation and telomere clustering. We demonstrate that Dos1 and Dos2 act in the RNAi-dependent heterochromatin pathway and that Dos1 physically interacts with Rik1, suggesting a mechanism by which Clr4 activity might be recruited.

## Results

### Isolation of Mutants with Diffuse GFP-Swi6 Pattern

In this study, we utilized a strain containing GFP fused to the N terminus of Swi6 (GFP-Swi6) under the control of an attenuated nmt promoter and integrated at *ars1*$^+$ in the genome [30]. GFP-Swi6 has proven to be a good monitor for chromosome behavior in both mitotic and meiotic mutants because it binds to heterochromatin in telomeric, centromeric, and the mating-type regions throughout the cell cycle and does not compromise heterochromatin formation [30, 31]. In vegetative cells, all three centromeres cluster together next to the spindle pole body (SPB), whereas telomeres attach to the nuclear envelope distal to the centromeres. As a result, three to four bright GFP-Swi6 spots are seen. During the meiotic horsetail stage, telomeres cluster at the leading edge of the nucleus with a single spot of GFP-Swi6 while at the centromere GFP-Swi6 forms several spots. The nucleus is visible in both mitotic and meiotic cells because the GFP-Swi6 signal is faintly diffuse within the nucleoplasm. Cells containing GFP-Swi6 were mutagenized by random insertion of the *ura4*$^+$ gene, and mutants were visually examined (X.T. and W.Z.C., unpublished data). Two mutants were recovered from the screen with diffuse GFP-Swi6 localization during meiosis rather than discrete foci. GFP-Swi6 was also diffuse in the nuclei of mutant cells during mitosis, indicating that the recruitment of Swi6 and heterochromatin organization were defective (Figure 1).

The locations of the *ura4*$^+$ insertions were determined with a competitive PCR approach and found to be in SPCC613.12c and SPCC970.07, respectively. These two genes both encode hypothetical proteins and were named *dos1*$^+$ and *dos2*$^+$, respectively. The 1906 bp *dos1*$^+$ (SPCC613.12c) open reading frame (ORF) encodes 638 amino acids including a WD-repeat domain, and *dos2*$^+$ (SPCC970.07) encodes 636 amino acids with a hypothetical zinc finger domain. Comparison with GenBank suggested that Dos1 is unique to *S. pombe*, whereas Dos2 shares weak homology with *Drosophila melanogaster* dhc62B (identities 29%, positives 43%) and *Homo sapiens* NCOA4 (identities 25%, positives 43%). Null deletion strains were generated by replacing the entire coding sequence of *dos1*$^+$ and *dos2*$^+$ with either *ura4*$^+$ or a G418-resistance cassette via homologous recombination [32]. Gene disruptions were verified, and strains carrying the disrupted allele were viable, indicating that *dos1*$^+$ and *dos2*$^+$ are not essential genes. Microscopic examination revealed that all of the deletion strains had the same diffuse GFP-Swi6 phenotypes as the original insertion mutants. Moreover, the diffuse GFP-Swi6 pattern in *Δdos1* and *Δdos2* was rescued by transformation with pREP2 plasmids carrying wild-type *dos1*$^+$ and *dos2*$^+$, respectively, verifying that disruption of these two genes was responsible for the phenotype.

*Δdos1Δdos2* double mutants were not lethal and had the same GFP-Swi6 phenotype as the single mutants (data not shown). In addition, overexpression of *dos1*$^+$ in *Δdos2* or of *dos2*$^+$ in *Δdos1* could not rescue the diffuse GFP-Swi6 phenotype (data not shown). These data are consistent with Dos1 and Dos2 acting in the same pathway with respect to localization of Swi6.

### *Δdos1* and *Δdos2* Are Defective in Mitotic and Meiotic Chromosome Segregation

Deletions of *dos1*$^+$ and *dos2*$^+$ did not cause a temperature-sensitive growth phenotype; however, significantly longer cells than the wild-type were frequently seen (data not shown). To determine whether the mu-

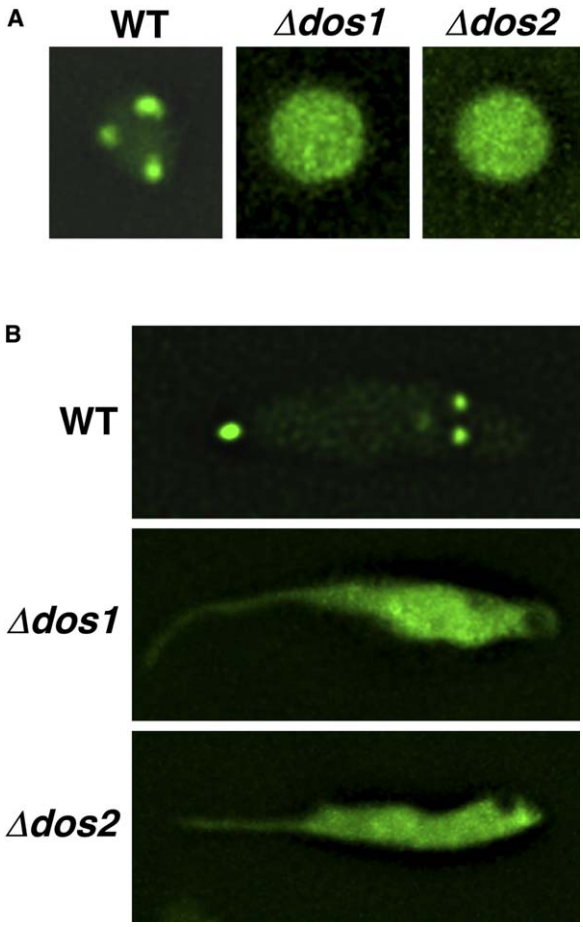


Figure 1. GFP-Swi6 Is Delocalized in *Δdos1* and *Δdos2* Mutants

(A) GFP-Swi6 forms three to four green foci in mitotic wild-type cells (YS106), reflecting localization at the telomeres and centromeres. This localization is lost in *Δdos1* and *Δdos2*.

