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Sir2 Regulates Histone H3 Lysine 9 Methylation and Heterochromatin Assembly in Fission Yeast

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Summary

Hypoacetylated histones are a hallmark of heterochromatin in organisms ranging from yeast to humans [1]. Histone deacetylation is carried out by both NAD$^+$-dependent and NAD$^+$-independent enzymes. In the budding yeast *Saccharomyces cerevisiae*, deacetylation of histones in heterochromatic chromosomal domains requires Sir2, a phylogenetically conserved NAD$^+$-dependent deacetylase [2–5]. In the fission yeast *Schizosaccharomyces pombe*, NAD$^+$-independent histone deacetylases are required for the formation of heterochromatin [6, 7], but the role of Sir2-like deacetylases in this process has not been evaluated. Here, we show that spSir2, the *S. pombe* Sir2-like protein that is the most closely related to the *S. cerevisiae* Sir2, is an NAD$^+$-dependent deacetylase that efficiently deacetylates histone H3 lysine 9 (K9) and histone H4 lysine 16 (K16) in vitro. In *sir2*Δ cells, silencing at the donor mating-type loci, telomeres, and the inner centromeric repeats (*imr*) is abolished, while silencing at the outer centromeric repeats (*otr*) and rDNA is weakly reduced. Furthermore, Sir2 is required for hypoacetylation and methylation of H3-K9 and for the association of Swi6 with the above loci in vivo. Our findings suggest that the NAD$^+$-dependent deacetylase Sir2 plays an important and conserved role in heterochromatin assembly in eukaryotes.

Results and Discussion

Isolation and Biochemical Characterization of spSir2

The *S. pombe* genome encodes three Sir2-like proteins [8], one of which is an uncharacterized open reading frame (SPBC16D10.07c) with the highest degree of sequence similarity to the budding yeast Sir2. We therefore named this gene *sir2*$^+$. The *sir2*$^+$ open reading frame was amplified by PCR and was fused in frame to GST in a bacterial expression vector. Following purification and cleavage with a site-specific protease to remove GST (Figure 1A), we tested the recombinant spSir2 protein's deacetylation activity. The Sir2 family of enzymes couple deacetylation to hydrolysis of NAD$^+$ and release of nicotinamide with a 1:1 stoichiometry [9–11]. Acetyl-lysine-dependent release of nicotinamide therefore was used as an assay for deacetylation. spSir2 efficiently deacetylated an H4 peptide with acetyl-lysine at position 16 (AcK16) and an H3 peptide with acetyl-lysine at position 9 (AcK9), compared to H4 peptides with acetyl-lysine at positions 5 (AcK5), 8 (AcK8), and 12 (AcK12) (Figure 1B). The specificity of spSir2 for H4-AcK16 is similar to the substrate specificity of *S. cerevisiae* Sir2 and human SirT1 [3, 9] and supports the assignment of spSir2 to the Sir2/SirT1 subfamily of NAD$^+$-dependent deacetylases.

spSir2 Is Required for Silencing at the Mating-Type Locus and Centromeric DNA Regions

In *S. cerevisiae*, Sir2 is required for silencing of all heterochromatic regions, including the mating-type loci [12]. Here, we tested if Sir2 is similarly required for silencing of the mating-type genes in *S. pombe*. The mating-type region of *S. pombe* consists of three genes: an expressed gene *mat1* that determines the mating type of the cell, and two silent donor loci, *mat2* and *mat3* (Figure 2A) [13]. A 20-kb chromosomal domain including the donor loci, their flanking sequences, and an 11-kb interval between them (*K*-region) is maintained in a silent state. We deleted *sir2*$^+$ in strains with the *ade6*$^+$ reporter gene inserted immediately to the left of the *mat2* locus (*L(BglII)::ade6*$^+$) (Figure 2A) [14]. As expected, *sir2*$^+$ cells containing the *L(BglII)::ade6*$^+$ insert formed red or sectored colonies, indicating that the *ade6*$^+$ reporter was silenced (Figure 2C) [14]. In contrast, *sir2*Δ *L(BglII)::ade6*$^+$ cells formed white colonies, indicating loss of silencing of the reporter gene (Figure 2C).

