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Isw1 acts independently of the Isw1a and Isw1b complexes in regulating transcriptional silencing at the ribosomal DNA locus in *Saccharomyces cerevisiae*

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Summary

Transcriptional silencing of Pol II-transcribed genes in *Saccharomyces cerevisiae* occurs at the *HM* loci, telomeres and ribosomal DNA (rDNA) locus. Gene silencing at these loci requires histone-modifying enzymes as well as factors that regulate local chromatin structure. Previous work has shown that the ATP-dependent chromatin remodeling protein Isw1 is required for silencing of a marker gene inserted at the *HMR* locus, but not at telomeres. Here we show that Isw1 is required for transcriptional silencing of Pol II-transcribed genes in the ribosomal DNA locus. Our results indicate that Isw1 associates with the rDNA and that this interaction is not altered in cells lacking other members of the Isw1a and Isw1b chromatin remodeling complexes. Further, the association of Isw1 with the rDNA is not altered in cells lacking the histone deacetylase Sir2 or the histone methyltransferase Set1, two factors that are required for gene silencing at the rDNA. Notably, the loss of transcriptional silencing at the rDNA in cells lacking Isw1 is correlated with a change in rDNA chromatin structure. Together, our data support a model in which Isw1 acts independently of the previously characterized Isw1a and Isw1b complexes to maintain a heterochromatin-like structure at the rDNA that is required for gene silencing.

Keywords

Isw1; gene silencing; ribosomal DNA; transcription; recombination

The maintenance of heterochromatic domains in eukaryotic cells is vital to appropriate gene expression and genomic stability. In the budding yeast *Saccharomyces cerevisiae*, heterochromatin-like domains are present at the homothallic mating (*HM*) loci, telomeres and the ribosomal DNA (rDNA) locus. Genes transcribed by RNA polymerase II (Pol II) that are present at these loci are silenced at the level of transcription^{1; 2}.

The ribosomal DNA locus is located on chromosome *XII* and comprises 150 to 200 copies of the ribosomal RNA genes. Each rDNA repeat consists of the RNA polymerase I-transcribed 35S rRNA gene and a non-transcribed spacer (NTS). The NTS is divided into NTS1 and NTS2 by the RNA polymerase III-transcribed 5S rRNA gene (Figure 1a). When Ty1 elements or other Pol II-transcribed genes are inserted into the rDNA, they become silenced at the level of

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transcription^{3; 4; 5}. Recently, Pol II transcription units were identified in the rDNA NTS. These transcription units are silenced in a manner similar to other Pol II-transcribed marker genes in the rDNA^{6; 7}.

Histone-modifying enzymes, including proteins that are required for histone ubiquitylation, methylation and deacetylation, play critical roles in Pol II transcriptional silencing^{8; 9}. In *S. cerevisiae*, methylation of histone H3 on lysine 4 by Set1 is required for transcriptional silencing at the rDNA and telomeres^{10; 11; 12; 13; 14; 15}. In addition, the NAD-dependent histone deacetylase Sir2 is required for silencing by maintaining low levels of acetylated histones H3 and H4 at the *HM* loci, telomeres and rDNA^{3; 4; 5; 7; 10; 16; 17; 18}. Recently, it was shown that the deacetylase activity of Sir2 reduces the association of Pol II with the rDNA, and thereby limits the level of K4 methylated histone H3 at the rDNA⁷.

Factors involved in DNA topology and chromatin remodeling modulate transcriptional silencing in *S. cerevisiae*^{3; 19; 20; 21; 22}. The Swi/Snf chromatin remodeling complex is required for silencing of Pol II-transcribed genes at the rDNA and telomeres, but not the *HM* loci²¹. The expression of Pol II marker genes located in the rDNA increases significantly in cells that lack Snf2 and Snf5, two members of the Swi/Snf complex. Notably, in *snf2Δ* cells, recombination is decreased between the rDNA repeats. These data suggest that loss of the Swi/Snf remodeling complex results in altered chromatin at the rDNA that is more open to the Pol II transcription machinery, but less accessible to recombination factors²¹.

The Imitation Switch or ISWI class of ATP-dependent chromatin remodeling complexes is distinct from the family of Swi/Snf complexes. Members of these two classes differ in their characteristic chromatin binding motifs, nucleosome remodeling activities and biological function^{23; 24}. In *S. cerevisiae*, there are two members of the ISWI class of chromatin remodelers, Isw1 and Isw2. Isw2 contributes largely to gene repression in *S. cerevisiae*. It is a negative regulator of early meiotic genes during vegetative growth and α -specific genes in *MAT α* strains^{25; 26}. Isw1 has been implicated in both gene repression and expression²⁷. Isw1 has been shown to exist as a monomer or as a member of two different complexes *in vivo*²⁸. The Isw1a complex is composed of Isw1 and Ioc3, while the Isw1b complex consists of Isw1, Ioc2, and Ioc4. Unlike an Isw1 monomer, both the Isw1a and Isw1b complexes exhibit nucleosome-dependent ATPase activity and can reposition nucleosomes *in vitro*, suggesting that Isw1 requires additional factors to function *in vivo*^{28; 29}. Expression profile analyses suggest that both the Isw1a and Isw1b complexes contribute to gene activation, as well as gene repression²⁸. Studies measuring the expression of the inducible genes *PHO8* and *MET16* in *S. cerevisiae* showed that the Isw1a complex functions in gene repression by positioning nucleosomes at promoter sequences thereby preventing transcription, while the Isw1b complex promotes transcription elongation and termination by interacting with coding sequences^{30; 31; 32; 33}.

