Heterochromatin consists of highly ordered nucleosomes with characteristic histone modifications. There is evidence implicating chromatin remodeling proteins in heterochromatin formation, but their exact roles are not clear. We demonstrate in Saccharomyces cerevisiae, the Fun30p and Isw1p chromatin remodeling factors are similarly required for transcriptional silencing at the HML locus, but they differentially contribute to the structure and stability of HML heterochromatin. In the absence of Fun30p, only a partially silenced structure is established at HML. Such a structure resembles fully silenced heterochromatin in histone modifications, but differs markedly from both fully silenced and derepressed chromatin structures regarding nucleosome arrangement. This structure likely represents an intermediate state of heterochromatin that can be converted by Fun30p to the mature state. Moreover, Fun30p removal reduces the rate of de novo establishment of heterochromatin, suggesting that Fun30p assists the silencing machinery in forming heterochromatin. We also find evidence suggesting that Fun30p functions together with, or after, the action of the silencing machinery. On the other hand, Isw1p is dispensable for the formation of heterochromatin structure, but is instead critically required for maintaining its stability. Therefore, chromatin remodeling proteins may rearrange nucleosomes during the formation of heterochromatin, or serve to stabilize/maintain heterochromatin structure.
The high regularity of nucleosomes in heterochromatin may be achieved during chromatin replication when new nucleosomes are formed. Alternatively, or in addition, preexisting nucleosomes may be repositioned by chromatin remodeling factors to form ordered arrays. None of the Sir proteins has chromatin remodeling activity. However, there is evidence for the involvement of three chromatin remodeling proteins Isw1p, Snf2p and Fun30p in transcriptional silencing. Isw1p is required for HMR but not telomeric silencing, whereas Snf2p is required for telomeric but not HM silencing (20,21). Fun30p, on the other hand, is required for silencing at both HMR and telomeric loci, and is associated with heterochromatin (22). Whereas the chromatin remodeling activities of Isw1p and Snf2p have been long established, Fun30p has only recently been shown to possess histone H2A/H2B dimer exchange and nucleosome sliding activities (23). How any of these factors contributes to heterochromatin structure is not known.

In this report, we examined the roles of Fun30p and Isw1p in heterochromatin at the HML locus. We found that although Fun30p and Isw1p are similarly required for efficient HML silencing, they differentially contribute to the structure and maintenance of HML heterochromatin.

**Experimental Procedures**

**Yeast strains.**

Yeast strains are listed in Table 1. Each strain is numbered according to the order of its first appearance. Replacement of a gene with the *kanMX* or *natMX* marker was achieved by transforming the parental strain to geneticin- or nourseothricin-resistant with a PCR-generated fragment composed of *kanMX* or *natMX* bracketed by 5'- and 3'-flanking sequences of the coding region of the gene to be disrupted. Replacing a gene with the *HIS3* or *URA3* marker was achieved by transforming the parental strain to histidine or uracil prototrophy with a PCR-generated fragment composed of *HIS3* or *URA3* gene bracketed by 5’- and 3’-flanking sequences of coding region of the gene to be disrupted. Strain 15 was made by transforming strain 7 to geneticin-resistant with Thh1111-digested plasmid pUC-SK. pUC-SK was made by inserting the *sir3-8* allele and *kanMX* cassette into pUC19.

**Analysis of DNA topology**

Cells were grown in YPR medium (1% yeast extract, 2% bacto-peptone and 2% raffinose) to log or stationary phase. Galactose (2%) was added to the culture that was further incubated for 2.5 hr to induce the expression of *P*gal*10-FLP1*. Nucleic acids were isolated using the glass bead method and fractionated on an agarose gel supplemented with 26 µg/ml chloroquine. After Southern blotting, the DNA circles were detected by an *HML*-specific probe. The profile of topoisomers from specific samples was obtained using the NIH image software.

**Chromatin mapping with micrococcal nuclease**

Chromatin mapping was carried out as previously described (25). About 1 x 10^6 log phase cells were made into spheroplasts by zymolyase treatment. The spheroplasts were permeabilized with NP-40 as described (28). About 2 x 10^8 permeabilized spheroplasts were treated with MNase at 120 or 160 units/ml at 37°C for 5 minutes. The reaction was stopped by 0.5% SDS and 25 mM EDTA, and DNA was isolated. An aliquot of DNA was determined to contain fragments indicative of the presence of nucleosome ladders by gel electrophoresis. An aliquot of permeabilized spheroplasts not treated with MNase was used to isolate genome (naked) DNA that was digested with MNase at 7.5 units/ml. DNA from each sample was then digested with PvuII, EcoRI or NgolIV and run on a 1.0% agarose gel. Relevant fragments were visualized by hybridization with probe 1, 2 or 3 after Southern blotting.

**Chromatin immunoprecipitation (ChIP)**

ChIP was carried out primarily as described (29). Briefly, crosslinked chromatin from 50 ml mid-log phase cell culture was sonicated with Branson Sonifier 450 to yield DNA fragments of an average size of 500 bp. According to the A_260_, 120 U of whole-cell extract was used for immunoprecipitation with antibody against total histone H3 (C terminus, Abcam), H3 acetylated at lysines 9 and 14 (H3-K9,14-Ac, simplified as H3-Ac) (Upstate), H4-K5,8,12,16-Ac (H4-Ac) (Upstate), H3 tri-methylated at K79 (H3-K79-Me) (Abcam), or Myc (Roche). For semiquantitative
PCR reactions, the proper amount of input or immunoprecipitated chromatin DNA to use was predetermined to be in the linear range by serial dilution analysis. PCR primers used were HML-1F (5'-GAACTAGCTTCTCCGGATGGCA-3') and HML-1R (CCCTTATCTACTTGCCTCTTTTGTT) for ChIP experiments with antibodies against histone H3, H3-Ac, H4-Ac and H3-K79-Me, HML-2F (AAATCTTTCACGTCTTTTCTGTG) and HML-2R (CTCACGTTACAGAATTTTCG), and HMR-F (TCCCGTTCAAGTTATGAGC) and HMR-R (TCGGAAATCGAGAATCTTCGT), as well as ACT1-F (GCGCTAGAACATACCAGAATCC) and ACT1-R (GATCCTTTTCCTTCCCAATCTCTC) for ChIP with Myc antibody. To correct for potential variations in nucleosome occupancy, the abundance of H3-Ac, H4-Ac or H3-K79-Me was calculated as the ratio of signal (IP/input) obtained using antibody against H3-Ac, H4-Ac or H3-K79-Me over that obtained using antibody against total H3. The means of data from three independent experiments together with corresponding standard deviations were presented.