We also introduced the *sir2*Δ mutation into a strain containing the *ura4*$^+$ reporter gene inserted in the *K*-region of the mating-type locus (*Kint2::ura4*$^+$) (Figure 2A) [15]. The expression of the *Kint2::ura4*$^+$ was assayed by growth on nonselective (N/S), selective (AA-URA), and counter-selective (FOA, 5-fluoroorotic acid) media (Figure 2D). *sir2*$^+$ cells carrying *Kint2::ura4*$^+$ grew poorly on medium lacking uracil but grew well on FOA-containing plates, indicating that the *Kint2::ura4* was silenced efficiently (Figure 2D, row 1). In contrast, *sir2*Δ *Kint2::ura4*$^+$ cells grew on medium lacking uracil and failed to form colonies on FOA-containing plates, indicating that Sir2 was required for silencing of the *Kint2::ura4*$^+$ (Figure 2D, row 2). Thus, *sir2*$^+$ was required for silencing of two different reporter genes inserted at different locations in the mating-type region.

During mitotic divisions, a switching competent cell (*h*^{90}) switches to the opposite mating type in about 90% of cell divisions [13]. This nonrandom utilization of donor loci during mating-type switching, termed directionality, depends on maintenance of a special chromatin structure at the silent mating-type (*mat2/3*) region [15, 16].

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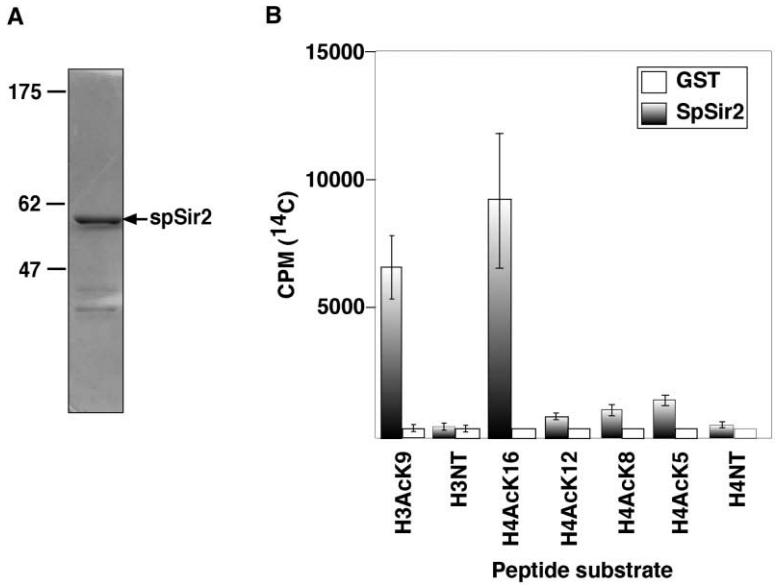

Figure 1. SPBC16D10.07c, the *S. pombe* Sir2-like Protein with the Highest Degree of Sequence Similarity to *S. cerevisiae* Sir2, Is an NAD$^+$-Dependent Deacetylase

(A) A Coomassie-stained gel showing the recombinant *S. pombe* Sir2 protein used in deacetylation assays.

(B) Deacetylation of histone H3 and H4 amino-terminal peptides by spSir2 showing preferential deacetylation of H3-AcK9 and H4-AcK16. Deacetylation was carried out as described previously [9, 11] (see the Supplemental Data for details). Counts per minute (CPM) on the *y* axis show the release of ^{14}C-labeled nicotineamide, which is a product of NAD$^+$-dependent deacetylation. H3NT and H4NT refer to unacetylated H3 and H4 peptides, respectively, used as negative controls.

Several *trans*-acting factors such as histone deacetylase Clr3, H3-K9-specific histone methyltransferase Clr4, and Swi6 (a homolog of mammalian HP1) protein are involved in silent chromatin assembly at the *mat2/3* interval [6, 17–19]. Furthermore, mutations in most of these and other *trans*-acting factors affect the efficiency of mating-type switching [15, 17, 20]. Switching-proficient colonies stain black after exposure to iodine va-