Little is known about the contribution of Isw1 to silent chromatin in *S. cerevisiae*. Unlike Swi/Snf, Isw1 is required for silencing of Pol II marker genes inserted at the *HMR* locus, but not at telomeres³⁴. In this study, we show that Isw1 associates with the rDNA and regulates silencing of Pol II-transcribed genes in the rDNA. The role of Isw1 at the rDNA is independent of the previously identified Isw1a and Isw1b complexes. We demonstrate that the loss of rDNA silencing in cells lacking Isw1 is associated with changes in chromatin structure in the NTS1 region of the rDNA. Our data support a direct role for Isw1 in the maintenance of silent chromatin at the rDNA and raise the possibility that Isw1 acts as a member of a novel Isw1 chromatin-remodeling complex to regulate gene silencing at the rDNA.

A requirement for Isw1 in rDNA silencing

To examine the requirements for Isw1a and Isw1b complexes in transcriptional silencing at the rDNA, we isolated and measured steady-state RNA from a Ty1*his3AI* element located in NTS1 of the rDNA (Figure 1a) from wild-type cells and deletion mutants lacking individual members of the Isw1a or Isw1b complexes. The Ty1*his3AI* marker is transcribed by Pol II, and when present at the rDNA, it is silenced at the level of transcription in wild-type cells³. Ty1*his3AI* mRNA was detected by hybridization with a radiolabeled probe specific for Ty1*his3AI* transcripts (Figure 1b, upper panel). In wild-type cells, we observed low levels of Ty1*his3AI* mRNA, characteristic of transcriptional silencing at the rDNA (Figure 1b, upper panel, WT). However, in cells lacking *ISW1*, the amount of Ty1*his3AI* mRNA was 4.7-fold greater than that observed in wild-type cells (Figure 1b, upper panel, *isw1Δ*), indicating a requirement for Isw1 in transcriptional silencing at the rDNA. This is consistent with unpublished work from our lab, where Isw1 was identified in a genetic screen for factors that are required for rDNA silencing (M. Elfline, personal communication). As a control, we measured steady-state Ty1*his3AI* mRNA levels in cells lacking Set1, a histone methyltransferase that is required for transcriptional silencing at the rDNA^{10; 11; 14; 15}. Ty1*his3AI* mRNA levels were 6.5-fold more abundant in *set1Δ* cells relative to wild-type cells (Figure 1b, upper panel, *set1Δ*). These data demonstrate that the increase in Pol II transcription at the rDNA observed in *isw1Δ* cells is comparable to the increase observed in other rDNA silencing mutants.

The levels of Ty1*his3AI* mRNA were not increased in cells lacking other members of Isw1a (Figure 1b, upper panel, *ioc3Δ*) or Isw1b (Figure 1b, upper panel, *ioc2Δ* and *ioc4Δ*). Thus, while Isw1 is required for transcriptional silencing at the rDNA, other members of the Isw1a and Isw1b complexes are not required. Interestingly, we found that Ty1*his3AI* transcript levels were consistently lower in *ioc2Δ* cells and *ioc4Δ* cells relative to wild-type cells, indicating an increase in silencing at the rDNA in the absence of the Isw1b complex.

There are approximately 30 endogenous Ty1 retrotransposons dispersed throughout the *S. cerevisiae* genome³⁵. We measured the level of mRNA from these genomic Ty1 elements in wild-type cells and deletion mutants to verify that the differences observed in the level of Ty1*his3AI* transcript in the deletion mutants were specific to gene silencing at the rDNA and not due to genome-wide changes in Ty1 element transcription. The level of total Ty1 transcript in the cells lacking *ISW1* (Figure 1b, middle panel, *isw1Δ*) was similar to the level in wild-type cells (Figure 1b, middle panel, WT). This finding is consistent with previous studies that showed that deletion of *ISW1* does not affect transcription of Ty1 elements³⁶, and confirms that Isw1 plays a specific role in transcriptional silencing at the rDNA. The levels of total Ty1 transcript in the *IOC* gene deletion mutants were also similar to those in wild-type cells (Figure 1b, middle panel).

