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Detection of an altered heterochromatin structure in the absence of the nucleotide excision repair protein Rad4 in *Saccharomtyces cerevisiae*

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Rad4p is a DNA damage recognition protein essential for global genomic nucleotide excision repair in *Saccharomyces cerevisiae*. Here, we show that Rad4p binds to the heterochromatic HML locus. In a yeast mutant lacking Rad4p, an increased level of SIR complex binding at the HML locus is accompanied by an altered, more compact heterochromatin structure, as revealed by a topological analysis of chromatin circles released from the locus. In addition, gene silencing at the HML locus is enhanced in the rad4Δ mutant. Importantly, re-expression of Rad4p in the rad4Δ mutant restores the altered heterochromatin structure to a conformation similar to that detected in wild-type cells. These findings reveal a novel role of Rad4p in the regulation of heterochromatin structure and gene silencing.

Nucleotide excision repair (NER) is an important evolutionarily conserved repair pathway that removes helix-distorting DNA lesions. For example, mutations in genes of the pathway can cause the Xeroderma pigmentosum skin cancer predisposition syndrome.[1] NER can detect DNA lesions either in a transcription-coupled manner or in a genome-wide process.[2] The Xeroderma pigmentosum C (XPC) protein recognizes bulky DNA lesions and plays an essential role in initiating global genomic nucleotide excision repair (GG-NER).[1] GG-NER is responsible for the repair of DNA lesions throughout the genome, including actively transcribed and untranscribed regions. This is in contrast to the transcription coupled NER pathway,[2] which only removes DNA damage in the transcribed strand of an active gene. The yeast XPC ortholog, Rad4p, is responsible for recognizing DNA damage in NER, and its cellular levels are tightly regulated by ubiquitination and proteasomal degradation.[3,4] In RNA polymerase II-transcribed regions in *S. cerevisiae*, Rad4p is essential for both GG-NER and transcription coupled repair (TC-NER).[5] Analysis of the crystal structure of Rad4p reveals that in addition to binding damaged DNA, Rad4p can also bind undamaged DNA due to the flexibility of β-hairpin domains.[6] While Rad4p binding to damaged DNA initiates NER, the significance of Rad4p binding to undamaged DNA remains unknown. However, evidence is emerging that the Rad4p counterpart in humans, XPC, binds to gene promoter regions and acts as a transcription factor.[7]

DNA in eukaryotes is packed into chromatin. Euchromatin and heterochromatin are two forms of chromatin structure. The transcriptionally silent mating HM loci of the *S. cerevisiae* genome represent the yeast equivalent of metazoan heterochromatin.[8,9] Heterochromatin plays important roles in both gene regulation and maintenance of chromosome stability. In contrast to euchromatin structure that is permissive for gene expression, heterochromatin adopts a condensed higher order structure that silences gene transcription. The silent information regulator (SIR) complex, containing proteins Sir2p, Sir3p, and Sir4p, mediates heterochromatin formation at the mating type loci.[10-12] The SIR complex or Sir3p alone can compact nucleosomal arrays in vitro.[10,12,13] In addition, Sir3p alone can produce a hypercondensed chromatin structure in vitro,[13] and overexpression of Sir3p is toxic to yeast cells.[14] These findings suggest that unregulated heterochromatin compaction mediated by the SIR complex could be detrimental to the cell. Here, we show that the primary structure of heterochromatin is regulated by a novel mechanism involving the NER protein Rad4p. Our data show that Rad4p resides at the native silent *HML* locus in *S. cerevisiae* and modulates the levels of SIR proteins at *HML*.