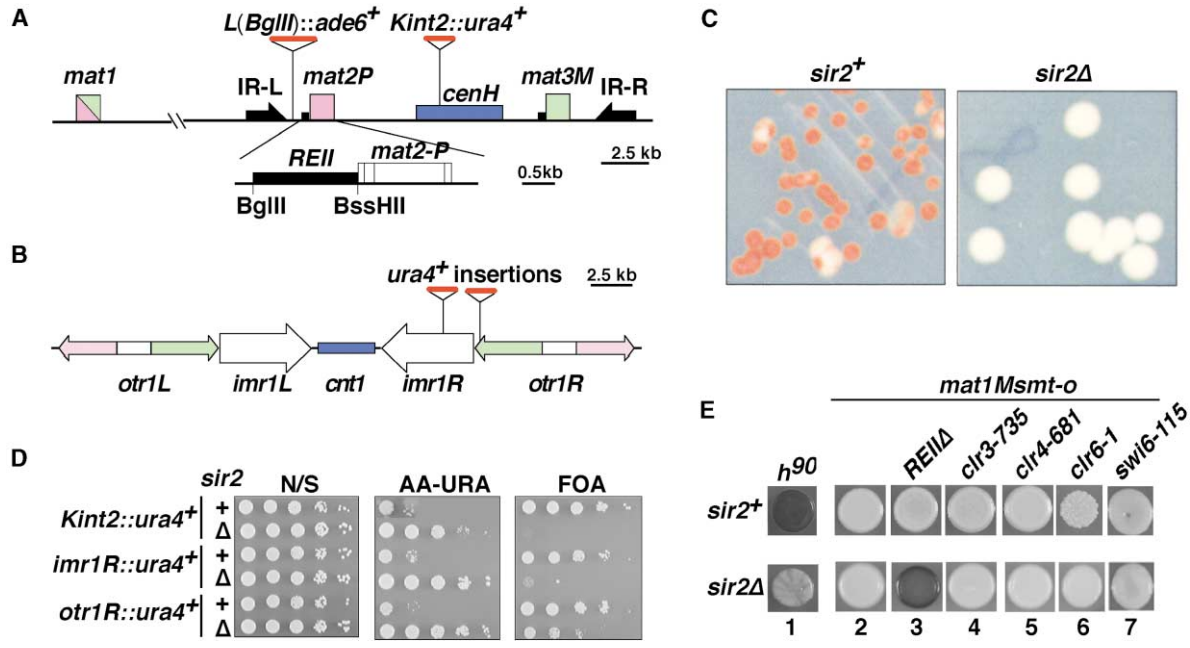

Figure 2. Sir2 Is Required for Silencing at the Mating Loci and Centromeres

(A and B) Schematic diagrams of the (A) mating-type region and (B) *cen1*. The positions of *ade6*$^+$ and *ura4*$^+$ reporter genes used in this study are indicated.

(C) Colony color of *L(BglII)::ade6*$^+$ in *sir2*Δ and wild-type cells. The white colonies in the right panel indicate that deletion of *sir2*$^+$ results in loss of silencing of the *ade6*$^+$ reporter.

(D) Effect of *sir2*Δ on *Kint2::ura4*$^+$, *imr1R::ura4*$^+$, and *otr1R::ura4*$^+$ expression. Expression of the *ura4*$^+$ reporter was assayed by spotting 10-fold serial dilutions of cultures onto the indicated medium.

(E) *sir2*$^+$ regulates mating-type switching and silencing of donor mating-type loci. Unlike the *h*^{90} *sir2*$^+$ cells, the *h*^{90} *sir2*Δ cells do not stain dark when exposed to iodine vapors, indicating they are defective in mating-type switching (column 1). In nonswitchable mating-type background (*mat1M-smt-o*), deletion of *sir2*$^+$ in combination with *REII*Δ (column 3), but not with *clr3-735*, *clr4-681*, *clr6-1*, or *swi6-115*, results in dark staining after exposure to iodine vapors, which is indicative of abortive haploid meiosis caused by the expression of both mating types (columns 4–7). Cells were spotted and grown on sporulation medium at 26°C for 3 days and were exposed to iodine vapors.