Results

**FUN30 and ISW1 are required for efficient transcriptional silencing at the HML locus**

Although heterochromatin at HML and HMR loci is formed via a common mechanism, the extent and regulation of transcriptional silencing at each locus is not identical. For example, HMR silencing is more dependent on the nearby telomere than HML silencing, while HML silencing is preferentially affected by certain histone mutations (30,31). Although FUN30 and ISW1 are required for HMR silencing, it has not been examined whether they are also required for HML silencing (20,22). To address this question, we deleted FUN30 and ISW1 individually from strain 1 bearing a URA3 reporter in the middle of HML (Fig. 1A; Table 1). URA3 expression makes cells sensitive to 5-fluoroorotic acid (FOA), so URA3 silencing can be measured by cell growth on FOA-containing medium (32). We found that deletion of FUN30 and ISW1 reduced URA3 silencing to similar degrees (Fig. 1A, compare 2 and 3 with 1). Therefore, Fun30p and Isw1p are required for full transcriptional silencing at HML.

The fact that neither FUN30 deletion (fun30Δ) nor isw1Δ completely eliminated HML silencing as sir3Δ does (Fig. 1A, compare 2 and 3 with 1s) made us wonder whether Fun30p and Isw1p were redundantly required for HML silencing. We showed that simultaneously deleting both FUN30 and ISW1 caused a decrease in URA3 silencing that is slightly more severe than the reduction of silencing caused by fun30Δ or isw1Δ alone (Fig. 1A, compare 4 with 2 and 3). Therefore, Fun30p and Isw1p seem to play partially overlapping roles in HML silencing.

To complement the assay of URA3 silencing by monitoring cell growth on FOA containing medium, we also used Northern blotting to directly measure the level of URA3 mRNA in wild type and sir3Δ strains, as well as fun30Δ and isw1Δ single and double mutants. As expected, URA3 mRNA was abundant in the sir3Δ strain in which silencing was abrogated, but was hardly detectable in Sir3Δ (WT) strain where URA3 at HML was silenced (Fig. 1B, note the abundance of URA3 mRNA in WT strain was 11 fold less than that in sir3Δ strain). In the fun30Δ or isw1Δ strain, URA3 mRNA level was 5 fold less than that in WT strain, but 2 fold more than that in sir3Δ strain (Fig. 1B), supporting the notion that FUN30 and ISW1 are required for full transcriptional silencing. URA3 mRNA in the fun30Δ isw1Δ double mutant was 2 fold more abundant than that in either single mutant (Fig. 1B), suggesting that FUN30 and ISW1 play partially overlapping roles in HML silencing. In summary, these results are fully consistent with results of monitoring cell growth on FOA medium (Fig. 1A and 1B).

**FUN30 but not ISW1 is required for the high negative supercoiling of silent HML DNA**

As both Fun30p and Isw1p have chromatin remodeling activities (23,33), they may contribute to the formation of the special heterochromatin structure. We tested this notion using a DNA topology based assay. In eukaryotes, formation of each nucleosome constrains on average one negative supercoil on nucleosomal DNA (34). This number is reduced (to as low as 0.8) if histones in the nucleosome are acetylated (35,36). Therefore, the topology of DNA spanning a specific region is
a measure of the state of local chromatin. We and others have previously developed a method to examine DNA topology at a particular locus by excising the region as a circular minichromosome via site-specific recombination in vivo, and isolating the DNA circle whose supercoiling can be determined by gel electrophoresis in the presence of a DNA intercalator (Fig. 1B) (27,37). Using this method, it was found that DNA from HML or HMR is more negatively supercoiled when the locus is silenced than when it is derepressed (24,27,37), which is a reflection of the high regularity/density of nucleosomes as well as low histone acetylation in heterochromatin (10,11,38,39).

To test if FUN30 and ISW1 play roles in heterochromatin structure at HML, we deleted them individually from strain 5 in which the modified HML locus excluding the E and I silencers are flanked by two copies of FRT (Flp1p recombination target), the recognition site for the site-specific recombinase Flp1p (Fig. 1B). Induction by galactose of a pGal1p-FLP1 gene integrated elsewhere in the genome would lead to the expression of Flp1p and recombination between the FRT sites, resulting in the excision of an HML circle (Fig. 1B). After being deproteinized, the supercoiling of the circle could be examined by gel electrophoresis in the presence of the DNA intercalator chloroquine (Fig. 1C, strain 5). Deletion of SIR3, which completely disrupts heterochromatin, reduced the negative supercoiling of HML circle by a linking number change (ΔLk) of 9 (Fig. 1C, compare the centers of topoisomer distributions in lanes 5 and 9; note that more negatively supercoiled circles migrate more slowly under the condition used).

We showed that fun30Δ reduced the negative supercoiling of HML DNA by a linking number change of ~2 (ΔLk = ~2) (Fig. 1C, compare 5 and 6), suggesting a role of Fun30p in heterochromatin structure. It is obvious that the fun30Δ-induced change in HML DNA topology is significantly smaller than the ΔLk of 9 caused by the complete disruption of heterochromatin by sir3Δ (Fig. 1C, compare 6 and 7 with 5), which is consistent with the fact that fun30Δ does not completely abolish HML silencing (Fig. 1A). Unlike fun30Δ, isw1Δ did not reduce the supercoiling of HML DNA (Fig. 1C, compare 7 with 5). On the other hand, a small portion of HML circles from isw1Δ cells had a topology similar to circles from sir3Δ cells (Fig. 1C, compare 7 and 9). We have shown previously that silent HML circles lacking silencers would gradually lose their high negative supercoiling and assume a topology similar to HML circles in sir cells when the host cells progress in the cell cycle, which suggests that the heterochromatin dissociated from silencers is subject to disruption during cellular proliferation (27). Therefore, the minor population of sir circles from isw1Δ cells was the result of disruption of heterochromatin on HML circle during the 2.5 hour induction of circle excision when cells continued to grow. The fact that no sir circles existed in samples from WT and fun30A strains suggest that HML heterochromatin is less stable in isw1Δ cells than in WT or fun30Δ cells.

HML circles from the fun30Δ isw1Δ strain consisted two major populations, one with a topology similar to fun30Δ circles and the other similar to sir circles (Fig. 1C, compare 8 with 6 and 9). This indicates that the effect of fun30Δ on the topology of HML DNA is dominant over than of isw1Δ, and that fun30Δ and isw1Δ have a synthetic destabilizing effect on the HML heterochromatin.