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Results

Detection of Rad4 at the heterochromatic *HML* locus

Previously we employed the transcriptionally inactive *HML* locus as a model chromatin template to first link chromatin remodeling activities to NER.[15] Surprisingly, using chromatin immunoprecipitation (ChIP), PCR primers specific for the *HML* locus (**Fig. 1A**), and an antibody recognizing Rad4p developed by Sigma for our laboratory (**Fig. 1B**), we consistently detected the presence of Rad4p at the *HML* locus in the absence of exogenous DNA damage (**Fig. 1C and D**). As positive controls, both Sir2p and Sir3p were detected at the silent *HML* locus (**Fig. 1**). However, Rad4p and Sir2p/Sir3p proteins were not detected in the repressed *GAL1–10* gene promoter region, which was used as a negative control (**Fig. 1C**). Interestingly, Rad4p was also detected at telomeres (**Fig. 1E**), where the binding of SIR complex is also essential for telomeric silencing.[16] These findings raise the possibility that Rad4p may have a role in the regulation of heterochromatin structure.

Increased levels of Sir proteins detected at *HML* in the *rad4*Δ cells

Since the SIR complex establishes and maintains heterochromatin structure at the *HML* locus,[10,11] we examined if the amount of Sir2p bound at the *HML* locus is altered when Rad4p is absent. Interestingly, ChIP analysis revealed that an increased level of Sir2p is present at *HML* in *rad4*Δ cells, when compared with wild-type cells (**Fig. 2A**). Comparable results were obtained from two sets of isogenic yeast strains with different genetic backgrounds. We estimated by real-time PCR that the level of Sir2p detected at *HML* increases more than 2-fold in *rad4*Δ cells (**Fig. 2B**, bar graph), while a western blot demonstrated that the cellular levels of Sir2p are not affected by the absence of Rad4p (**Fig. 2B**, bottom panel). In addition, an increased level of Sir3p was also detected at *HML* in the absence of Rad4p (**Fig. 2C**), whereas the cellular levels of Sir3p are not affected by the absence of Rad4p (**Fig. 2C**, WB panel). Taken together, these data suggest that Rad4p, residing at the silent *HML* locus, may modulate heterochromatin structure and gene silencing established by the SIR complex.

Altered heterochromatin conformation at the *HML* locus in the *rad4*Δ cells

Consistent with the notion that Rad4p interferes with the binding of SIR complex at *HML*, the following observations indicate that the heterochromatin conformation at the silent *HML* locus is altered in the absence of Rad4p. It is known that formation of each nucleosome confers on average one negative supercoil on