(B) At the horsetail stage of meiosis, foci at the leading edge of wild-type nuclei reflect localization of GFP-Swi6 at the telomeres, whereas foci at the distal end of the nucleus reflect localization at the centromeres. In the mutants, GFP-Swi6 is delocalized during the horsetail stage.

tants had chromosome-segregation defects, serial dilutions of the *Δdos1* and *Δdos2* strains were plated on rich medium containing 10 μg/ml of the microtubule-destabilizing drug thiobendazole (TBZ). Both strains were hypersensitive to TBZ (see Figure S1B in the Supplemental Data available with this article online), indicating that chromosome segregation was disrupted. To directly examine the mitotic chromosome segregation, we visualized the spindle by using tubulin-GFP distribution, and we counterstained the cells with DAPI. Lagging chromosomes were rarely observed in the wild-type (1%), whereas 23% and 27% of cells in *Δdos1* and *Δdos2*, respectively, showed unsegregated lagging chromosomes (Figure 2A). In addition, the mutants often had multiple or fragmented nuclei. To determine whether the lagging chromosomes were due to the defects in the SPB, we examined the two null mutants by using the SPB markers, Sad1-CFP and Pcp1-GFP. Organization and behavior of the SPB were found to be normal in both *Δdos1* and *Δdos2* (data not shown).

Chromosome segregation during meiosis also was examined. Normal meiosis results in an ascus that contains four similar-sized spores. However, we found that 22% of *Δdos1* and 28% of *Δdos2* sporulated mutants contained fewer than four spores and that the spores varied in size and shape (see Table S1), suggesting that meiotic chromosome segregation was defective. Even in mutant asci that appeared to have normal spores, careful examination of the asci revealed that more than 20% contained an aberrant number of nuclei as indicated by diffuse GFP-Swi6 (Figure 2B) and DAPI (data not shown).

### Meiotic Telomere Clustering Is Disrupted in *Δdos1* and *Δdos2* Mutants

In wild-type cells at the meiotic horsetail stage, telomeres cluster together near the SPB at the leading edge of the nucleus as it becomes elongated. However, in heterochromatin mutants, such as in *rik1* and *clr4*, meiotic telomere clustering is disrupted. To examine meiotic telomere clustering in *Δdos1* and *Δdos2*, we crossed a line carrying the endogenous tagged Taz1-GFP, a marker for the telomere, into *Δdos1* and *Δdos2* mutants and examined Taz1-GFP localization in the mutants stained by DAPI. During the horsetail stage, a single Taz1-GFP spot was observed at the leading edge in wild-type cells. In *Δdos1* and *Δdos2* mutants, two separated Taz1-GFP spots were frequently observed, and some spots failed to localize to the leading edge (Figure 3A), indicating that meiotic telomere clustering was aberrant. The telomere-clustering defects in *Δdos1* appeared more severe than in *Δdos2*. Similar to the *rik1* mutant [12], we found that nuclei during the horsetail stage in the two mutants often appeared lumpy rather than smoothly elongated (Figure 1B). To determine whether the telomere-clustering defect was caused by dysfunction of SPB, we examined the SPB in horsetail nuclei by using Sad1-CFP. We found that the SPBs in *Δdos1* and *Δdos2* strains were always located close to the leading edge of horsetail (data not shown), the same as the wild-type, indicating that the SPB functioned normally during the horsetail stage. The failure to obtain normal telomere clustering suggests that normal telomere structure or function is required during meiosis for this process to occur.

### Mitotic Centromeric Cohesion Is Defective in *Δdos1* and *Δdos2* Mutants

Proper chromosome segregation depends on the interaction between spindle microtubules and the kinetochore. Accurate chromosome segregation also requires cohesin, which mediates sister-chromatid cohesion. Swi6 interacts with cohesin to preserve proper centromere function [28, 29]. In RNAi mutants, centromeric cohesion during mitosis is defective [26, 27, 33, 34]. To see whether the observed chromosome missegregation resulted, at least in part, from defects in centromeric cohesion, we crossed *Δdos1* and *Δdos2* with a strain that expressed LacI-GFP at the LacO array inserted at the *lys1* locus that is linked to centromere1 (*cen1*-GFP). In wild-type cells, a single GFP spot was usually observed