The results from the Northern analysis of the single deletion mutants (Figure 1b) do not eliminate the possibility that the Isw1a and Isw1b complexes act redundantly in transcriptional silencing at the rDNA. If this is the case, then silencing at the rDNA will be maintained in cells lacking a single *IOC* gene, *IOC3*, *IOC2*, or *IOC4*, as we observed in Figure 1b. To test whether the Isw1a and Isw1b complexes are redundant in their roles in rDNA silencing, we constructed individual strains that lack genes encoding members of both complexes. In one strain, *IOC3* and *IOC2* were deleted (Figure 1c, *ioc3Δ ioc2Δ*) and in a second strain, *IOC3* and *IOC4* were deleted (Figure 1c, *ioc3Δ ioc4Δ*). Previous studies have shown that the Isw1a complex does not form in cells lacking Ioc3 and the Isw1b complex does not form in cells lacking Ioc2 or Ioc4²⁸. Northern analysis revealed that the level of Ty1*his3AI* mRNA was similar in the *ioc3Δ ioc2Δ* and *ioc3Δ ioc4Δ* double mutants and wild-type cells (Figure 1c, upper panel). Interestingly, the reduced expression of Ty1*his3AI* observed in the single *ioc2Δ* and *ioc4Δ* cells

(Figure 1b) was restored to wild-type levels in the double mutants. While we did observe that total Ty1 transcript levels in *ioc3Δ ioc2Δ* and *ioc3Δ ioc4Δ* double mutants were slightly increased relative to levels in wild-type cells (Figure 1c, middle panel), transcript levels from Ty1*his3AI* in the rDNA were not affected in the double-deletion cells (Figure 1c, upper panel). These data suggest that the Isw1a and Isw1b complexes do not function redundantly at the rDNA and that Isw1 acts independently of these two complexes to regulate transcriptional silencing at the rDNA. Consistent with these findings, there is evidence that Isw1 acts independently of the Isw1a complex in silencing at the *HMR* locus. Specifically, cells lacking Isw1 exhibit a severe loss of silencing phenotype at a genetically marked *HMR* locus, while cells lacking Ioc3 are much less defective in silencing at that locus³⁴.

To confirm our Northern assays, we examined the role of Isw1a and Isw1b complex members in silencing of a *URA3* marker gene located in the 25S coding region of the 35S rRNA gene (Figure 1a) using a plate growth assay (Figure 1d). Wild-type, *isw1Δ*, *ioc3Δ*, *ioc2Δ*, *ioc4Δ* and *set1Δ* cells with an *mURA3* marker gene in the rDNA were assayed for growth on media containing 5-fluoroorotic acid (5-FOA)⁴. The gene product of the *mURA3* gene, orotidine-5'-phosphate decarboxylase, converts 5-FOA to 5-fluorouracil, which is toxic to cells. Wild-type cells, in which the *mURA3* gene is silenced, grew relatively well on media containing 5-FOA (Figure 1d, rDNA, SC + 5-FOA). Conversely, *isw1Δ* cells grew approximately 25-fold less than wild-type cells on media containing 5-FOA, indicating increased expression of the *mURA3* gene in the *isw1Δ* cells (Figure 1d, rDNA, SC + 5-FOA). As a control, we examined the growth of *set1Δ* cells on media containing 5-FOA. *set1Δ* cells grew approximately 10-fold less than wild-type cells reflecting the requirement for Set1 in transcriptional silencing at the rDNA (Figure 1d, rDNA, SC + 5-FOA). Cells lacking *IOC3*, *IOC2* or *IOC4* grew like the wild-type cells on media containing 5-FOA (Figure 1d, rDNA, SC + 5-FOA). Growth of wild-type, *set1Δ*, *isw1Δ*, *ioc3Δ*, *ioc2Δ* and *ioc4Δ* cells with an *mURA3* marker gene located outside the rDNA locus was similar on media lacking uracil (Figure 1d, *leu2Δ1*, SC - URA), indicating that Isw1 does not affect *mURA3* expression at a euchromatic locus, but specifically regulates silencing of the *mURA3* gene when inserted in the rDNA. These data confirm the results of the Northern studies using Ty1*his3AI* (Figures 1b and 1c) that Isw1, and not Ioc3, Ioc2, or Ioc4, is required for rDNA silencing.

While we identified a requirement for Isw1 in silencing of Pol II-transcribed reporter genes in NTS1 and the 35S rRNA gene, we found that Isw1 is not required for silencing of a marked Ty1 element located in NTS2 (data not shown). Similarly, Fob1, a protein required for blocking replication fork migration and promoting recombination in the rDNA, exhibits local effects on transcriptional silencing in the rDNA³⁷. Specifically, Fob1, which localizes to replication fork barrier sequences in NTS1, is required for transcriptional silencing in NTS1, but not in NTS2. Accordingly, our silencing data (Figure 1), as well as Isw1 localization and chromatin structural data presented in Figures 2 and 3, suggest that Isw1 acts at specific regions of the rDNA to regulate chromatin structure and repress Pol II transcription.