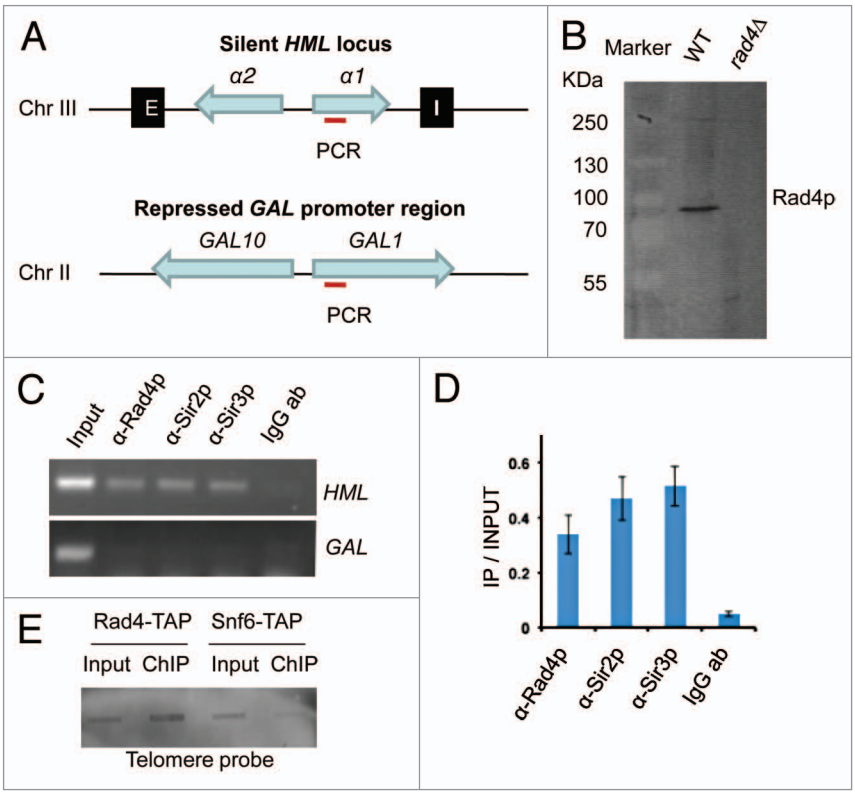

Figure 1. Rad4p resides at the silent *HML* locus. (**A**) Schematic illustration of the *HML* locus and the *GAL1–10* promoter region. *HML*- and *GAL*-specific PCR primers used in the ChIP experiments are indicated. (**B**) Development of an antibody specific for Rad4p. A western blot shows the detection of Rad4p in BY4741 wild-type cells, but not in *rad4* cells. (**C**) Detection of Rad4p, Sir2p, and Sir3p at the *HML* locus by ChIP. *HML*-specific PCR amplification of immunoprecipitated DNAs separated on an agarose gel is presented. Immunoprecipitation of DNA fragments cross-linked to proteins Rad4p, Sir2p, and Sir3p was performed using antibodies specific to these proteins. A preimmune antibody (IgG ab) was used as a control. As a negative control, *GAL* promoter region was compared with the *HML* locus. (**D**) A bar graph shows real-time PCR quantitation of the *HML* ChIP signals. Data are shown as mean ± *s.d.* for 4 replicates (two biological replicates). (**E**) Detection of Rad4p at telomeres. Cells expressing TAP-tagged Rad4p and Snf6 were cross-linked and ChIP was performed using IgG beads. Southern blot detection of the enrichment of telomeres was performed using probe 5′-TGGGTGTGGTGTGTGGGTGTGGTG-3′.