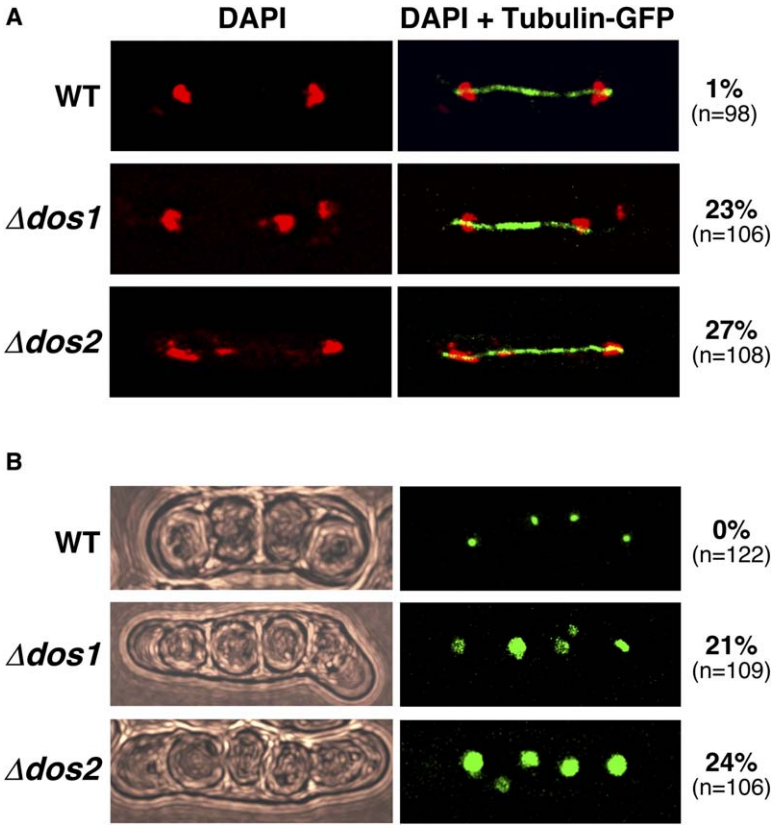


Figure 2. Mitotic and Meiotic Chromosome Segregations Are Impaired in the *Δdos1* and *Δdos2* Mutants

(A) Segregation of chromosomes during late mitotic anaphase in the wild-type, *Δdos1*, and *Δdos2* as visualized by DAPI staining (red) and tubulin-GFP (green). Values to the right of each panel indicate the proportion of cells displaying a segregation defect.

(B) Strains were sporulated on SPAS medium and subject to deconvolution light microscopy. The left panel shows asci sporulated from *Δdos1* and *Δdos2* strains. The right panel shows the aberrant number of nuclei in mutant asci, as visualized with GFP-Swi6. Values to the right of each panel indicates proportion of asci with four spores and aberrant nuclei number.

because fission yeast spends most of the cell cycle in the interphase, and the sister chromatids of chromosome I are paired at the centromere. In *Δdos1* and *Δdos2* mutants, more than 30% of the cells contained two GFP spots, indicating that centromeric cohesion was severely defective in interphase mutant cells (Figure 3B). The lagging chromosomes observed during anaphase (Figure 2A) could result either from problems

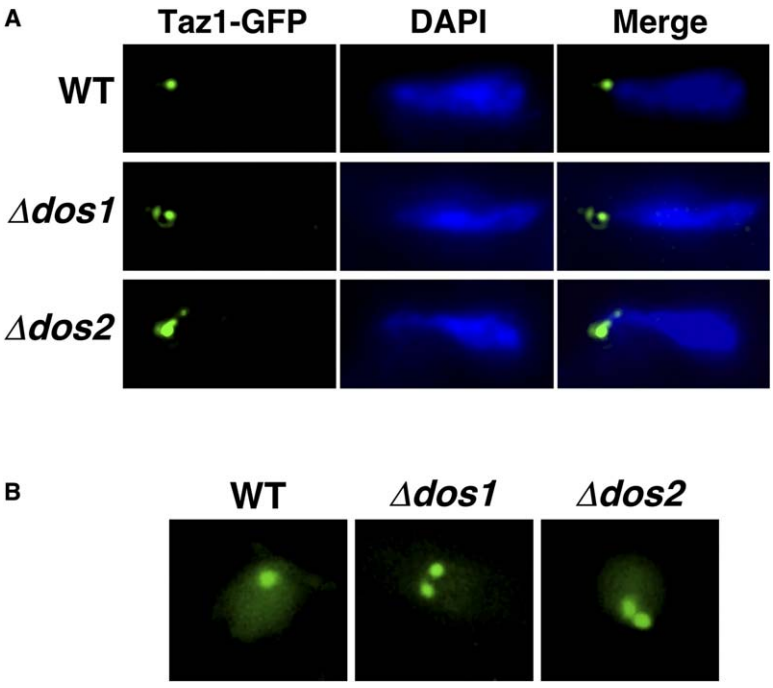


Figure 3. Meiotic Telomere Clustering and Mitotic Centromere Cohesion Are Disrupted in *Δdos1* and *Δdos2* Mutants

Telomeres were visualized by Taz1-GFP, and *cen1* was visualized with LacI-GFP expressed at the LacO array inserted at the *lys1* locus that is linked to centromere1 (*cen1*-GFP).

(A) In wild-type (WT), Taz1-GFP clusters into one spot at the leading edge of the horsetail. In *Δdos1* and *Δdos2,* two separated Taz1-GFP spots were seen, indicating defective telomere clustering. The horsetail nuclei were visualized by DAPI staining.

(B) *cen1*-GFP formed one spot in wild-type (WT) but two spots in more than 30% of the *Δdos1* and *Δdos2* mutant cells. This phenotype indicates that centromeric cohesion is defective in the mutant cells.