Isw1 associates with rDNA

While the silencing phenotype observed in *isw1Δ* cells (Figure 1) indicates that Isw1 functions in rDNA silencing, it does not discriminate between a direct or indirect role. Association of Isw1 with the rDNA would support a model in which Isw1 plays a direct role in regulating silent chromatin. To determine if Isw1 associates with the rDNA, we performed chromatin immunoprecipitation (ChIP) studies using a C-terminally myc-tagged version of Isw1 (*ISW1::c-myc*) and antisera specific for the myc epitope (Figure 2). Previous studies have shown that the C-terminally myc-tagged form of Isw1 was functionally equivalent to wild-type Isw1 protein³⁶. We also performed ChIP analysis using cells with a non-tagged version of Isw1, which provided a measurement of background for the experiments. The relative amounts

of rDNA that associated with Isw1 were determined using quantitative real-time PCR (qPCR) and slot-blot analysis. Data from our qPCR analyses (Figure 2a) demonstrated that Isw1 associates with the rDNA throughout the locus at levels that were approximately 6 to 25-fold higher than the no-tag control, consistent with a recent report examining a role for Isw1 in RNA Pol I transcription³⁸. Interestingly, our results revealed the highest association of Isw1 at a region downstream of the 5S gene in NTS1 (Figure 2a). This is consistent with our Northern data indicating a requirement for Isw1 in silencing the Ty1*his3AI* reporter gene located in NTS1 (Figure 1b). As a control, we examined Isw1 association with sequences upstream of the *INO1* gene (Figure 2a), which encodes a protein involved in inositol biosynthesis, and found that Isw1 associated with *INO1* promoter sequences 14-fold above background, a level similar to those reported previously³⁸.

In our slot blot analyses, blots were hybridized to a radiolabeled DNA probe specific for the entire non-transcribed spacer region of the rDNA. Data from these studies revealed that the myc-tagged version of Isw1 associates with the NTS region of the rDNA at levels that were approximately four-fold above background (Figure 2b-d), further supporting a direct role for Isw1 in rDNA silencing. Interestingly, the mammalian ISWI chromatin remodeling complex NoRC also functions directly at the rDNA locus and is required for transcriptional silencing of Pol I-transcribed rRNA genes^{39; 40}. While our data suggest that Isw1 plays a direct role in silencing of Pol II-transcribed genes in the rDNA, it is unlikely that Isw1 contributes significantly to the regulation of Pol I transcription in *S. cerevisiae* since *isw1Δ* cells exhibit growth characteristics similar to wild-type cells (data not shown). Furthermore, Jones *et al.* have recently shown that Pol I transcription initiation and rRNA levels were unaffected in triple-deletion mutants lacking *ISW1* and genes encoding chromatin remodelers Isw2 and Chd1³⁸. Their study reported that Pol I termination is unaltered in *isw1Δ* cells, although they detected a Pol I termination defect in the triple mutant, suggesting that Isw1, Isw2 and Chd1 are redundant with respect to their involvement in Pol I termination. A possible role for Isw1 in Pol I termination, a process that occurs in or near NTS1, is consistent with our data demonstrating a function for Isw1 at NTS1 where we detected a high level of Isw1 (Figure 2a) and a requirement for Isw1 in rDNA silencing (Figure 1b). Since loss of *ISW1* alone does not affect RNA Pol I transcription³⁸, it is unlikely that the increased Pol II transcription in the rDNA that we observed in cells lacking *ISW1* is due to changes in Pol I transcription.

To complement the data showing that Isw1 regulates rDNA silencing independently of the Isw1a and Isw1b complexes (Figure 1), we examined the association of Isw1::c-myc with the rDNA NTS in cells lacking the *IOC3*, *IOC2* or *IOC4* genes. Data from these ChIP studies suggest that loss of the Isw1a or Isw1b complex does not alter the association of Isw1::c-myc with the rDNA NTS (Figure 2b). These findings support our model that Isw1 acts independently of the Isw1a and Isw1b complexes in transcriptional silencing at the rDNA. Furthermore, these results suggest that the increased rDNA silencing observed in *ioc2Δ* cells and *ioc4Δ* cells (Figure 1b) is not the result of increased association of Isw1 with the rDNA. The increase in silencing observed in *ioc2Δ* and *ioc4Δ* cells may reflect changes in the expression of Isw1b-dependent genes that encode factors involved in rDNA function or structure.