**Contribution of Fun30p to the special structure of heterochromatin.**

Our finding that fun30Δ reduces the negative supercoiling of HML DNA (Fig. 1C) suggests that fun30Δ alters the structure of heterochromatin. To directly test if Fun30p plays a role in the unique primary structure of heterochromatin, we mapped nucleosomes within HML in fun30Δ and FUN30 cells by micrococcal nuclease (MNase) digestion and indirect end labeling. As MNase preferentially digests linker DNAs connecting the nucleosomes, indirect end labeling of MNase digested DNA can reveal the borders of nucleosomes in the region of interest thereby allowing the inference of the positions of nucleosomes (40). DNA from chromatin treated with MNase was isolated and subjected to digestion by EcoRI, PvuII or NgoMIV at the HML locus (Fig. 2A and 3A), followed by electrophoresis and Southern blotting. DNA fragments ending at the EcoRI, PvuII and NgoMIV restriction sites were detected
by probes 1 through 3, respectively (Fig. 2A and Fig. 3A). In the wild type (WT) strain, 20 positioned nucleosomes could be inferred from the MNase digestion pattern of the 3.3 kb HML sequence bracketed by the E and I silencers (Fig. 2B, 2C and 3B, filled ovals labeled 1 through 20). The positions of these nucleosomes generally matched those of the 20 positioned nucleosomes (numbered 1 through 20) at HML inferred from previous chromatin mapping results obtained by Weiss and Simpson and us (Fig. 2A) (11,12). We numbered the 20 nucleosomes following Weiss and Simpson’s early designations (11). Note the region between nucleosomes 9 and 10 containing the UAS of the $\alpha$ genes was free of nucleosomes (Fig. 2B). Deletion of SIR2 caused salient changes in the profile of MNase sensitive sites across most of HML (Fig. 2B and C, compare sir2Δ with WT; note the differences in intensities of MNase digestion sites between sir2Δ and WT lanes as indicated by open diamonds). These changes affected the borders of nucleosomes 4 through 16 to various extents, confirming that Sir complex promotes nucleosome rearrangement during the formation of heterochromatin structure at HML (11,12). We noted that the lower parts of the Southern blots shown in Fig. 2B were overexposed, which might have obscured changes in intensities of MNase sites there. To address this issue, we also presented the same blots that had been exposed for a shorter time in Fig. S1. This more clearly revealed the sir2Δ-induced changes around the shared borders of nucleosomes 3 and 4 (Fig. S1, compare sir2Δ with WT).

We showed that deletion of FUN30 induced multiple alterations in the MNase digestion profile at HML (Fig. 2B and 2C and Fig. S1, note the unique presence or change in intensity of MNase sites in fun30Δ lanes vs. WT lanes as indicated by black dots). Part of the alterations affected the borders of nucleosomes 5 to 8 in such a way that they suggest a fun30Δ-induced reduction in the translational dynamics of these nucleosomes (Fig. 2B and Fig. S1, compare fun30Δ and WT, note that sites coinciding with the borders of nucleosomes 5 to 8 were more accessible to MNase in fun30Δ vs. WT cells).

FUN30 deletion induced the appearance of two strong MNase digestion sites in the regions covered by nucleosomes 4 and 9, suggesting a repositioning/sliding of these nucleosomes to positions 4’ and 9’, respectively (Fig. 2B and S1). As a consequence, nucleosomes 4’ and 5’ were separated by a relatively big gap, so were nucleosomes 8’ and 9’ (Fig. 2B and S1). The presence of these gaps may be related to the reduced silencing function of HML heterochromatin in fun30Δ cells (Fig. 1A).

A series of changes caused by fun30Δ affected the borders of nucleosomes 15'-19 in such a way that they suggest a fun30Δ-induced sliding en bloc of nucleosomes 15-19 (and 20 by inference) toward the HML-E silencer to positions denoted 15’–20’ (Fig. 2C, compare fun30Δ and WT).

Importantly, the changes in MNase digestion (hence the inferred alterations in nucleosome positioning) within HML caused by fun30Δ generally did not overlap with those induced by sir2Δ (Fig. 2B and 2C). Therefore, in the absence of Fun30p, chromatin at HML adopts a unique structure that is different from both the fully silent heterochromatin structure (in WT strain) and the fully derepressed chromatin structure (in sir2Δ strain).

Mapping chromatin in the region surrounding the HML-I silencer revealed 4 nucleosomes (designated –1 through –4) positioned immediately outside of the border of HML defined by HML-I (Fig. 3B). Deletion of SIR2 renders two sites more accessible to MNase digestion with one in nucleosome –1 and the other around the shared border of nucleosomes –1 and –2 (Fig. 3B, bands in sir2Δ lanes indicated by open diamonds). These changes suggest that nucleosomes –1 and –2 can adopt alternative positions in derepressed chromatin. Therefore, formation of Sir-dependent heterochromatin involves the repositioning/stabilization of nucleosomes –1 and -2. Interestingly, fun30Δ induced extensive changes in MNase digestion pattern in the region spanning nucleosomes –1 through –4 (Fig. 3B, right, note the unique presence or enhancement of MNase sites in fun30Δ lanes vs. WT lanes as indicated by black dots). These changes suggest that in the absence of FUN30, nucleosomes -1 through -4 can slide en bloc toward the HML-I silencer to adopt alternative positions –1’ through –4’ (Fig. 3B, right). Again, these changes did not overlap with the changes...
induced by sir2Δ.

In summary, we found evidence indicating that fun30Δ leads to the change in the positioning and/or stability of at least 12 nucleosomes (#4 to #9 and #15 to #20) within HML and 4 nucleosomes outside of HML (#-1 to #-4) (Fig. 2 and 3). Therefore, Fun30p plays a major role in the primary structure of HML heterochromatin. In the absence of Fun30p, heterochromatin seems to adopt an altered/intermediate state that is distinct from the fully silenced state (in WT cells) and the completely derepressed state (in sir2Δ cells).

**Isw1p does not significantly contribute to heterochromatin structure.**

We found that, unlike fun30Δ, isw1Δ did not cause substantial changes in HML chromatin (Fig. 2B, 2C and 3B, compare isw1Δ and WT lanes), which was correlated with the lack of effect of isw1Δ on HML DNA supercoiling (Fig. 1C). One relatively obvious change induced by isw1Δ was the moderate increase in MNase sensitivity of a site coinciding with the shared border of nucleosomes 17 and 18 (Fig. 2C). Note that this change coincides with one of many alterations induced by fun30Δ (Fig. 3B, compare fun30Δ and isw1Δ). Therefore, unlike Fun30p, Isw1p seems to make minimum contribution to the primary structure of HML heterochromatin.