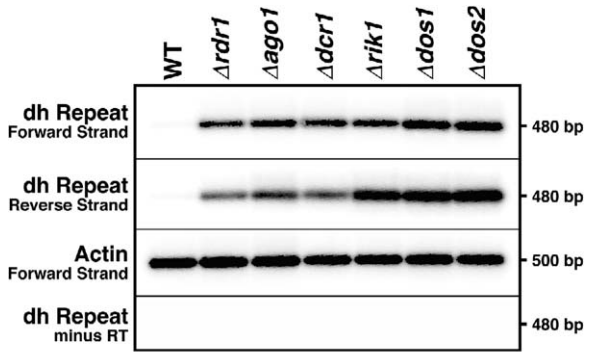


Figure 4. *Δdos1* and *Δdos2* Accumulate Heterochromatic Transcripts

Transcripts derived from the *dh* centromeric repeat were detected in total RNA from each strain with semiquantitative strand-specific PCR. Total RNA was prepared from DG21 (WT), DG124 (*Δrdr1*), ZB20 (*Δago1*), DG690 (*Δdcr1*), DG763 (*Δrik1*), FL70 (*Δdos1*), and FL71 (*Δdos2*). Actin was used as a positive control. RNA samples not subjected to first-strand cDNA synthesis were also amplified with the *dh* primers as a negative control (minus reverse transcriptase).

in sister-chromosome cohesion or defects in the kinetochore.

## Dos1 and Dos2 Are Required for Heterochromatic Silencing

To examine centromeric silencing in *Δdos1* and *Δdos2* mutants, strains with an *ade6*$^+$ reporter gene integrated in the pericentromeric outer repeat (*otr*) were crossed with each mutant strain [35]. Growth on media lacking adenine was substantially enhanced relative to the wild-type, indicating that the *otr::ade6*$^+$ gene loses silencing in the absence of Dos1 or Dos2 (see Figure S2A). To examine the effect of *dos1* and *dos2* deletions on silencing at the mating-type region, we used a strain with a *ura4*$^+$ reporter gene inserted in the mating-type region [36]. Wild-type strains grow well on counterselective FOA media (5 fluoro-orotic acid, toxic to *ura4*$^+$ cells), whereas growth of *Δdos1* and *Δdos2* strains was inhibited on FOA (see Figure S2B). Similar effects on telomeric silencing were observed with *his3*$^+$ and *ura4*$^+$ genes integrated adjacent to telomere TEL1L and TEL2L regions [37]. These wild-type strains are not able to grow on minimal media because the *his3*$^+$ and *ura4*$^+$ genes are normally silenced; however, *Δdos1* and *Δdos2* mutant strains grew well, indicating that the silencing at telomeres was also defective (see Figure S2A).

The pericentromeric repeats themselves are also transcribed in fission yeast, and accumulation of the transcripts is regulated in a strand-specific manner. The "reverse" strand is always transcribed in wild-type cells, but transcripts are not readily detected because they are rapidly processed by RNAi. On the other hand, the "forward" strand is transcriptionally silenced via Swi6 in a process that also depends on the RNAi machinery [16]. As a result, transcripts from both strands are detected in RNAi mutants [16]. We therefore tested

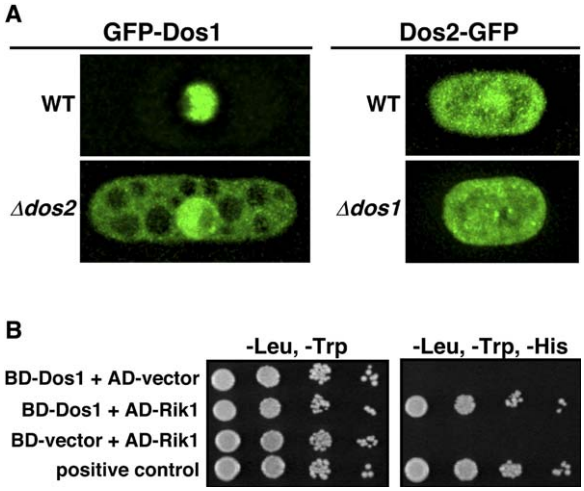


Figure 5. Dos1 Is Localized Preferentially to the Nucleus via Dos2 and Interacts Directly with Rik1

(A) Cells expressing GFP-Dos1 (left panel) and Dos2-GFP (right panel) under the *nmt* inducible promoter were grown on minimal EMM medium for 24 hr at 30°C. More than 20 cells from each genotype were analyzed. GFP-Dos1 was delocalized from the nucleus in the *Δdos2* mutant and diffuse in the cytoplasm but not in vacuoles. The average ratio of GFP-Dos1 fluorescence intensity between the nucleus and cytoplasm was 2.8:1 in the wild-type but only 1.8:1 in the *Δdos2* mutant (FL149). However, the pattern of Dos2-GFP in *Δdos1* strains (FL150) was not affected.

(B) Yeast two-hybrid assay. A positive direct interaction between Dos1 and Rik1 is indicated by growth on media lacking leucine, tryptophan, and histidine (-Leu, -Trp, -His). TA-BLR and DB-STM were used as a positive control [47]. BD denotes GAL4 binding domain fusion, and AD denotes GAL4 activation domain fusion.

whether *Δdos1* and *Δdos2* mutants accumulate transcripts from the pericentromeric repeats by strand-specific RT-PCR. In wild-type cells, centromeric transcripts are hardly detectable, whereas transcripts from both the forward and reverse strands accumulated to high levels in *Δdos1* and *Δdos2*, indicating that silencing at the centromere was abolished in the mutants (Figure 4). Neither *Δdos1Δdcr1* nor *Δdos2Δdcr1* strains displayed any synthetic lethality, and both had the same diffuse GFP-Swi6 phenotype as *Δdos1* or *Δdos2* alone (data not shown).