Sir2 and Set1 are not required for Isw1 binding to the rDNA

The recruitment of ISWI remodeling complexes has been shown to be dependent on the level of acetylated histone H4 present at target loci. In *Drosophila*, ISWI interacts preferentially with chromatin enriched with deacetylated histone H4 *in vitro* and *in vivo*^{41; 42}. This interaction is proposed to maintain the balance between transcriptional expression and repression seen in male X-chromosome compensation⁴². Similarly, in mammals, the ISWI remodeler, SNF2H, binds deacetylated histone H4 to establish repressive chromatin at genes targeted by the thyroid hormone receptor⁴³. Recently, in *S. cerevisiae*, it was shown that

isw1Δ cells with lysine-to-arginine substitutions in the N-terminal tail of histone H4 exhibit temperature-sensitive growth, indicating that Isw1 interacts genetically with acetylated histone H4⁴⁴. To examine the role of acetylated histones in the recruitment of Isw1 to the rDNA, we measured the association of Isw1::c-myc with the rDNA NTS in wild-type cells and cells lacking the histone deacetylase Sir2. The results of the ChIP experiments indicate that the association of Isw1 with the rDNA NTS in *sir2Δ* cells was similar to that observed in wild-type cells (Figure 2c). Thus, while the deacetylase activity of Sir2 is required for rDNA silencing, it is not required for the association of Isw1 with the rDNA NTS.

In *S. cerevisiae*, methylation of histone H3 on lysine 4 by the methyltransferase Set1 is required for recruitment of Isw1 to a number of actively transcribed genes^{30; 32}. In addition, K4-methylated H3 is present at the rDNA and telomeres and is required for transcriptional silencing at these chromosomal domains^{10; 11; 14}. Together, these data raise the possibility that K4-methylated histone H3 regulates the interaction of Isw1 with the rDNA. To test this, we performed ChIP on *set1Δ* cells that lack K4-methylated histone H3. The fraction of rDNA NTS associated with Isw1::c-myc in *set1Δ* cells was similar to that observed in wild-type cells (Figure 2d), indicating that the association of Isw1 with the rDNA does not require K4-methylated histone H3.

Deletion of *ISW1* results in changes in chromatin structure at the rDNA

The association of Isw1 with rDNA and its requirement for transcriptional silencing prompted us to examine whether cells lacking *ISW1* have altered chromatin structure at the rDNA. We performed micrococcal nuclease (MNase) accessibility studies on wild-type, *isw1Δ*, *ioc3Δ*, *ioc2Δ* and *ioc4Δ* cells (Figure 3). In this study, we included a strain lacking the gene encoding the histone deacetylase Sir2, a known regulator of rDNA chromatin structure and gene silencing. For this analysis, we focused on the NTS region of the rDNA, where changes in chromatin structure were identified previously in rDNA-silencing mutants^{5; 7; 45}. Comparison of the MNase digestion patterns from wild-type and *isw1Δ* cells revealed a change in MNase accessibility in NTS1 downstream of the 5S rRNA gene (Figure 3). In Figure 3, the intensity of the upper band of the doublet marked by the filled circles is reduced in *isw1Δ* cells relative to wild-type cells. This decrease in MNase accessibility is localized to the region of NTS1 where we observed association of Isw1 with the rDNA (Figure 2a) and may reflect local changes in chromatin structure in the absence of Isw1 that allow transcription factors to bind these sequences and block MNase accessibility. Notably, the MNase digestion pattern of the rDNA NTS in *isw1Δ* cells was different from patterns observed in *sir2Δ* cells (Figure 3), where we observed changes in MNase accessibility in other portions of the rDNA NTS, as has been reported previously^{5; 7}. This is in agreement with our ChIP data that indicated that Sir2 is not required for the interaction of Isw1 with the rDNA (Figure 2c). The MNase digestion patterns for *ioc3Δ*, *ioc2Δ* and *ioc4Δ* cells were similar to the pattern for wild-type cells (Figure 3), consistent with our transcriptional silencing and ChIP data suggesting that Isw1 acts independently of the Isw1a and Isw1b complexes at the rDNA (Figures 1 and 2b).

Our MNase accessibility data reflect an average MNase digestion pattern of ~150 - 200 rDNA repeats present at the rDNA locus. Differences in MNase accessibility at the rDNA when comparing wild-type and *isw1Δ* cells are expected to be difficult to detect. Studies profiling chromatin structure by MNase accessibility suggest that differences in the MNase digestion patterns between wild-type and *isw1Δ* cells should be detected approximately once per 5,000 base pairs in the *S. cerevisiae* genome²⁷. Thus, the difference that we observed in the MNase cleavage patterns between wild-type and *isw1Δ* cells (Figure 3) reflects the participation of Isw1 in the formation of silent chromatin at the rDNA.