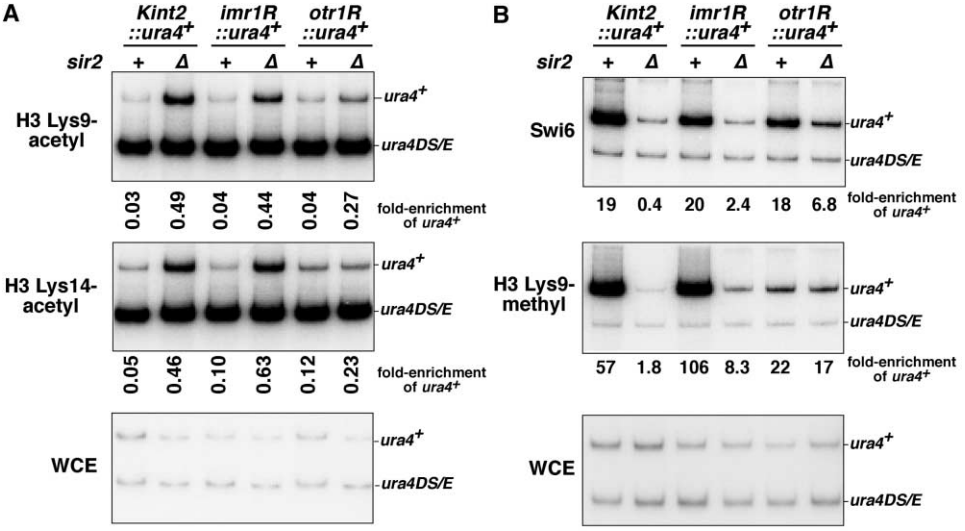

Figure 3. Sir2 Is Required for Hypoacetylation of H3-K9 and -K14, Methylation of H3-K9, and Swi6 Localization at the *mat* Locus and Centromeres

(A) ChIP experiments showing increased acetylation of H3-K9 and -K14 at *Kint2::ura4*$^+$, *imr1R::ura4*$^+$, and *otr1R::ura4*$^+$ in *sir2*Δ compared to wild-type cells.

(B) *sir2*Δ causes a strong reduction in methylation of H3-K9 and Swi6 localization at the *mat* locus and the inner (*imr*) centromeric repeats. ChIP experiments were performed as described previously [31]. DNA from immunoprecipitated or whole-cell crude extract (WCE) fractions was analyzed by a competitive PCR strategy, whereby one set of primers amplifies the *ura4*$^+$ reporter gene and a control set of primers amplifies a *ura4DS/E* minigene at the endogenous euchromatic location. The ratio of *ura4*$^+$ to *ura4DS/E* signals present in ChIP and WCE was used to calculate the relative fold enrichment. Relative fold enrichment values are shown beneath each lane.

pors, because they accumulate a starch-like compound during sporulation, while inefficiently switching cells stain sparingly. *sir2*Δ was introduced into a switching competent background and its effect on mating-type switching was analyzed by the iodine vapor staining assay. We observed a strong reduction in staining of *sir2*Δ compared to *sir2*$^+$ cells, which indicated a reduced rate of switching to the opposite mating type in *sir2*Δ cells (Figure 2E, column 1).

Redundant pathways involving *cis*-acting elements and *trans*-acting factors regulate silencing of donor mating-type loci in fission yeast [13]. Deletion of a 1.5-kb *cis*-acting region (*REII*) proximal to *mat2-P* potentiates the effect of mutations in all other genes that regulate chromatin structure throughout the *mat2/3* interval [14, 17] (Figure 2A). In a nonswitchable mating-type (*mat1M-smto*) background, the combination of deletion of the *REII* element and a mutation in one of the *trans*-acting factors leads to simultaneous expression of both mating-type information and causes an abortive meiosis. This phenomenon, described as haploid meiosis phenotype, is also detectable by the iodine vapor assay, and, in nonswitching *mat1M-smto* background, the intensity of iodine staining indicates the level of haploid meiosis [17]. We combined the *sir2*Δ mutant with *REII*Δ and found that the *sir2*Δ *REII*Δ double mutant had a strong haploid meiosis phenotype (Figure 2E). We also analyzed the cumulative effect of *sir2*Δ in combination with mutations in *clr3*, *clr4*, *clr6*, and *swi6*. In contrast to the *sir2*Δ *REII*Δ double mutant, we found that pairwise combination of *sir2*Δ with each of the above mutations did not show a cumulative effect on the haploid meiosis phenotype (Figure 2E). We therefore conclude that *sir2*$^+$ might participate in the same silencing pathway or in multiple dependent pathways as *clr3*$^+$, *clr4*$^+$, *clr6*$^+$, and *swi6*$^+$.