**Fun30p and Isw1p do not play redundant roles in heterochromatin structure.**

It is possible that although Isw1p does not contribute significantly to heterochromatin structure on its own, it synergizes with Fun30p to facilitate the formation of heterochromatin. To test this hypothesis, we mapped HML chromatin in isw1Δ fun30Δ double mutant. We found that the pattern of MNase digestion in the double mutant was identical with that in the fun30Δ mutant (but not with that in the isw1Δ mutant) (Fig. 2B, 2C and 3B, compare isw1Δ fun30Δ with fun30Δ and isw1Δ lanes). Therefore, fun30Δ has a dominant effect over isw1Δ on HML heterochromatin structure, and Fun30p and Isw1p do not cooperate to contribute to heterochromatin structure at HML.

**Fun30p does not remodel derepressed HML chromatin**

Results from the above experiments strongly suggest an important role of Fun30p in the formation of the primary heterochromatin structure at HML. It is possible that Fun30p helps to create a chromatin configuration prior to heterochromatin formation that is favorable for the spreading of Sir complex. This model predicts that Fun30p modulates HML chromatin in the absence of Sir complex. We tested this model by comparing HML chromatin structure in fun30Δ to that in FUN30 strain in the sir2Δ background. We found no obvious differences between sir2Δ and sir2Δ fun30Δ strains regarding the profile of MNase digestion across the entire HML locus (Fig. 4B through 4D, compare fun30Δ and FUN30). Therefore, fun30Δ does not affect derepressed HML chromatin structure in the absence of Sir complex, which argues against the model that Fun30p facilitates the formation of heterochromatin structure by modulating derepressed chromatin in preparation for Sir complex spreading.

**FUN30 is required for de novo establishment of fully silent heterochromatin structure.**

Given that fun30Δ makes heterochromatin assume an altered/intermediate state, but does not affect derepressed chromatin at HML, we propose that Fun30p is specifically involved in the formation of heterochromatin. We examined whether fun30Δ affected de novo establishment of heterochromatin. This was achieved by monitoring the kinetics of the transition from derepressed to silenced state of a circular HML minichromosome following the activation of the Sir complex in a strain bearing sir3-8, a temperature sensitive allele of SIR3 (41). Sir3-8p is nonfunctional at 30°C but functional at 23°C (41). The state of the HML minichromosome was measured by examining the negative supercoiling of the DNA.

A pair of isogenic sir3-8 FUN30 and sir3-8 fun30Δ strains were constructed in which HML including the silencers was flanked by FRTs, and P_{GAL-FLP1} was integrated elsewhere in the genome (Fig. 5A). Each strain was first grown to log phase at 30°C in YPR medium. Galactose was then added to the culture that was further incubated for 2.5 hr to induce P_{GAL-FLP1} and the excision of the HML circle. Cells were then shifted to YPD (YP + glucose) and further grown at 23°C. The short half-
life of Flp1p and the stringent repression by glucose of \( P_{Gal}\)-FLP1 ensured that HML circles were excised exclusively during the 2.5 hr of galactose induction (27,42,43). The HML circle isolated at 30°C assumed a topology characteristic of HML circle from a sir strain (Fig. 5B, compare lanes 1 and 9 with the sir lane), which verifies the inactivity of sir-3-8 at 30°C.

HML circles were isolated from aliquots of culture taken at a series of time points after the temperature shift from 30°C to 23°C. We found that in FUN30 cells, starting around hour 4, the HML circles became more and more negatively supercoiled (Fig. 5B, lanes 5 through 8), which is indicative of the formation of heterochromatin on these circles. The gradual nature of the increase of negative supercoiling found in the HML DNA (Fig. 5B) suggests that formation of fully silenced chromatin was a stepwise process.

HML circles in fun30Δ cells also became more negatively supercoiled after incubation at 23°C (Fig. 5B, lanes 9 through 16), suggesting that heterochromatin was able to form on these circles in the absence of Fun30p. However, the level of negative supercoiling of HML circle did not increase significantly after hour 6 (Fig. 5B, lanes 14 through 16), which is different from the gradual increase of negative supercoiling found in the FUN30 background (Fig. 5B, lanes 6 through 8). As a consequence, the final level of supercoiling of HML circle (after prolonged incubation at 23°C) was lower in fun30Δ cells than in FUN30 cells (Fig. 5B and 5C, compare fun30Δ and FUN30 samples at 20 hr). These data are consistent with the notion that in fun30Δ cells, an altered/intermediate state of heterochromatin is formed, which fails to be further converted to the fully silent state of heterochromatin (as can be formed in FUN30 cells). In addition, we showed that the rate of conversion of HML circles from derepressed state to silent state was reduced by fun30Δ (Fig. 5B and 5C, note that at hour 4 the residual population of sir- circles in fun30Δ cells was significantly larger than that in FUN30 cells). Therefore, Fun30p is required for efficient de novo establishment of fully silenced heterochromatin.

Heterochromatin in fun30Δ cells retains characteristic histone modifications

Yeast heterochromatin is characterized by histone hypoacetylation and hypomethylation (9,13,14,38). We wondered whether the altered/intermediate state of heterochromatin formed in fun30Δ cells is different from fully silenced heterochromatin in FUN30 cells regarding the status of histone acetylation and methylation. To address this question, we measured the occupancy of acetylated histones H3 and H4 (H3-Ac and H4-Ac) and histone H3 tri-methylated at lysine 79 (H3-K79-Me) at HML in fun30Δ, FUN30 (WT) and sir2Δ cells by chromatin immunoprecipitation (ChIP). Three independent experiments were performed. The abundance of an HML sequence in immunoprecipitated chromatin fragments was measured by PCR, and a representative gel picture was shown in Fig. 6A. The intensity of each band was quantified and normalized against input control. To correct for potential variations in nucleosome occupancy in different strains, the occupancy of H3-Ac, H4-Ac or H3-K79-Me in each strain was presented as the ratio of ChIP signal (IP/input) obtained using an antibody against the respective modified histone over that obtained using an antibody against total histone H3.

As expected, the abundances of H3-Ac and H4-Ac at HML in WT cells were markedly lower than their respective counterparts in sir2Δ cells (Fig. 6A through 6C), confirming Sir-dependent histone hypoacetylation in heterochromatin. The levels of H3-Ac and H4-Ac at HML in fun30Δ cells were similar to those in WT cells (Fig. 6A through 6C). Moreover, fun30Δ and WT cells were similar regarding the abundance of H3-K79-Me at HML (Fig. 6A and 6D). Therefore, fun30Δ does not affect the hypoacetylation of histone H3 and H4 and the hypomethylation of H3-K79 in heterochromatin.