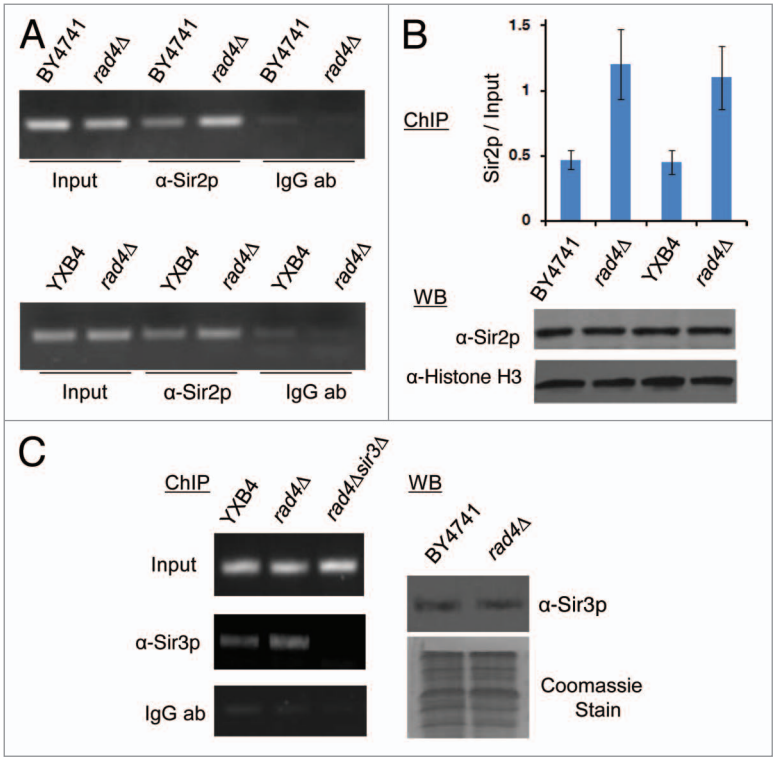

Figure 2. Deletion of *RAD4* leads to increased SIR complex binding at the *HML* locus. (**A**) Increased Sir2p binding at *HML* in the absence of Rad4p. ChIP was used to examine the levels of Sir2p bound at *HML*. *HML*-specific PCR amplification separated on agarose gels is presented. (**B**) qPCR quantitation of the ChP signals. The bar graph shows the quantitative real-time PCR data as mean ± *s.d.* for 4 replicates (2 biological replicates). Bottom panel shows a western blot (WB) of total cell extracts to examine of Sir2p expression in *wt* and *rad4* cells. (**C**) Increased Sir3p binding at the *HML* locus in *rad4Δ* cells. Right panel: ChIP detection of increased Sir3p binding at *HML*. A pre-immune antibody (IgG ab) and a *sir3Δ* strain were used as negative controls. PCR was performed using *HML*-specific primers. Left panel: Comparable Sir3p expression in yeast cells with or without the presence of Rad4p. Total cell extracts from YXB4 (*wt*) and *rad4Δ* cells were probed for Sir3p by western blot (WB).

nucleosomal DNA, and DNA supercoiling can be quantitated by measuring the linking number (*Lk*).[17,18] The topology of DNA spanning a specific region in the chromosome reflects the conformation of local chromatin structure. Previous studies, including one by one of the authors in this study, have established a method to examine DNA topology at a particular genomic locus using site-specific recombination in vivo to produce non-replicating chromatin circles.[19,20] In the yeast strains we used,[19] two FRT (Flp1p recombination target) sequences are inserted in direct orientation at positions flanking *HML* (**Fig. 3A**). Galactose induction of the site-specific recombinase Flp1p expression leads to recombination between the two FRTs and subsequent excision of *HML* from the yeast chromosome III as chromatin circles (**Fig. 3B**). Topoisomers of chromatin circles can be separated on agarose gels in the presence of chloroquine. Chloroquine intercalation into DNA causes unwinding of the negatively supercoiled *HML* circles purified from yeast cells. This causes positive twisting in the closed *HML* DNA circles that can be converted to positive writhe. At the chloroquine concentration we used (30 μg/ml), all DNA circles are observed in agarose gels as positively supercoiled DNA circles. Therefore, more negatively supercoiled DNA circles prior to chloroquine intercalation would migrate more slowly in agarose gels as chloroquine-intercalated positively supercoiled molecules.[21] Different topologies of the *HML* chromatin circles isolated from isogenic YXB4 (wild-type) and *rad4*Δ cells were observed with a linking difference (ΔLk) of ~1 (**Fig. 3C**). Surprisingly, *HML* circles isolated from *rad4*Δ cells are more negatively supercoiled than circles isolated from YXB4 cells. Together with the observation that more Sir proteins are bound at *HML* in *rad4*Δ cells (**Fig. 2**), our data suggest that Rad4p regulates the structure of heterochromatin by opposing the binding of the SIR complex to chromatin.

Opposing effects of Rad4p and Sir3p in the HML circle topology

In contrast to the more negatively supercoiled *HML* circles isolated from *rad4*Δ cells (**Fig. 3C and D**, lane 1 vs. lane 2), *HML* circles from *sir3*Δ cells are less negatively supercoiled (**Fig. 3D**, lane 1 vs. lane 3). Thus, Rad4p and Sir3p have opposite effects on the *HML* heterochromatin structure. We note that the *HML* heterochromatin structure is completely disrupted in *sir3*Δ cells,[22,23] since Sir3p is essential for the formation and maintenance of the silent *HML* chromatin.[11,24,25] Significantly, Rad4p specifically regulates heterochromatin conformation, since no alteration of the *HML* circle topology was detected when the *RAD4* gene was deleted from *sir3*Δ cells (**Fig. 3D**, lane 3 vs. lane 4). Importantly, in our analysis we used yeast strains containing a promoter-less *HML* locus to exclude any effect of transcription on *HML* circle topology (**Fig. 3A**; **Table S1**). Thus, the