Fission yeast centromeres are assembled into heterochromatic structures [13]. Moreover, factors affecting silencing at the mating-type region have also been shown to affect centromeric silencing [6, 21]. To investigate whether *sir2*$^+$ affects centromeric silencing, we combined *sir2*Δ with the *ura4*$^+$ reporter integrated at two locations within *cen1*: the outer repeat (*otr*) and the inner repeat (*imr*) regions [21] (Figure 2B). Deletion of *sir2*$^+$ caused derepression of *ura4*$^+$ at both loci (Figure 2D). However, this effect was much stronger at the *imr* repeats than at the *otr* repeats. While *sir2*Δ cells carrying *imr1R::ura4*$^+$ did not form colonies on FOA-containing plates, mutant cells carrying *otr1R::ura4*$^+$ showed appreciable growth on FOA plates (Figure 2D). These results indicated that Sir2 was required for silencing at the *S. pombe* centromeric DNA regions.

Role of spSir2 in Assembly of Silent Chromatin

We next investigated the effect of deletion of *sir2*$^+$ on histone acetylation at the different heterochromatin regions described above by using the chromatin immunoprecipitation (ChIP) assay with acetylation site-specific antibodies. Compared to wild-type cells, in *sir2*Δ cells, acetylation of K9 was increased by 16-, 11-, and 7-fold at the *ura4*$^+$ reporter inserted in the mating locus (*Kint2::ura4*$^+$), the inner (*imr1R::ura4*$^+$), and the outer centromeric repeats (*otr1R::ura4*$^+$), respectively (Figure 3A). Similarly, the acetylation of H3-K14 at *ura4*$^+$ was increased by 9-, 6-, and 2-fold at the *ura4*$^+$ reporter in each of the above strains in *sir2*Δ compared to wild-type cells (Figure 3A). Thus, loss of silencing in *sir2*Δ