Association of Fun30p with the HML and HMR loci

We tested if Fun30p could be cross-linked to HML locus by ChIP. The C terminus of endogenous FUN30 in strain 10 was tagged with Myc to make strain 18. Strain 18 was phenotypically indistinguishable from 9, and Fun30p-Myc was expressed as determined by Western blotting (data not shown). An anti-Myc antibody was used to perform ChIP on strain 18.
The abundance of sequences from the silent \textit{HML} and \textit{HMR} loci, as well as active \textit{ACT1} locus in the immunoprecipitated chromatin fragments was measured by PCR. Three independent experiments were performed, and a representative gel picture was shown in Fig. 7A. The intensity of each band was quantified and normalized against input control. The mean of data from all of the experiments was graphed in Fig. 7B. We found that Fun30p-Myc to be preferentially enriched at the silent \textit{HML} and \textit{HMR} loci (Fig. 7B). This is consistent and extends an earlier study showing association of Fun30 with \textit{HMR} locus (22).

\textbf{Isw1p plays a key role in maintaining the stability of \textit{HML} heterochromatin}

The fact that a small portion of silencer-free \textit{HML} circles in \textit{isw1Δ} cells assumes a topology similar to \textit{sir} circles (Fig. 1C) suggests that Isw1 is important for the stability of \textit{HML} chromatin. To further examine the role of Isw1 in maintaining heterochromatin structure, we examined the effect of \textit{isw1Δ} on the kinetics of the loss of the silent state of silencer-free \textit{HML} circle after its excision from the genome (27).

Strains 5 (wild type) and 7 (\textit{isw1Δ}) were grown in parallel to stationary phase before being treated with galactose for 2.5 hr to induce the excision of silencer-free \textit{HML} circles. Cells were then washed and diluted with fresh glucose medium, which inhibited \textit{Pgal10-FLP1} and allowed cell cycle progression to resume. The topology of the \textit{HML} circle was followed during further cell growth in glucose medium. As cells were not growing (in stationary phase) when circles were being excised, the silent state on the \textit{HML} circle was not disrupted. This was reflected by the fact that \textit{HML} circle in wild type or \textit{isw1Δ} cells lacks \textit{sir} topoisomers at 0 hr time point (Fig. 8, lanes 1 and 6). In wild type cells, as expected, the proportion of topoisomers with high negative supercoiling characteristic of heterochromatin (\textit{SIR'}) gradually decreased, whereas that with lower negative supercoiling characteristic of derepressed chromatin (\textit{sir'}) increased as a function of growth time (Fig. 8B, compare lanes 2 through 5 with 1). The rate of conversion of the \textit{HML} circle from the \textit{SIR'} to \textit{sir'} state was substantially higher in \textit{isw1Δ} cells than that in wild type cells (Fig. 8B, compare \textit{isw1Δ} and wild type). For example, \textit{sir'} topoisomers were readily detectable by hour 2 in \textit{isw1Δ} cells but were not present even by hour 4 in wild type cells (Fig. 8B, compare lane 7 with 3). By hour 6, most of \textit{HML} circles had been converted to the \textit{sir'} state in \textit{isw1Δ} cells, while the majority of \textit{HML} circles maintained the \textit{SIR'} state in wild type cells (Fig. 8B, compare lane 10 with 4). These results demonstrate a critical role of Isw1p in the stability of \textit{HML} heterochromatin.

We also examined if \textit{fun30Δ} affected the stability of \textit{HML} chromatin. We showed that the rate of loss of silent state of \textit{HML} circles in \textit{fun30Δ} cells was higher than that in wild type cells (Fig. 8B, compare lanes 12 – 17 with 1 – 5), albeit to a lesser extent relative to that in \textit{isw1Δ} cells (Fig. 8B, compare lanes 12 – 17 with 6 – 11). This result suggests that \textit{HML} chromatin in \textit{fun30Δ} cells is less stable than that in wild type cells.

We further examined whether Isw1p was involved in the maintenance of the stability of \textit{HML} chromatin in \textit{fun30Δ} cells. We showed that the loss rate of the silent state of \textit{HML} circle in the \textit{fun30Δ isw1Δ} double mutant was markedly greater than that in \textit{fun30Δ} and \textit{isw1Δ} single mutants (Fig. 8B, compare lanes 18 to 24 with 12 to 17; note that by hour 2, nearly all \textit{HML} circles had been converted to the \textit{sir'} state in the \textit{fun30Δ isw1Δ} double mutant, but no \textit{sir'} topoisomers were detectable in \textit{fun30Δ} cells). Therefore, Isw1p is also required for the stability of the intermediate state heterochromatin formed in the absence of Fun30p. In other words, Fun30p and Isw1p play redundant roles in the maintenance of heterochromatin stability.

\textbf{Discussion}

Establishment of heterochromatin in eukaryotes involves rearrangement of the primary chromatin structure and potentially formation of high-order structures (2-4). Chromatin remodeling activities have been implicated in the formation of heterochromatin in different model organisms. In \textit{Drosophila}, the chromatin remodeling factor dATRX is involved in heterochromatin function and co-localizes with the heterochromatin protein HP1 (5). In fission yeast, a Snf2p homolog Mit is required for heterochromatin at the silent mating locus, whereas the histone chaperone and remodeling complex FACT is required for
centromeric-heterochromatin function (7,8). In the budding yeast *S. cerevisiae*, chromatin remodeling proteins Fun30p, Isw1p and Snf2p each have been implicated in heterochromatin at one or more loci (20-22). However, the roles of chromatin remodeling factors in the structure and maintenance of heterochromatin remain unresolved. We show in this report that Fun30p plays a key role in nucleosome rearrangement associated with the formation of heterochromatin, whereas Isw1p is critically required for the stability of heterochromatin.

Deletion of *FUN30* results in extensive changes in the primary structure of heterochromatin pertaining to nucleosome positioning at *HML* (Fig. 2 and 3). However, these changes are fundamentally different from those caused by *sir2Δ* that completely disrupts heterochromatin (summarized in Fig. 9). These results, together with the fact that partial *HML* silencing is maintained in *fun30Δ* cells (Fig. 1A), suggest that in the absence of Fun30p, a partially silent, intermediate state of heterochromatin that differs from both the fully silent state and fully derepressed state of chromatin is formed. This intermediate state is associated with hypoacetylation and hypomethylation of histones (Fig. 6), two hallmarks of heterochromatin (9,13,14,38). Why the putative intermediate state of heterochromatin in *fun30Δ* cells is less efficient in silencing relative to heterochromatin in *FUN30* cells? We found that *fun30Δ* creates relatively large gaps between two adjacent nucleosomes in two instances (Fig. 2B, see the gap between 4’ and 5’, and that between 8’ and 9’). Given that large gaps in the nucleosome array are disruptive to heterochromatin function (25), it is possible that these *fun30Δ*-induced gaps in *HML* heterochromatin are at least part of the reason for the decrease in silencing. Therefore, Fun30p may serve to remove gaps in chromatin thereby contributing to efficient silencing mediated by heterochromatin.