Several studies have linked ISWI chromatin remodelers with RNA Pol I and RNA Pol III transcription. The mammalian ISWI remodeling complex NoRC is required for silencing of Pol I-transcribed genes in the rDNA^{39; 40}. In humans, SNF2h has been implicated in the transcription on RNA Pol III-transcribed genes. As a member of the WICH complex with the Williams syndrome transcription factor, SNF2h associates with the Pol III-transcribed 5S RNA gene⁴⁶. Interestingly, WICH also interacts with the nucleolar protein Myb-bp1a, which shares sequence similarity with *S. cerevisiae* Pol5, a protein involved in transcriptional regulation of ribosomal RNA genes^{46; 47; 48}. In *S. cerevisiae*, affinity purification studies have identified an interaction between Isw1 and the RNA Pol I and RNA Pol III subunit, Rpc40⁴⁹. Furthermore, Isw1 has been linked to Pol I transcription termination in *S. cerevisiae*³⁸. Given these findings and our ChIP and MNase data that implicate Isw1 in chromatin structure in NTS1 downstream of 5S RNA gene, it is tempting to speculate that RNA Pol I or Pol III transcription machinery contributes to the recruitment of Isw1 to the rDNA. Notably, Isw2 is recruited to Pol III-transcribed tRNA genes by the RNA Pol III transcription factor subunit, Bdp1⁵⁰.

Loss of *ISW1* does not significantly alter mitotic stability at the rDNA locus

The repetitive nature of the rDNA is expected to contribute to mitotic instability due to recombination. Interestingly, the proteins that are required for transcriptional silencing at the rDNA vary in their ability to repress recombination at the rDNA. Some rDNA silencing proteins, like Sir2, repress recombination at the rDNA (Table 1)^{3; 51; 52}, whereas others, including Set1 and Snf2, have no effect or an antagonistic effect on mitotic stability of the rDNA^{10; 21}. To determine if Isw1 contributes to mitotic stability of the rDNA, we compared recombination rates at the rDNA in wild-type and *isw1Δ* cells by measuring the mitotic stability of a Ty1*HIS3* element within the rDNA. The rate of loss of the *HIS3* marker from the rDNA in cells lacking *ISW1* was 1.4-fold higher than the rate of loss observed in wild-type cells (Table 1), suggesting that Isw1 makes a minor contribution to mitotic stability at the rDNA. Conversely, cells lacking *SIR2* exhibited a 5-fold increase in the rate of loss of the *HIS3* marker from the rDNA relative to wild-type cells (Table 1). From these data, we conclude that the change in rDNA chromatin structure observed in *isw1Δ* cells (Figure 3), which is associated with loss of transcriptional silencing (Figure 1), is not sufficient to significantly increase recombination at the rDNA.

The loss of transcriptional silencing and the modest increase in recombination at the rDNA that we observed in *isw1Δ* cells are similar to phenotypes detected in cells with conditional mutations in the essential gene *ESA1*⁵³. Esa1 is the catalytic subunit of the histone acetylation complex NuA4⁵⁴. The results of studies examining the role of Esa1 in gene silencing at the rDNA suggest that the histone acetylase activity of NuA4 and the histone deacetylase activity of Sir2 maintain the appropriate levels of acetylated and deacetylated histones at the rDNA to create repressive chromatin at that locus⁵³. Our data showing that Isw1 associates with the rDNA in cells lacking Sir2 (Figure 2c) suggest that Isw1 functions independently of Sir2 in rDNA silencing. Genetic studies probing the roles of Isw1 and the NuA4 complex in cellular stress response concluded that Isw1 and members of the NuA4 complex function in non-overlapping pathways to regulate stress-inducible genes⁴⁴. Future studies to investigate interactions between Isw1 and NuA4 with respect to transcriptional silencing and mitotic stability at the rDNA will provide additional insight into how chromatin remodelers, together with histone-modifying enzymes, contribute to rDNA structure and function.

The data from our studies support a model in which Isw1 plays a direct role in maintaining a repressive chromatin structure at the rDNA in *S. cerevisiae*. Our studies show that cells lacking Isw1 exhibit altered chromatin structure at the rDNA in NTS1, which is associated with increased transcription by Pol II. Our model is corroborated by data demonstrating the

association of Isw1 with the rDNA. Interestingly, in cells in which the Isw1a and Isw1b complexes cannot form, Isw1 association with the rDNA is maintained. These findings are in agreement with proteomic analyses of protein complexes in *S. cerevisiae*^{49; 55} and individual genetic studies^{28; 34} that suggest Isw1 may form additional chromatin remodeling complexes beyond the previously characterized Isw1a and Isw1b complexes. Indeed, data from studies examining silencing at the *HMR* locus³⁴ and results from the work presented here raise the possibility of novel Isw1 complexes required for the maintenance of heterochromatin-like structures in *S. cerevisiae*. Further studies are needed to identify and characterize these putative remodeling complexes and examine their potential roles in the maintenance of both repressive and active chromatin throughout the *S. cerevisiae* genome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Isw1 is required for rDNA silencing and acts independently of the Isw1a and Isw1b complexes