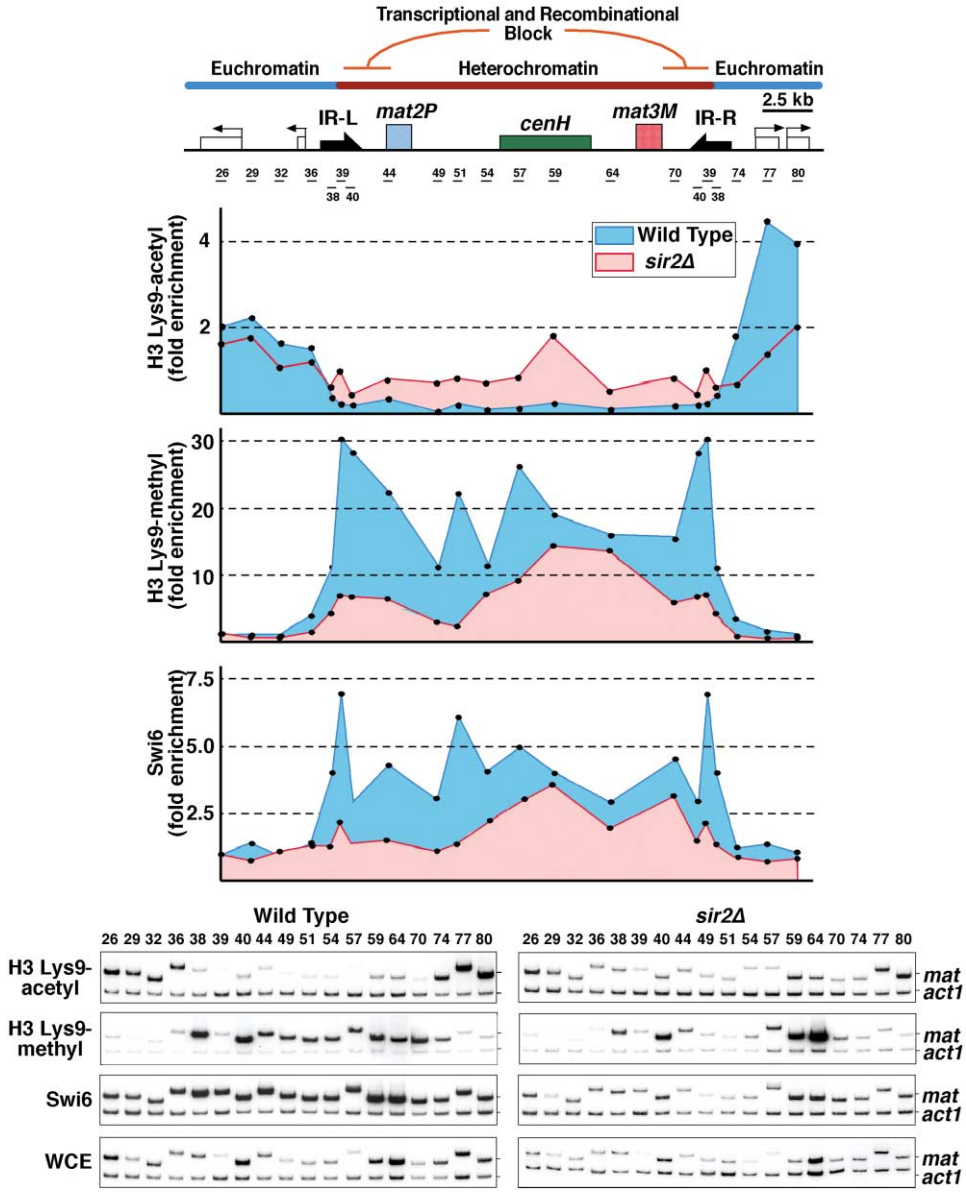

Figure 4. High-Resolution Map of H3-K9 Acetylation, H3-K9 Methylation, and Swi6 Localization at the *mat2/3* Interval

A physical map of the silent mating-type locus with the location of primers used is shown (top). High-resolution mapping of histone modifications and Swi6 was carried out as described previously [24] in wild-type and *sir2*Δ cells with antibodies to acetylated or dimethylated H3-K9 or Swi6. Quantification of the ChIP data is plotted in alignment with a map of the *mat* locus shown on top (see also Figure S2).

cells, at each of the above loci, correlates with increased levels of histone H3 acetylation.

Swi6 is an essential structural component of heterochromatin at the mating-type region and centromeres [19, 22]. Because *sir2*$^+$ and *swi6*$^+$ function in the same genetic pathway for silencing and deacetylation of H3-K9 and their methylation is required for localization of Swi6 to silent chromatin, we evaluated the role of Sir2 in localization of Swi6 to silent chromatin. ChIP analysis showed that the level of Swi6 associated with *Kint2::ura4*$^+$ was reduced 47-fold in *sir2*Δ compared to wild-type cells (Figure 3B). Moreover, the level of Swi6 associated with *imr1R::ura4*$^+$ and *otr1R::ura4*$^+$ reporters was reduced 8- and 3-fold, respectively, in *sir2*Δ compared to wild-type cells (Figure 3B). These observations suggested that *sir2*$^+$ was required for the localization of Swi6 to the silent mating-type region and contributed to a lesser extent to localization of Swi6 to centromeric DNA regions. It has been shown previously that localization of Swi6 at heterochromatic loci depends on H3-K9 methylation by Clr4 [19, 23]. Therefore, we examined the effect of deleting *sir2*$^+$ on H3-K9 methylation. Histone H3-K9 methylation levels at *Kint2::ura4*$^+$ and *imr1R::ura4*$^+$ were strongly reduced in *sir2*Δ compared to *sir2*$^+$ cells (32- and 13-fold, respectively) (Figure 3B). Consistent with its weaker effect on silencing of *otr1R::ura4*$^+$, deletion of *sir2*$^+$ had only a weak effect on methylation of this *ura4*$^+$ reporter (Figure 3B).

Previous studies have shown that the *mat2/3* interval displays an altered pattern of histone modifications