Fun30p might directly contribute to the formation of heterochromatin structure by the following three mechanisms that are not mutually exclusive. First, Fun30p may modulate chromatin before Sir complex spreading. As the Sir complex deacetylates and propagates along chromatin, the structure of the preexisting chromatin has the potential of influencing the efficiency of Sir complex spreading. It is believed that a highly regular array of nucleosomes is more inducive to Sir protein spreading than a random array. In fact, we have shown that large gaps between nucleosomes are inhibitory to the propagation of Sir complex (25). Fun30p may reposition nucleosomes into a better-ordered array that is more favorable for Sir complex spreading. However, this notion is not supported by our finding that Fun30p does not modulate *HML* chromatin structure in a *sir* background (Fig. 4). Secondly, Fun30p may remodel or reposition nucleosomes simultaneously with the spreading of Sir complex along chromatin (Fig. 9, curved arrow). Fun30p may remodel a nucleosome immediately after it is deacetylated by Sir complex, or vice versa (i.e., Sir complex deacetylates and binds the nucleosome immediately after it is remodeled by Fun30p). Thirdly, Fun30p may remodel chromatin after Sir complex spreading. This model implies that, without Fun30p, Sir complex spreading (perhaps with the assistance of another yet to be identified chromatin remodeler(s)) only results in the formation of an immature or intermediate state of heterochromatin. Fun30p remodels such a chromatin structure thereby converting it into the final mature heterochromatin that is fully silenced (Fig. 9, sequential arrows designated “Sir complex” and “Fun30p”). The second and third models, especially the third one, are in line with our finding that partially silenced heterochromatin with a unique structure is formed at *HML* in the absence of Fun30p (Fig. 9, b).

There is evidence suggesting that the establishment of yeast heterochromatin involves discrete ordered steps that are regulated by the cell cycle (44-46). When *de novo* formation of heterochromatin is allowed to begin in G1 phase, gene silencing does not occur in G1-phase, limited silencing happens in S-phase, partial silencing is achieved during G2/M, and it is not until telophase of mitosis that robust silencing takes place (44,45). It is possible that *intermediate chromatin structures* are formed in the different stages of heterochromatin formation before the adoption of a final/mature structure. Fun30p might be involved in a specific stage(s) of the process of heterochromatin formation. Given that Fun30p is known to be subject to phosphorylation by the
cyclin-dependent kinase Cdk1p (47), it is possible that the role of Fun30p in the formation of heterochromatin is subject to cell cycle regulation.

Our finding that fun30Δ affects heterochromatin but not derepressed chromatin at HML raises the important question of how Fun30p function is specifically directed to heterochromatin. Many chromatin remodeling proteins associate with other factors to form multisubunit complexes (48). These complexes contain conserved motifs such as the SANT, bromodomain and chromodomain that recognize distinct features of chromatin. Therefore, these domains are responsible for targeting chromatin remodeling complexes to specific chromatin structures/loci. Fun30p belongs to a subfamily of Snf2p-like ATPases that contain one or two copies of a putative CUE (Coupling of Ubiquitin conjugation to ER degradation) motif that is similar to the UBA (Ubiquitin Associated domain) motif that binds ubiquitin (22,49). As there is evidence suggesting an involvement of protein ubiquitination in transcriptional silencing, it is possible that the CUE motif of Fun30p helps to target Fun30p activity to heterochromatin regions by recognizing a ubiquitinated component of heterochromatin.

Deletion of ISW1 reduces HML silencing to a similar extent as fun30Δ does. However, isw1Δ causes minimum change in fun30Δ that does not affect the supercoiling of HML DNA (Fig. 1 - 3), suggesting that Isw1p does not contribute significantly to the primary structure of heterochromatin as Fun30p does. Instead, Isw1p is critically required for the stability of heterochromatin (Fig. 8). This is in line with the fact that Isw1p has the ability to assemble or stabilize nucleosomes (33). Exactly how Isw1p helps to maintain the stability of heterochromatin remains unclear. Based on the fact that Isw1p contains a SANT motif that can recognize unmodified histone tails (50), it is possible that Isw1p is targeted to, and remodels, nucleosomes deacetylated by Sir2p, making them more stable during or after the propagation of Sir complex. The ISWI complex in Drosophila regulates high-order chromatin structure (6). Isw1p might also help to maintain yeast heterochromatin by stabilizing high-order chromatin structures.

The fact that a partially silent chromatin structure that is different from derepressed chromatin in nucleosome arrangement is still formed in the absence of Fun30p (and Isw1p) (Fig. 2 and 3) suggests that other chromatin remodeling activities may also be involved in forming heterochromatin structure. Besides Fun30p and Isw1p, there are several other known chromatin remodeling proteins including Isw2p, Chd1p, Ino80p and Rad54p in yeast (51). Although deleting any one of them does not significantly affect heterochromatic gene silencing (X.Z., Q.Y. and X.B., unpublished results), it is formally possible that these factors play redundant roles in heterochromatin.

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Footnotes
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**Abbreviations:** ChIP, Chromatin immunoprecipitation; FOA, 5-fluoroorotic acid; FRT, Flp1p recombination target; HML, homothallic mating locus left; HMR, homothallic mating locus right.