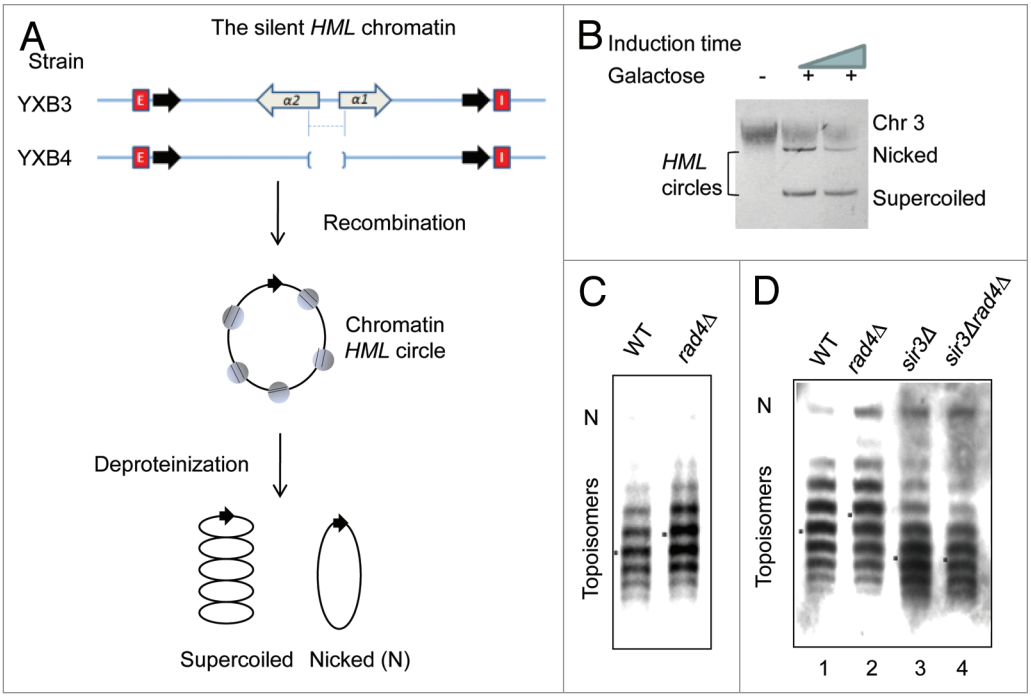

Figure 3. Altered *HML* circle topology in the absence of Rad4p. (**A**) Chromatin circle formation in vivo. In strain YXB3,[19] two FRT sequences (Flp1p recombination target) (filled arrows) are inserted in direct orientation at positions flanking *HML*. Recombination between FRTs by the site-specific recombinase Flp1p leads to the excision of *HML* as a chromatin circle. Strain YXB4 is identical to YXB3 except that α*1* and α*2* gene promoters are deleted.[19] (**B**) Galactose induction of the *HML* chromatin circles. DNA samples were isolated before and after galactose induction, separated on an agarose gel and detected by a Southern blot using an *HML*-specific probe: chromosome 3 (Chr3), nicked and supercoiled *HML* circles were indicated. (**C**) Deletion of *RAD4* alters *HML* circle topology. DNA was isolated and separated on an agarose gel in the presence of chloroquine (30 μg/ml). Shown is a Southern blot using an *HML*-specific probe to label the *HML* topoisomers. Nicked circles (N) and the Gaussian center of topoisomer distribution (dots) are indicated. (**D**) Rad4p and Sir3p have opposing effects on *HML* chromatin topology. Shown is a southern blot using an *HML*-specific probe to label the *HML* topoisomers.

topological difference between *HML* circles isolated from wild-type and *rad4*Δ cells may be attributed exclusively to a change in chromatin structure.

Re-expression of Rad4p in *rad4*Δ cells restores *HML* heterochromatin structure to a topology similar to that in wild-type cells

To test if Rad4p can restore the altered heterochromatin structure observed in *rad4*Δ cells, the *RAD4* gene under the control of its native promoter was cloned into a low copy *CEN* plasmid and introduced into wild-type (YXB4) and *rad4*Δ cells. Expression of Rad4p in wild-type cells had a small, but reproducible effect on *HML* circle topology. *HML* circles migrated faster in chloroquine gels when Rad4p was re-expressed in YXB4 cells (**Fig. 4A**), indicating that *HML* circles are less negatively supercoiled. Importantly, re-expression of Rad4p in *rad4*Δ cells partially corrected the altered heterochromatin structure observed in *rad4*Δ cells (**Fig. 4A**, lane 3 vs. 4). Taken together, these findings indicate that Rad4p controls heterochromatin conformation at *HML* by regulating the levels of SIR complex assembled at the *HML* locus. In the absence of Rad4p, increased SIR complex binding at *HML* results in a more negatively supercoiled, i.e., more compact, heterochromatin structure.