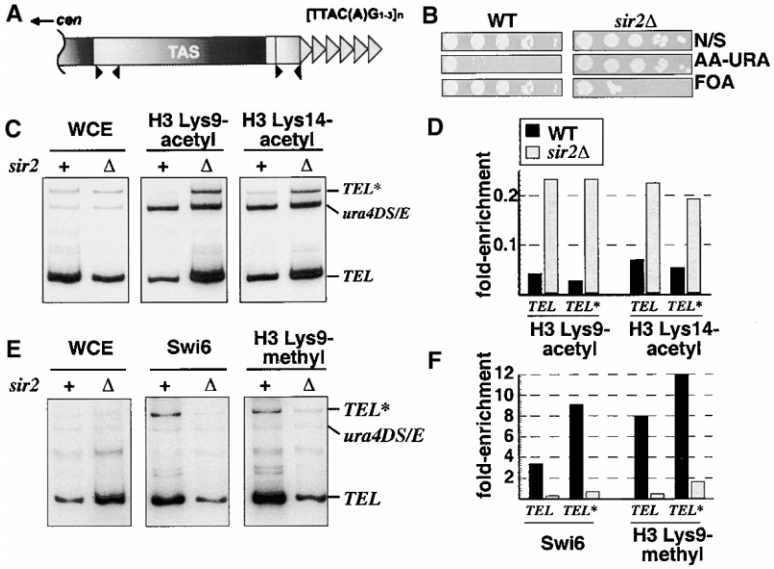

Figure 5. Deletion of *sir2*$^+$ Disrupts Silencing, Increases H3-K9 and H3-K14 Acetylation, and Reduces H3-K9 Methylation and Swi6 Localization at Telomeres

(A) A diagram of a telomere including TAS, short repeats (gray triangles), and locations of PCR primers (black triangles) used in ChIP analyais is shown.

(B) Silencing of *TEL::ura4*$^+$ was assayed by serial dilution of cultures on the indicated medium.

(C–F) ChIP experiments showing that deletion of *sir2*$^+$ results in increased acetylation of (C and D) H3-K9 and -K14, decreased localization of Swi6, and decreased methylation of (E and F) H3-K9 in a telomeric region. DNA isolated from WCE or immunoprecipitated fractions was subjected to multiplex PCR to analyze for telomere-associated sequences (TAS) as well as *ura4DS/E* sequences, used as control. The PCR product marked with an asterisk resulted from primers binding to an additional site within the TAS.

compared to surrounding euchromatic regions [24]. Here, we examined the role of Sir2 in deacetylation of histones throughout the *mat2/3* interval. Deletion of *sir2*$^+$ resulted in a general increase in the level of H3-K9 acetylation throughout the mating-type region (Figure 4). In comparison to the greatly increased levels of acetylation at the *ura4*$^+$ reporter gene (Figure 3), the effect of *sir2*Δ on histone acetylation at the native *mat2/3* region sequences is relatively modest. This result likely reflects differences in chromatin structure at the actively transcribed *ura4*$^+$ locus versus transcriptionally inert sequences at the *mat2/3* interval, except the centromere homology region *cenH* that is transcribed at low levels [25]. Similar results were obtained for H3-K14 acetylation (data not shown).

We next mapped Swi6 localization and H3-K9 methylation patterns at the mating-type locus in *sir2*$^+$ and *sir2*Δ cells. In *sir2*Δ cells, we observed a general decrease in Swi6 localization and H3-K9 methylation throughout the heterochromatic interval of the mating-type locus (Figure 4). However, the high-resolution mapping data presented in Figure 4 showed the presence of significant amounts of H3-K9 methylation and Swi6 localization in the *cenH* region in *sir2*Δ cells. This is in contrast to our observations for the *ura4*$^+$ reporter inserted in the *cenH* region, where deletion of *sir2*$^+$ abolished H3-K9 methylation and Swi6 localization (Figure 3B). Because the *ura4*$^+$ reporter inserted in the *cenH* region is strongly expressed in *sir2*Δ cells (Figure 2) and lacks heterochromatin-specific modifications, we propose that the spreading of Swi6 and H3-K9 methyl modification into the *ura4*$^+$ gene requires Sir2. Alternatively, expression of *ura4*$^+$ in *sir2*Δ mutant cells directly affects H3-K9 at the *cenH* region. Taken together, these results suggest that *sir2*$^+$ plays an important role in establishing the histone code for silencing of the mating-type region and that this role involves the deacetylation of H3-K9, which is required for the methylation of H3-K9 and localization of Swi6 to chromatin. In particular, Sir2 appears to play a crucial role in spreading of H3-K9 methylation and Swi6 to regions outside of *cenH* at the silent mating-type interval.