## Dos2 Is Required for Nuclear Localization of Dos1, which Interacts with Rik1

To examine the localization of Dos1 in wild-type cells, we tagged the *dos1*$^+$ gene with GFP at the N terminus (GFP-Dos1) by homologous recombination at the endogenous locus. Exponentially growing cells were examined by fluorescence microscopy. GFP-Dos1 was concentrated in the nucleus; however, the signal was weak. To confirm this, we placed an in-frame fusion of GFP and Dos1 under a thiamine-inducible *nmt* promoter on a plasmid vector and transformed it into a *Δdos1* strain. The *Δdos1* mutant phenotype (GFP-Swi6 delocalization and TBZ sensitivity) was rescued by thiamine induction, demonstrating that the GFP-Dos1 fusion protein was functional (see Figure S1). As expected, the GFP-Dos1 signal in the wild-type appeared

predominantly nuclear at all stages of the cell cycle (Figure 5A). Endogenous *dos2*$^+$ under its own promoter was also fused to GFP at its C terminus (Dos2-GFP) by homologous recombination. The Dos2-GFP signal was too weak to be observed, presumably due to low expression. Following a similar approach to that for GFP-Dos1, overexpression of Dos2-GFP with an inducible construct indicated that Dos2-GFP was localized in both the nucleus and cytoplasm (Figure 5A). This fusion construct was also able to rescue the *Δdos2* mutant phenotype, demonstrating that it forms a functional protein (see Figure S1).

Because Dos2-GFP was found in both the nucleus and cytoplasm, whereas GFP-Dos1 was primarily nuclear, we next examined the possibility that Dos2 may be involved in localizing Dos1 to the nucleus. To test this, we expressed GFP-Dos1 and Dos2-GFP in *Δdos2* and *Δdos1* mutants, respectively, by using the inducible vectors described above. GFP-Dos1 localization was first examined in both the wild-type and *Δdos2* background, whereby more than 20 cells for each genotype were analyzed. The average ratio of GFP-Dos1 fluorescence intensity between the nucleus and cytoplasm was 2.8:1 in the wild-type, but only 1.8:1 in the *Δdos2* mutant, indicating that GFP-Dos1 was delocalized from the nucleus in the *Δdos2* mutant (Figure 5A). In contrast, the pattern of Dos2-GFP in the *Δdos1* background was the same as that in the wild-type. These results are consistent with a pathway in which Dos2 regulates the nuclear localization of Dos1.

To determine whether Dos1 and Dos2 are involved in protein-protein interactions, we performed a directed yeast two-hybrid assay with each gene fused to the GAL4 DNA binding domain and a panel of strains carrying the GAL4 activation domain fused to Dos1, Dos2, Ago1, Rdr1, Dcr1, Rik1, Swi6, and Clr4. Dos2 failed to interact with any of these proteins; however, Dos1 displayed a strong direct interaction with Rik1 (Figure 5B).

### Dos1 and Dos2 Are Required for Heterochromatic Histone Modification and RNA Interference

To determine the nature of the silencing defect in *Δdos1* and *Δdos2*, we examined the association of modified histone H3 with centromeric chromatin in each of the mutant strains by using chromatin immunoprecipitation (ChIP). H3K9me was completely lost from centromeric repeats in both *Δdos1* and *Δdos2*, whereas histone H3K4me was increased compared to that in the wild-type (Figure 6). The loss of H3K9me observed in *Δdos1* and *Δdos2* was comparable to that observed in *Δrik1* (Figure 6) and *Δclr4*, and these losses are more severe than in RNAi mutants [14]. These results indicate that Dos1 and Dos2 are required for heterochromatic histone modification to a similar extent as Rik1 and Clr4.

In order to determine the role, if any, of Dos1 and Dos2 in RNAi, the accumulation of small interfering RNAs (siRNAs) derived from the centromeric repeats was examined by Northern blots. siRNAs between 22 and 26 nucleotides were readily detected in wild-type cells with probes corresponding to regions matching sequenced siRNAs [17]. As expected, these siRNA were absent in the RNAi mutants *Δrdr1*, *Δago1*, and *Δdcr1*, indicating the siRNAs were products of the RNAi machinery. These siRNAs were also lost in *Δdos1* and *Δdos2*, suggesting that the RNAi machinery was defective in these mutants (Figure 7). Interestingly, a similar loss of siRNAs was also observed in *Δrik1*. These results demonstrate that Dos1 and Dos2 are required for both RNAi processing and heterochromatin formation at centromeric repeats, in a similar manner to Rik1 and Clr4.