(a) A single rDNA repeat. Non-transcribed spacer (NTS) is divided into NTS1 and NTS2 by the 5S rRNA gene (5S, black triangle). Thick black line, 35S rRNA gene; bent line with arrowhead indicates direction of Pol I transcription. Open triangles, location of *Ty1his3AI* and *mURA3* reporter genes. Oval, autonomously replicating sequences (ARS). (b) Northern analysis to measure steady-state *Ty1his3AI* (upper panel), total Ty1 (middle panel) and *PYK1* (lower panel) transcript levels in wild-type (WT), *isw1Δ*, *set1Δ*, *ioc3Δ*, *ioc2Δ* and *ioc4Δ* cells. Northern analysis of total RNA isolated from yeast cells was performed as described⁵⁶. Blots were quantified on a Molecular Dynamics Storm 860 phosphorimager using

ImageQuant software. Ty1*his3AI* and total Ty1 transcript levels were normalized to *PYK1* RNA levels. Numbers below Ty1*his3AI* and total Ty1 panels are the average mutant:WT ratios of the normalized transcript levels. The average mutant:WT ratios ($\pm$ SE) of Ty1*his3AI* RNA normalized to *PYK1* RNA were *isw1Δ*:WT 4.7 ($\pm$ 0.9, n=5); *set1Δ*:WT 6.5 ($\pm$ 0.6 range, n=2); *ioc3Δ*:WT 1.0 ($\pm$ 0.2, n=3); *ioc2Δ*:WT 0.4 ($\pm$ 0.05, n=3); *ioc4Δ*:WT 0.5 ($\pm$ 0.07, n=3). The average mutant:WT ratios ($\pm$ SE) of total Ty1 RNA normalized to *PYK1* RNA were *isw1Δ*:WT 1.3 ($\pm$ 0.2, n=5); *set1Δ*:WT 0.8 ($\pm$ 0.3, range n=2); *ioc3Δ*:WT 1.5 ($\pm$ 0.3, n=3); *ioc2Δ*:WT 1.3 ($\pm$ 0.3, n=3); *ioc4Δ*:WT 1.5 ($\pm$ 0.5, n=3). (c) Northern analyses were performed and quantified as in (b) to measure Ty1*his3AI* (upper panel), total Ty1 (middle panel) and *PYK1* (lower panel) RNA in wild-type cells and double-deletion mutants, *ioc3Δ ioc2Δ* and *ioc3Δ ioc4Δ*. The average mutant:WT ratios ($\pm$ SE; n=3) of Ty1*his3AI* RNA normalized to *PYK1* RNA were *ioc3Δ ioc2Δ*:WT 0.8 ($\pm$ 0.1); *ioc3Δ ioc4Δ*:WT 0.9 ($\pm$ 0.1). The average mutant:WT ratios ($\pm$ SE; n=3) of total Ty1 RNA normalized to *PYK1* RNA were *ioc3Δ ioc2Δ*:WT 1.7 ($\pm$ 0.1); *ioc3Δ ioc4Δ*:WT 1.5 ($\pm$ 0.2). (d) A plate assay examining growth on media containing 5-FOA indicates that *Isw1* is required for silencing of *mURA3* at the rDNA. Cultures of wild-type, *isw1Δ*, *set1Δ*, *ioc3Δ*, *ioc2Δ* or *ioc4Δ* cells with *mURA3* at the rDNA were grown to saturation. Ten-fold serial dilutions were made in sterile water and spotted onto synthetic complete (SC) + 5-FOA and SC agar (left panels). Robust growth of cells on media containing 5-FOA reflects silencing of the *mURA3* marker gene in the rDNA, while limited growth reflects a loss of silencing and expression of the *mURA3* gene. Saturated cultures of wild-type, *isw1Δ*, *set1Δ*, *ioc3Δ*, *ioc2Δ* or *ioc4Δ* cells with *mURA3* inserted at the *LEU2* locus (*leu2Δ1*) were serially diluted and plated on synthetic complete media lacking uracil (SC-URA) and SC agar (right panels). Cell growth was similar for all strains, indicating that expression of *mURA3* outside the rDNA is not altered in the deletion strains. Cells were plated on SC media to insure that comparable numbers of wild-type and mutant cells were analyzed. Photographs were taken after 3 days of growth at 30°C. The results of a representative plate assay are shown (n = 5).

Figure 2. Association of Isw1 with the rDNA NTS

(a) Schematic of a single rDNA repeat. Solid lines labeled NTS1-a, NTS1-b, 5S, NTS2, 35S-a, 35S-b, 35S-c and 35S-d represent the location of PCR products used in ChIP analysis. Other labels as in Figure 1. ChIP studies using a myc-tagged form of Isw1 and antisera specific for the myc epitope (10 μ L of anti-c-myc (A-14):sc-789; Santa Cruz Biotechnology) were performed on wild-type cells (WT *ISW1::c-myc*, black bars). A non-tagged version of Isw1 (WT - no tag, gray bars) was evaluated to provide a measurement of background signal. ChIPs were performed and analyzed by quantitative real-time PCR as previously described^{7, 10}. Percent immunoprecipitations (%IP) were determined by dividing α -myc IP signal by input signal. The values of the average %IP ($\pm$ SE) for three independent experiments for the nine