Interestingly, the distribution of H3-K9 methylation at the *mat2/3* region of *sir2*Δ cells closely resembles that of *swi6* mutant cells. In both cases, H3-K9 methylation seems to be primarily restricted to the *cenH* nucleation center [25] (Figure 4). Based on these observations, it is possible that the recruitment of H3-K9 methylation at the *cenH* might occur via a Swi6- and Sir2-independent mechanism, but these proteins are required for the efficient spreading of H3-K9 methylation and heterochromatin formation across the rest of the *mat2/3* interval.

Role of spSir2 in Silencing at Telomeres

We also investigated the possible role of *sir2*$^+$ in gene silencing at fission yeast telomeres. In *sir2*Δ cells, silencing of the *ura4*$^+$ inserted in the telomeric region (*TEL::ura4*$^+$) was strongly reduced, as indicated by robust growth on medium lacking uracil and poor growth on FOA medium (Figure 5B). At the telomeric-associated sequences (TAS), H3-K9 and -K14 acetylation levels were increased by 3- to 5-fold in *sir2*Δ compared to *sir2*$^+$ cells (Figures 5C and 5D). Moreover, Swi6 and H3-K9 methylation at telomeres was reduced 7- and 10-fold, respectively, in *sir2*Δ compared to *sir2*$^+$ cells (Figures 5E and 5F). These results indicate that Sir2 is required for efficient silencing at telomeres. We also observed a decrease in H3 Lys9 methylation, Swi6 localization, and silencing at ribosomal DNA (rDNA) in *sir2*Δ compared to *sir2*$^+$ cells (see Figure S3 in the Supplemental Data available with this article online).

Conclusions

The results presented here show that the fission yeast Sir2 protein is an NAD$^+$-dependent deacetylase that plays an important role in heterochromatin assembly. In particular, Sir2 has an essential role in silencing at the mating-type loci, telomeres, and the inner centromeric repeats. Our results suggest that the molecular function of Sir2 in silencing involves the NAD$^+$-dependent deacetylation of histone H3-K9 in its target chromatin domains. Deacetylation of this H3 lysine is believed to be required for methylation of H3-K9 by the Clr4 methyl-

transferase and the subsequent localization of Swi6 to silent chromatin. We note that our data do not rule out the possibility that Sir2 deacetylates other lysine residues, in histones or other proteins that may be involved in the assembly of heterochromatin in *S. pombe*.

The function of Sir2 in *S. pombe* has striking overlaps with its role in silencing in the distantly related *S. cerevisiae*. In both yeasts, Sir2 is required for silencing at the silent mating-type loci and telomeres. Many of the silencing proteins in *S. pombe* have homologs in *Drosophila* and mammals. These and previous observations suggest that Sir2-like deacetylases play a critical role in the assembly of heterochromatin in a wide spectrum of organisms. For example, *Drosophila Sir2* is required for the efficient silencing of a reporter gene in centric heterochromatin and for repeat-induced gene silencing [26, 27]. However, *Drosophila Sir2*, as well as the mouse *Sir2*α gene, are also involved in promoter-specific transcriptional repression [26, 28], suggesting broad functions for the Sir2-like deacetylases in multicellular eukaryotes.

Several possibilities can explain the requirement of both NAD^+-dependent and NAD^+-independent deacetylases in silencing in *S. pombe*. One possibility is that this requirement reflects the need for deacetylation of multiple lysine residues in the assembly process and is independent of the mechanism of deacetylation by these enzymes. Multiple deacetylases with different substrate specificities may simply be required to deacetylate different amino-terminal lysine residues. In this regard, Clr3 histone deacetylase has been shown to specifically deacetyate H3-K14 [7, 29]. The second possibility is that the unique mechanism of deacetylation by the Sir2 family, involving NAD^+, makes an important contribution to the assembly or regulation of heterochromatin. Yet another possibility is that there might be a division of labor among these different classes of histone deacetylases. In any case, the requirement for NAD^+-dependent deacetylation suggests that the assembly of heterochromatin in *S. pombe* is responsive to the metabolic state of the cell, as is the case in *S. cerevisiae* [30].

Supplemental Data

Supplemental Data including a description of sequence conservation of Sir2 proteins and the requirement of *S. pombe* Sir2 in histone methylation and silencing at the rDNA locus are available at http://www.current-biology.com/cgi/content/full/13/14/1240/DC1/.

Acknowledgments

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