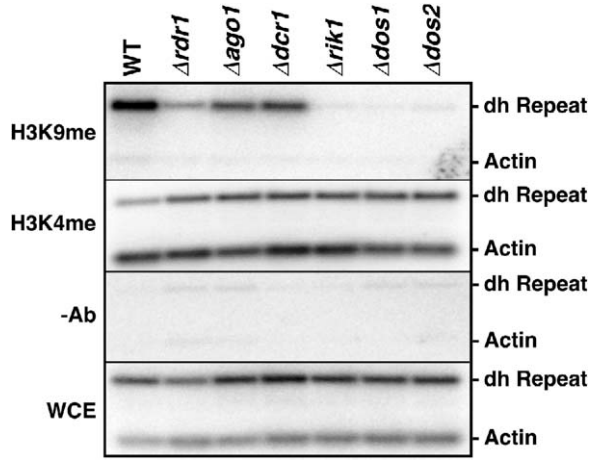


Figure 6. Dos1 and Dos2 Regulate Heterochromatic Histone Modifications

ChIP was carried out with antibodies against H3K4me2 and H3K9me2 or by mock immunoprecipitation (-Ab). DNA from whole-cell extracts (WCE) was diluted 1:50. The same strains were analyzed as described in Figure 4. Both the centromeric-*dh*-repeat fragment and actin control were amplified in each sample with multiplex PCR.

## Discussion

We have found two novel silencing genes, *dos1*$^+$ and *dos2*$^+$, which regulate RNAi-mediated heterochromatin formation. They arose in a genetic screen to identify genes that are required for chromatin function and integrity during meiosis. The screen was novel in that it was based on observing morphological changes in heterochromatin rather than function. The *Δdos1* and *Δdos2* mutants have diffuse GFP-Swi6 phenotypes and disrupted chromosome dynamics during mitosis and meiosis. They are required for the silencing of all of the heterochromatic regions. Our data also indicate that Dos1 directly interacts with Rik1, which is in turn found in a complex with Clr4.

As with RNAi and *clr4* and *rik1* mutants, disruption of *dos1*$^+$ and *dos2*$^+$ causes chromosome missegregation. Cohesin, which is required for sister-chromatid cohesion and chromosome segregation, is enriched in both heterochromatic regions and the arms of chromatids. Swi6 interacts with cohesins to recruit them to heterochromatic domains [28, 29]. In *Δswi6* strains, cohesin binding at centromeric repeats is disrupted. Our direct observation of Swi6 delocalization in *Δdos1* and *Δdos2* suggests that the chromosome-segregation defects resulted, at least in part, from defects in centromeric cohesion, as well as a failure to properly attach microtu-

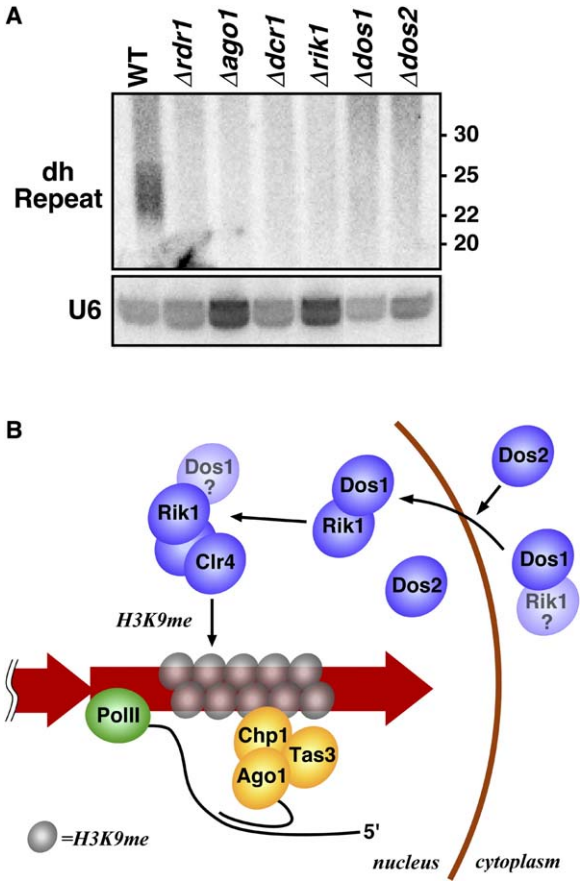


Figure 7. Centromeric Small RNAs Are Lost in *Δdos1* and *Δdos2*

(A) Total small RNAs were extracted from the strains described in Figure 4. Small RNAs spanning 22 to 26 nucleotides corresponding to the centromeric *dh* repeat were readily detected in the wild-type. These small RNAs were lost in the RNAi mutants (*Δrdr1*, *Δago1*, and *Δdcr1*), as well as in *Δrik1*, *Δdos1*, and *Δdos2*. The blots were reprobed for U6 snRNA as a loading control.

(B) Dos2 is required for nuclear localization of Dos1, which interacts with Rik1. Rik1 associates with Clr4 in a complex that mediates H3K9me in heterochromatin formation. Dos1 may regulate localization of Rik1 or formation/activity of the Rik1/Clr4 complex. Centromeric repeat DNA is shown as a red arrow, and gray circles represent histone H3 methylated at lysine 9 (H3K9me).

bules to the kinetochores, a failure due to either centromere-clustering defects and mislocalization or kinetochore malfunction.

Another important chromatin structure, the telomere, is not only essential for chromosome maintenance and faithful segregation but also facilitates homologous-chromosome pairing and recombination by forming a telomere cluster during meiosis. However, the mechanism for regulating telomere clustering is largely unknown. Four *S. pombe* telomere-clustering mutants, *dot1–dot4*, have previously been reported [31]. Recently, Clr4 and Rik1 have also been shown to be required for telomere clustering in a Swi6-independent manner [12]. On the other hand, RNAi mutants only have a mild telomere-clustering defect [26, 27]. Our data showed that disruption of *dos1*$^+$ and *dos2*$^+$, similar to that of *rik1*$^+$, causes severe defects in telomere clustering, indicating that they also play an important role in meiosis.