**Figure legends**

**Fig. 1. Effects of fun30Δ and isw1Δ on transcriptional silencing and DNA supercoiling at HML.** (A) FUN30 and ISW1 are required for efficient HML silencing. Top, schematic of the HML locus bearing a URA3 marker gene in strains 1 to 4 and 1s. HML-E and -I silencers are shown as filled boxes with white letters. Bottom, growth phenotypes of strains 1 to 4 and 1s. Cells of each strain were grown to log phase, and serial 10-fold dilutions were spotted and grown on synthetic medium with (+) or without (-) 1 mg/ml of 5-fluoroorotic acid (FOA). (B) Northern blot analysis of URA3 expression. Total RNA was extracted from log phase cells of each strain, and 10 μg was loaded in each lane. URA3 mRNA was detected by Northern blotting and hybridization with a radioactive probe made from the coding sequence of URA3. Note that in the strains tested here, the transcript from the nonfunctional *ura3-52* allele at the normal *URA3* locus on chromosome V is truncated, due to the insertion of a Ty element into the *URA3* coding sequence. Therefore, transcripts from both *URA3* gene at HML and *ura3-52* allele could be examined simultaneously. As the normal *URA3* locus is not subject to SIR-dependent silencing, *ura3-52* mRNA was used as an internal control for loading. The intensity of each band was quantified using NIH image, and the relative abundance of *URA3* mRNA in each strain was calculated as the ratio of *URA3* mRNA signal over *ura3-52* mRNA signal. (C) Strategy for examining the topology of HML DNA (3). Two FRT (Flp1p recombination target) sequences (filled arrows) are inserted to flank HML. Recombination between FRTs by the site-specific recombinase Flp1p excises HML as a circular minichromosome. (D) FUN30 but not ISW1 is required for the high negative supercoiling of HML DNA. DNA isolated from strains 5 (WT, wild type), 6 (*fun30Δ*), 7 (*isw1Δ*), 8 (*fun30Δ isw1Δ*), and 9 (*sir3Δ*) was subjected to agarose gel electrophoresis in the presence of 26 μg/ml of the DNA intercalator chloroquine. After Southern blotting, topoisomers of the HML circle were detected by an HML-specific probe. Under the condition employed here, more negatively supercoiled topoisomers migrate more slowly. The center of distribution of topoisomers from each strain is indicated by a dot. The nicked/relaxed and linear forms of the HML circle are indicated as N and L, respectively. Topoisomers of HML circle from WT, *fun30Δ* and *sir3Δ* strains are collectively designated SIR*, *fun30Δ* and *sir*, respectively.

**Fig. 2. Effects of fun30Δ and isw1Δ on the primary chromatin structure of HML.** (A) The HMLα locus. The HML-E and -I silencers and the α1 and α2 genes are shown. Filled bars indicate the sequences of probes 1 and 2 used in indirect end labeling experiments shown in (B) and (C), respectively. The positions of 20 nucleosomes inferred from a previous mapping experiment (11) are shown at the top. (B and C) Examination of HML chromatin in strains 10 (WT, wild type), 11 (*sir2Δ*), 12 (*fun30Δ*), 13 (*isw1Δ*) and 14 (*fun30Δ isw1Δ*) by MNase digestion and indirect end labeling. DNA isolated from MNase treated chromatin was digested with PvuII (B) or EcoRI (C) and fractionated on agarose gels. Genomic (naked) DNA from strain 10 (WT) was also treated with MNase and then digested with PvuII (B) or EcoRI (C). This sample is designated N. After Southern blotting, DNA fragments were detected by probe 1 near the PvuII site (B) or probe 2 near the EcoRI site (C). The relative positions of the α1 and α2 genes are shown on the left. The positions of inferred nucleosomes are indicated by filled ovals. Some bands representing MNase sensitive sites are indicated by open diamonds or black dots (see text for descriptions).

**Fig. 3. Effects of fun30Δ and isw1Δ on the primary chromatin structure around the HML-I silencer.** (A) Schematics of the region around HML-I. Filled bar indicate the sequence of probe 3 used in the indirect end labeling experiment shown in (B). The positions of nucleosomes 1 to 7 mapped previously
Fig. 4. Effects of fun30Δ on the primary chromatin structure of HML in the sirΔ background. (A) The HMLα locus. Filled bars indicate the sequences of probes 1 through 3 used in indirect end labeling experiments shown in (B) through (D), respectively. (B through D) Examination of HML chromatin in strains 11 (sir2Δ) and 15 (sir2Δ fun30Δ) by MNase digestion and indirect end labeling. MNase treated chromatin was digested with PvuII (B), EcoRI (C), or NgoMIV (D) and fractionated on an agarose gel. After Southern-blotting, DNA fragments ending at the PvuII (B), EcoRI (C), or NgoMIV (D) site were detected by hybridization with probe 3. The position of the HML-I silencer is shown on the left. The positions of inferred nucleosomes are indicated by filled ovals. Some bands representing MNase sensitive sites are indicated by open diamonds or black dots (see text for descriptions). Note the WT and fun30Δ lanes were aligned side-by-side on the right for better comparison and illustration of the positions of inferred nucleosomes.

Fig. 5. Effect of fun30Δ on de novo establishment of HML heterochromatin structure. (A) Strategy for examining the de novo establishment of heterochromatin on circular HML minichromosome. Shaded and filled circles denote nucleosomes in derepressed chromatin and heterochromatin, respectively. The HML locus including the E and I silencers is flanked by a pair of FRTs. See text for description of the scheme. (B) Examination of the kinetics of establishment of heterochromatin on HML circle in strains 16 (sir3-8 FUN30) and 17 (sir3-8 fun30Δ). Cells of each strain grown at 30°C in YPR medium were treated with galactose for 2.5 hr to induce excision of the HML circle. Cells were then shifted to fresh YPD (YP + glucose) medium and incubated at 23°C for up to 20 hours. Aliquots of the culture were harvested at time points 0, 1, 2, 3, 4, 6, 8 and 20 hr. DNA was isolated and fractionated by agarose gel electrophoresis in the presence of 26 µg/ml chloroquine. Under the conditions used, more negatively supercoiled topoisomers run more slowly. N and L, nicked and linear forms of the HML circle, respectively. The topoisomers corresponding to the silent and derepressed states of HML circles are designated SIRΔ and sirΔ, respectively. (C) The profiles of topoisomers in lanes 1, 5, 6, 8 and 9, 13, 14, 16 were determined using the NIH image software. The centers of distribution of topoisomers are marked by open circles.

Fig. 6. Deletion of FUN30 does not affect histone hypoacetylation and hypomethylation in heterochromatin at HML. (A) The abundance of an HML sequence in strain 10 (wild type, WT), 11 (sir2Δ) or 12 (fun30Δ) was measured by PCR before (input) and after chromatin IP with antibodies for acetylated histones H3 and H4 (H3-Ac and H4-Ac), histone H3 methylated at K79 (H3-K79-Me), as well as total H3 (H3). No Ab, samples from mock ChIP without using antibody. The gel picture of PCR products from one of three independent experiments is shown. The data were quantified and plotted in (B) through (D). The value of relative IP of H3-Ac, H4-Ac or H3-K79-Me was calculated as the ratio of signal (IP/input) for H3-Ac (B), H4-Ac (C), or H3-K79-Me (D) over that for total H3. The means of data from all three independent experiments together with corresponding standard deviations were presented. The value for each WT sample is taken as 1.

Fig. 7. Fun30 is enriched at HML and HMR loci. (A) The abundance of HML, HMR and ACT1 sequences in strain 18 carrying FUN30-Myc was measured by PCR before (input) and (α-Myc) after chromatin IP with α-Myc antibody. No Ab, samples from mock ChIP without using antibody. The gel picture of PCR products from one of three independent experiments is shown. The data were quantified and plotted in (B). The value of relative IP was calculated as the ratio of IP signal over input signal. The
means of data from all three independent experiments together with corresponding standard deviations were presented. The value for HMR sequence is taken as 1.