We reported previously that Rad4p interacts with the SWI/SNF chromatin remodeling complex.[15] We next compared *HML* circle topology in *rad4*Δ (**Fig. 4B**, a positive control), a SWI/SNF mutant *snf6*Δ (**Fig. 4C**), and another NER mutant *rad16*Δ (**Fig. 4D**). It is interesting that deletion of *SNF6* led to a slightly more condensed *HML* heterochromatin structure, similar to that in *rad4*Δ cells (**Fig. 4C**), suggesting that SWI/SNF may also play a role in heterochromatin structure at the *HML* locus. In contrast, Rad16p has no detectable effect on the conformation of *HML* heterochromatin (**Fig. 4D**), suggesting that NER deficiency at *HML* is not the cause of heterochromatin conformational change detected in *rad4*Δ.

Gene silencing at the *HML* locus is strengthened in the *rad4*Δ mutant

The SIR complex is crucial for gene silencing at the HM loci. An increased level of SIR proteins and a more compact heterochromatin structure indicate that gene silencing should be strengthened in the absence of Rad4p. To test this possibility, we examined the expression of the *URA3* gene inserted into the *HML* locus in place of the *HML*α mating genes in an otherwise *ura3*$^-$ strain. Levels of *URA3* expression can be monitored by measuring cell survival rate in medium containing 5-fluoro-orotic acid (FOA). As observed previously[26] and as shown in **Figure 5**, expression of the *URA3* gene inserted in *HML* is silenced, as witnessed by the resistance of strains YXB61-I and YXB61-II to FOA, when compared with a *sir3*Δ mutant deficient in gene silencing (**Fig. 5A**). However, it is known that the distance over which E and I silencers at *HML* can act to completely silence gene expression

is limited.[27] Normally the distance between E and I is ~3 kb, and silencing is weakened if that distance is increased.[27] Consistent with this notion, *URA3* silencing at the *HML* locus is not complete, since ~15% *HML::URA3* yeast cells can grow and form colonies on FOA plates (**Fig. 5B**). Notably, about 35% cells can grow on FOA plates when *RAD4* gene was deleted, indicating lower levels of *URA3* expression in *rad4*Δ cells (**Fig. 5B**). These data strongly suggest that gene silencing at the *HML* locus is enhanced in *rad4*Δ cells.

Discussion

In this report, we show that the DNA damage recognition protein Rad4p binds to the heterochromatic *HML* locus and regulates the primary structure of heterochromatin. Rad4p appears to compete with the SIR complex for *HML* binding to modulate heterochromatin structure. In the absence of Rad4p, the primary structure of *HML* heterochromatin was altered in yeast cells. The altered heterochromatin conformation results in a more negatively supercoiled DNA topology, which is different from the less negatively supercoiled DNA topology observed in *Sir3*Δ cells. Importantly, gene silencing at the *HML* locus is enhanced in *rad4*Δ cells.

A novel role of Rad4p in heterochromatin structure and gene silencing

Our study reveals a novel function of the NER protein Rad4p in heterochromatin and gene silencing, unrelated to its role in DNA damage repair. Overexpression of Sir3p, which can compact nucleosomal arrays and produce a hypercondensed structure in vitro,[13] is toxic to yeast cells and causes chromosome instability.[14] Therefore, in addition to the regulation of SIR protein expression, additional mechanisms, such as the one we describe here, may be involved to regulate heterochromatin structure and prevent heterochromatin hypercondensation in the cell. We speculate that Rad4p binds directly to DNA and somehow antagonizes the binding of SIR proteins to prevent overloading of the SIR complex (**Fig. 6**). The crystal structure of the Rad4p shows that Rad4p does not bind directly to the damaged DNA