Similar to that silencing previously observed for *rik1* and *clr4* mutants [25], silencing of marker genes inserted within both the centromere and the mating-type locus was alleviated in *Δdos1* and *Δdos2*. RNAi single mutants typically show loss of only centromeric silencing; however, it is known that the RNAi machinery acts redundantly with the CREB-family proteins Atf1 and Pcr1 to also mediate heterochromatin formation at the mating-type locus [20, 21, 38]. The fact that silencing of marker genes inserted within all heterochromatic regions was alleviated in *Δdos1* and *Δdos2* mutants demonstrates the requirement for Dos1 and Dos2 in both RNAi- and CREB-mediated heterochromatic silencing, likely through their interactions with Rik1.

Analysis of transcripts derived from the centromeric repeats revealed a strong loss of heterochromatic silencing in both the *Δdos1* and *Δdos2* mutants and similar to that in *Δrik1*. Moreover, small RNAs corresponding to these repeats were also lost in *Δdos1*, *Δdos2*, and *Δrik1*, demonstrating that RNAi processing of the heterochromatic transcripts was disrupted. Rik1 is a central player in heterochromatic silencing in fission yeast. As with many of the proteins involved in RNAi and heterochromatic silencing, Rik1 has homologs in animals and plants but not in budding yeast. The C-terminal WD-propeller-repeat domain of Rik1 is shared by both the DNA damage binding protein DDB1 and the RNA binding protein CPSF, both of which are associated with a smaller subunit [13]. In the case of DDB1, the DDB2 subunit is thought to translocate DDB1 from the cytoplasm into the nucleus [39]. The fission-yeast homolog of DDB1 is not essential for viability, but *ddb1* deletion strains have pleiotropic nuclear defects including lagging chromosomes at anaphase and TBZ sensitivity, similar to *rik1* [40, 41]. In this study, Dos1 was found to directly interact with Rik1. One intriguing possibility is that this interaction resembles the association of DDB1 with DDB2. Although DDB2 is conserved in mammals and plants, homologs are absent in fission yeast. However, like DDB2, Dos1 encodes a WD-repeat protein. By analogy with DDB2, Dos1 may be required for Rik1 activity. The fact that *Δdos1* mutants resemble *Δrik1* mutants in all respects is consistent with this idea. *Δdos2* mutants also resemble *Δrik1*. We have found that Dos2 is required to localize Dos1, indicating that localization may in Rik1 function play a regulatory role comparable with the role that DDB2 plays in humans (Figure 7B). It is likely, however, that additional factors may also be involved in regulating Dos1 nuclear localization, as suggested by the fact that not all Dos1 localizes to the cytoplasm in *Δdos2* and that Dos1 did not directly interact with Dos2 in the yeast two-hybrid assay.

Although Dos1 and Dos2 have weak similarity to WD-repeat proteins and zinc finger proteins, respectively, clear homologs are not found in other organisms. As described above, these proteins may play a role in Rik1 function, a role that would normally be provided by proteins not found in fission yeast (such as DDB2). We have found that Dos1 and Dos2 also resemble Rik1 and Clr4 in that they are required for H3K9 dimethylation. Rik1 is known to associate with Clr4 in a complex [14],

although the properties of this complex have not been defined. During heterochromatin formation, it is possible that Rik1 recognizes RNA through its similarity with CPSF [24] and recruits Clr4 activity, explaining the requirement of each of these proteins for small interfering RNA. The observed interaction between Dos1 and Rik1 could indicate that Dos1 itself is also a component of the Rik1/Clr4 complex and thus directly influences Clr4 activity.

Localization of Swi6 to heterochromatin is mediated by H3K9me [15, 42], which depends upon Clr4 [10]. The association of the RITS and RDRC complexes with each other and with centromeric transcripts and siRNA also requires Clr4 [24, 43]. Our data demonstrate that Dos1 and Dos2 are required for heterochromatin formation and suggest a role for Dos1 and Dos2 in the formation/activity of the Rik1/Clr4 complex that mediates H3K9me (Figure 7B). Histone H3K9 methylation not only results in recruitment of Swi6 but also stabilizes RITS binding to the chromosome through the chromodomain of Chp1, which specifically binds to H3K9me [44]. The RITS complex may then recruit, or possibly prime, RDRC to synthesize dsRNA, which subsequently is processed by Dicer [24].

## Experimental Procedures

### Strains, Genetic Procedures, and Media

*Schizosaccharomyces pombe* strains used in this study are listed in Table S1. Standard media and genetic protocols for fission yeast were used [45], and yeast two-hybrid assays were performed as directed by the manufacturer (Clontech). Yeast extract with supplements (YES) was used as a complete culture unless otherwise mentioned, SPAS or MSA for conjugation and sporulation, and EMM2 as a minimum media. The mutant screen and gene cloning will be described elsewhere (X.T. and W.Z.C., unpublished data). Simply, $h^{90}$ wild-type cells bearing GFP-Swi6 were mutagenized by random insertion of a *ura4*$^+$ gene. Colonies isolated from the EMM2 media without uracil were visually examined by fluorescence microscopy. Competitive PCR was employed with a random primer and a primer complementary to *ura4*$^+$ in order to clone the *dos*$^+$ genes. The PCR products were sequenced. With the partial sequences obtained from the sequencing, the whole sequences of *dos1*$^+$ and *dos2*$^+$ were determined by searching GeneDB. *dos1*$^+$ and *dos2*$^+$ were deleted with a PCR-based gene-disruption method described previously [32].