primer sets are shown in the bar graph. PCR primer sequences are available upon request. (b) ChIPs were performed as in (a) on wild-type (WT *ISW1::c-myc*), *ioc3Δ* (*ioc3Δ ISW1::c-myc*), *ioc2Δ* (*ioc2Δ ISW1::c-myc*) and *ioc4Δ* (*ioc4Δ ISW1::c-myc*) cells and analyzed by slot blot, using a radiolabeled probe to the entire NTS region of the rDNA (rDNA NTS). Blots were quantified on a Storm 860 phosphorimager (Molecular Dynamics) using ImageQuant software. The average ratio of %IP (+/- range, n=2) of the rDNA NTS for *ISW1::c-myc* / no tag was 4.3 (+/- 0.3). The average ratio of %IP (+/- range, n=2) of the rDNA NTS in *ioc3Δ ISW1::c-myc* cells / no tag was 3.9 (+/- 0.3), in *ioc2Δ ISW1::c-myc* cells / no tag was 3.4 (+/- 0.3) and in *ioc4Δ ISW1::c-myc* cells / no tag was 3.6 (+/- 0.4). The open triangle represents serial dilutions of input DNA to insure that hybridization to the probe was linear with respect to the amount of DNA applied to the membrane. (c) ChIPs were performed and analyzed as in (b) on wild-type (WT *ISW1::c-myc*) and *sir2Δ* (*sir2Δ ISW1::c-myc*) cells. The average ratio of %IP (+/- range, n=2) of the rDNA NTS for *ISW1::c-myc* / no tag was 7.4 (+/- 2.9) and in *sir2Δ ISW1::c-myc* cells / no tag was 5.9 (+/- 2.8). (d) ChIPs were performed and analyzed as in (b) on wild-type (WT *ISW1::c-myc*) and *set1Δ* (*set1Δ ISW1::c-myc*) cells. The average ratio of %IP (+/- SE) of the rDNA NTS for *ISW1::c-myc* / no tag was 4.4 (+/- 1.5, n = 3) and the average ratio of %IP (+/- range) of the rDNA NTS in *set1Δ ISW1::c-myc* cells / no tag was 4.3 (+/- 1.7, n = 2).

Figure 3. Deletion of *ISW1* alters chromatin structure at the rDNA

Wild-type (WT), *isw1Δ*, *sir2Δ*, *ioc3Δ*, *ioc2Δ* and *ioc4Δ* spheroplasts were prepared and treated with micrococcal nuclease (MNase) according to Kent and Mellor⁵⁷. DNA was purified, digested with *EcoRI* and subjected to Southern analysis. Blots were probed with a radiolabeled PCR fragment that allowed indirect-end-labeling analysis of the rDNA NTS. A schematic of the region analyzed is shown on the left. Open circles represent location of previously identified positioned nucleosomes^{5; 7}. Open triangles, increasing amounts of MNase. N, naked DNA. Filled circles, doublet in which upper band exhibits decreased MNase accessibility in *isw1Δ* cells. Asterisks, portions of NTS with altered MNase accessibility in *sir2Δ* cells. DNA lengths (base pairs) are designated on the right. Other labels as in Figure 1.

Table 1Deletion of *ISW1* does not significantly affect recombination at the rDNA.

Strain Ty1 element (location)	Relevant genotype	His ⁻ auxotrophs / total cells analyzed	Loss of <i>HIS3</i> per generation ^a (+/- SE, n=3)
MBY1445 Ty1 <i>HIS3</i> -236 (in rDNA)	<i>ISW1</i>	183 / 1577	$0.9 (+/- 0.16) \times 10^{-3}$
MBY2222 Ty1 <i>HIS3</i> -236 (in rDNA)	<i>isw1</i> Δ	278 / 1829	$1.3 (+/- 0.31) \times 10^{-3}$
MBY2295 Ty1 <i>HIS3</i> -236 (in rDNA)	<i>sir2</i> Δ	1173 / 2103	$4.7 (+/- 0.53) \times 10^{-3}$

^aMitotic recombination at the rDNA was measured as previously described³. Loss of the Ty1*HIS3* element in the rDNA prevents growth of cells on media lacking histidine. Thus, the fraction of His⁻ cells relative to total cells after ~120 generations of growth in nonselective YPADT media provides an accurate measurement of mitotic recombination in the rDNA. A culture containing a mixture of His⁺ and His⁻ cells served as a control to show that there is no growth advantage of His⁺ cells or His⁻ cells in YPADT media. The fraction of His⁺ cells in a mixed culture at the beginning of the experiment was 0.75 and the fraction of His⁺ cells in the mixed culture after ~120 generations of growth was 0.74 (data not shown).