**Fig. 8. ISWI is required for maintaining the stability of HML heterochromatin.** (A) Strategy for examining the stability of heterochromatin on HML minichromosome dissociated from silencers. Filled and shaded circles denote nucleosomes in silent and derepressed chromatin, respectively. See text for description. (B) Examination of the kinetics of the conversion of silent HML circle (SIR⁺) to derepressed circle (sir⁻) in strains 5 (Wild type), 6 (fun30Δ), 7 (isw1Δ) and 8 (fun30Δ isw1Δ). Cells of each strain grown to stationary phase in YPR were treated with galactose for 2.5 hr to induce the excision of the HML circle. Cells were then shifted and diluted into fresh YPD medium and further incubated for 8 hr. Aliquots of culture were harvested at time points 0, 1, 2, 3, 4, 6 and 8 hr. DNA was isolated from the samples and fractionated by agarose gel electrophoresis in the presence of chloroquine. N and L, nicked and linear forms of the HML circle, respectively. Topoisomers corresponding to the silent and derepressed states of HML circles are designated SIR⁺ and sir⁻, respectively. Topoisomers of HML circle from the fun30Δ strain are designated fun30Δ.

**Fig. 9. Contribution of Fun30p to the special primary structure of HML heterochromatin.** Illustrated is a summary of chromatin mapping data shown in Fig. 2 and 3. The HML locus and nucleosomes 1 through 20 and –1 through –4 in silent (SIR⁺) HML heterochromatin are shown as filled ovals at the top. a. Nucleosome distribution in HML chromatin in the derepressed state (in the absence of Sir complex). Open circles represent the nucleosomes in the derepressed (sir⁻) state that differ in position and/or stability from their counterparts in the silent (SIR⁺) state. b. Nucleosome distribution in HML chromatin in an intermediate state (in the absence of Fun30p). Shaded circles represent the nucleosomes in the intermediate (fun30Δ) state that differ in position and/or stability from their counterparts in the silent (SIR⁺) state. c. Nucleosome distribution in HML chromatin in fully silenced heterochromatin state.
### Table 1. Yeast strains

| Number | Name     | Relevant genotype                                                                 | Reference/Source |
|--------|----------|-----------------------------------------------------------------------------------|------------------|
| 1      | YXB61-1  | MATa ura3-52 ade2-1 lys1-1 leu2-3,112 his5-1 can1-100 sir3::LEU2 HML::URA3 TRP1-SIR3-SUP4-o | Ref. 24          |
| 1s     | YXB61-1s | MATa ura3-52 ade2-1 lys1-1 leu2-3,112 his5-1 can1-100 sir3::LEU2 HML::URA3         | Ref. 24          |
| 2      | YQY691   | YXB61-1, fun30Δ::kanMX                                                             | This work        |
| 3      | YQY684   | YXB61-1, isw1Δ::kanMX                                                              | This work        |
| 4      | YQY709   | YXB61-1, fun30Δ::kanMX isw1Δ::natMX                                                | This work        |
| 5      | YXB6     | MATa ura3-52 ade2-1 lys1-1 his5-1 can1-100 [cir'] LEU2-GAL10-FLP1 E-FRT-hml::β2-FRT-1 | Ref. 27          |
| 6      | YQY715   | YXB6, fun30Δ::kanMX                                                               | This work        |
| 7      | YQY672   | YXB6, isw1Δ::kanMX                                                                | This work        |
| 8      | YXZ90    | YXB6, fun30Δ::kanMX isw1Δ::URA3                                                    | This work        |
| 9      | YXB6s    | YXB6, sir3::URA3                                                                  | Ref. 27          |
| 10     | CCFY101  | MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-289 ura3-1 hmrΔ::TRP1 Tel VR-URA3 RDN1::ADE2-CAN1 | Ref. 26          |
| 11     | YQY540   | CCFY101, sir2Δ::kanMX                                                              | This work        |
| 12     | YQY680   | CCFY101, fun30Δ::kanMX                                                             | This work        |
| 13     | YQY674   | CCFY101, isw1Δ::kanMX                                                              | This work        |
| 14     | YQY696   | CCFY101, isw1Δ::HIS3 fun30Δ::kanMX                                                 | This work        |
| 15     | YQY707   | CCFY101, sir2Δ::HIS3 fun30Δ::kanMX                                                 | This work        |
| 16     | YXB141   | YXB10s, sir3-8-kanMX                                                              | This work        |
| 17     | YQY708   | YXB141, fun30Δ::natMX                                                              | This work        |
| 18     | YQY717   | CCFY101, FUN30-Myc-kanMX                                                           | This work        |
Fig. 1

A

\[ \text{HML::URA3} \rightarrow \text{URA3} \rightarrow \text{I} \]

- FOA
+ FOA

| Strain # | WT | fun30Δ | isw1Δ | fun30Δ isw1Δ | sir3Δ |
|---------|----|--------|-------|--------------|-------|
| 1       | 1  | 2      | 3     | 4            | 1s    |

B

Relative abundance: 0.09 1 2 2 4

C

| FRT | HML | FRT |
|-----|-----|-----|

Excision

Silent circle

● Nucleosome in heterochromatin
○ Nucleosome in euchromatin

D

Strain # 5 6 7 8 9

SIR⁺ fun30Δ sir⁻
Fig. 2

A

EcoRI

probe 2

α2

HML

α1

probe 1

Pvull

200 bp

B

N

sir2Δ

WT

fun30Δ

WT

isw1Δ

fun30Δ

WT

fun30Δ

isw1Δ

fun30Δ

MNase

C

α2

2038

1478

991

895

α1

661

(bp)

N

sir2Δ

WT

fun30Δ

WT

isw1Δ

fun30Δ

WT

isw1Δ

fun30Δ

MNase

1308

1119

844

611

302

(bp)
Fig. 4
Fig. 5

A

B

C

Nonfunctional $sir3-8$

Derepressed circle

$30^\circ C$

$23^\circ C$

Functional $sir3-8$

Silenced circle

Excise

$FRT$

$HML$

$SIR^+$

$sir^-$

$SIR^+$

$sir^-$

$FUN30$

$fun30\Delta$

Hours after 30 to 23°C shift

Hours after 30 to 23°C shift

Lane

0 1 2 3 4 6 8 20

0 1 2 3 4 6 8 20

N L

0 4 6 20 hours
Fig. 6
Fig. 7

A

B

Relative IP

HML  HMR  ACT1

Input

No Ab

Myc

HML

HMR

ACT1