### GFP Fusion Proteins

A DNA fragment covering the *dos1*$^+$-ORF was amplified by PCR and inserted at the SalI-BamHI site of a pREP2 plasmid. A GFP-tag from a plasmid pFA6a-GFP-kanMX6 was amplified by PCR and inserted at the SalI site in the pREP2 plasmids containing *dos1*$^+$ gene to make pGFP-dos1. Similarly, the *dos2*$^+$ ORF was amplified and inserted at the BamHI site, whereas a GFP fragment was placed at the SmaI site of the pREP2 plasmid to make pGFP-dos2. pGFP-dos1 and pGFP-dos2 were transformed into wild-type cells to make strains overexpressing GFP-Dos1 or Dos2-GFP.

A pBluescript plasmid carrying an intact *ura4*$^+$ gene and its promoter at HindIII site was used to tag *dos*$^+$ genes under their native promoter at their endogenous sites. About 600 bp of genomic sequences flanking both sides of the *dos1*$^+$ and *dos2*$^+$ genes were amplified by PCR. The PCR products from upstream of *dos*$^+$ genes were inserted at the BamHI site of pBluescript, and the downstream fragments were inserted at SalI sites. *dos*$^+$ genes fused with GFP were amplified from pGFP-dos1 and pGFP-dos2, respectively, and each PCR product was inserted at the PstI site of pBluescript vectors carrying their flanking sequences. The ~5 kb fragments containing *dos*$^+$ genes, tags, and their flanking sequences were cut by NruI and transformed into homothallic cells. Transformants were isolated from the EMM2 media without uracil. Their endogenous locations were verified by PCR.

### Microscopy

Samples were analyzed by a fluorescence microscope (Olympus BX51) or a Delta Vision System (Applied Precision, Issaquah, WA) with an Olympus oil immersion objective lense (Uplan Apo 100x/NA 1.35). For Delta Vision acquisition, stacks of Z-axis sections were subjected to reiterative deconvolution, and final projections were processed with SoftWoRX2.50 (Applied Precision).

### Molecular Analyses

Total RNA was extracted from cells growing at exponential phase in yeast extract adenine (YEA) medium and treated with DNase with the RiboPure-Yeast Kit (Ambion). RT-PCR was carried out with 100 ng of total RNA in 25 μl reactions with a one-step RT-PCR kit (QIAGEN). Small RNA was prepared from cells growing at exponential phase in YEA medium with the mirVana miRNA Isolation Kit (Ambion), and Northern blots were performed as described [46]. Chromatin immunoprecipitation (ChIP) was performed as described ([16]) with anti-dimethyl H3 Lys-9 polyclonal (07-441) and anti-dimethyl H3 Lys-4 polyclonal (07-030) antibodies (Upstate Biotechnology). For RT-PCR and ChIP, a region of the centromeric *dh* repeat was amplified with the primers *dh*A_F (5′-AGGGTGCAAAGCAGGTAGAGA-3′) and *dh*A_R (5′-CCTCAGCAGTCCTTGGGAAATG-3′). Actin controls were amplified in the RT-PCR experiments with the primers ActA_F (5′-TACCCCATTGAGCACGGTAT-3′) and ActA_R (5′-GGAGGAAGATTGAGCAGCAG-3′), and in the ChIP experiments with the primers ActB_F (5′-ACTACCGCCGAACGTGAAAT-3′) and ActB_R (5′-CCTCATGAATACCGGCGTTT-3′). For the RT-PCR and ChIP experiments, PCR reactions were carried out with a minimal number of cycles, separated on 2% agarose gels and transferred to a charged nylon membrane (Hybond-N+, Amersham). $^{32}$P-labeled riboprobes were prepared with the T3/T7 MAXIscript kit (Ambion), and blots were imaged with a Fuji Phosphoimager (Fuji). RT-PCR, ChIP, and small RNA blots were hybridized with the *dh*A probe generated with the primers described above. As loading controls, RT-PCR and ChIP blots were also hybridized with the ActA probe, whereas small RNA blots were hybridized with an oligonucleotide complimentary to the *S. pombe* U6 snRNA (5′-ATGTCGCAGTGTCATCCTTG-3′) that was labeled with $^{32}$P via polynucleotide kinase (NEB) according to the manufacturer's instructions.

### Supplemental Data

Supplemental Data include two figures and two tables and are available with this article online at: http://www.current-biology.com/cgi/content/full/15/16/1448/DC1/.

### Acknowledgements

We thank Robin Allshire, Thomas Pollard, and Yoshinori Watanabe for providing strains; members of the Martienssen and Cande laboratories, particularly Ye Jin, for helpful discussions; and Meredith Sagolla for help with the microscopy. D.G. is a DOE-Energy Biosciences Research Fellow of the Life Sciences Research Foundation, and M.Z. was supported by a fellowship from the Spanish Ministry of Education. This research was supported by grants from the National Institutes of Health to R.M. (R01-GM067014) and Z.C. (R01-GM067992).

Received: May 30, 2005
Revised: June 30, 2005
Accepted: July 1, 2005
Published online: July 21, 2005

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