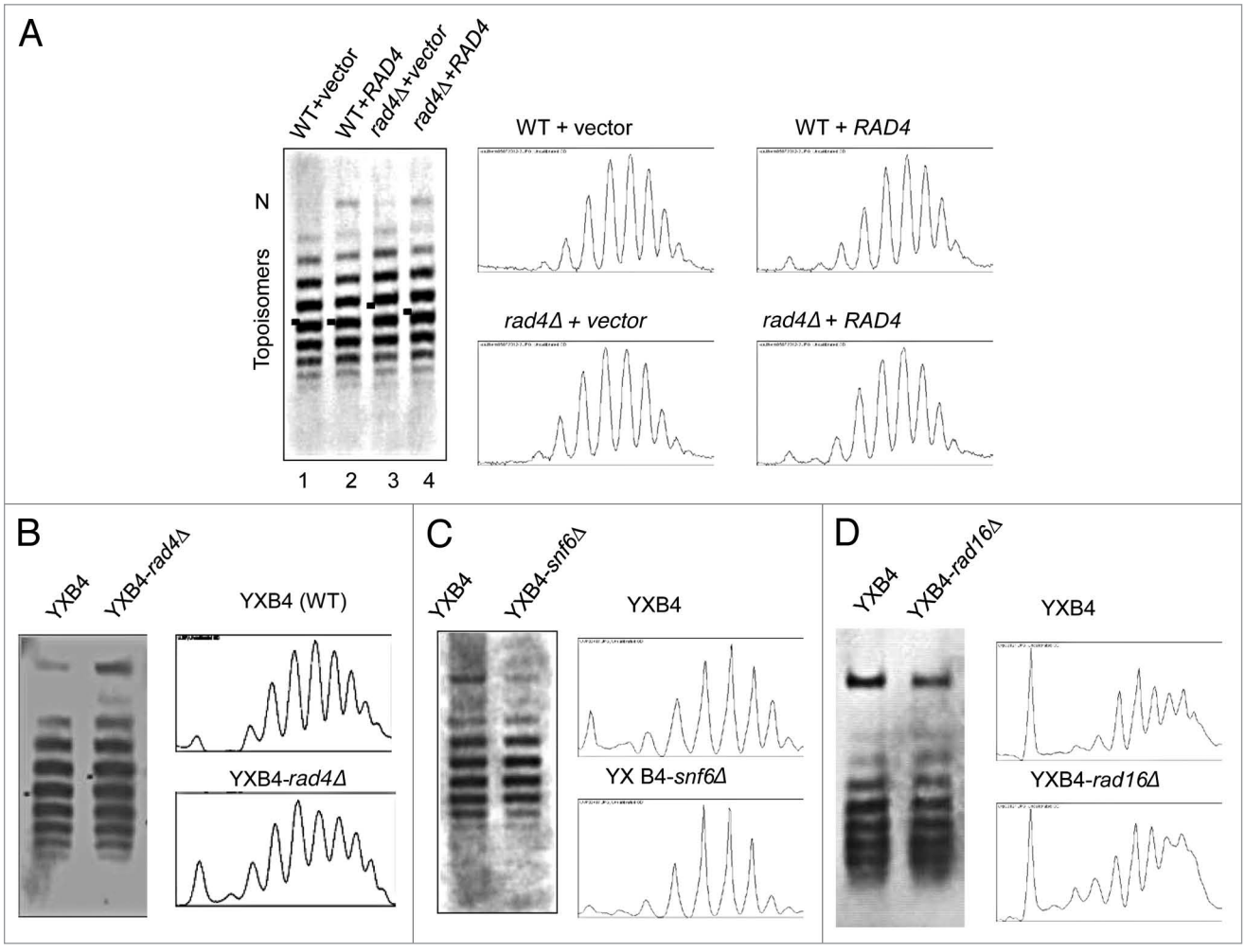

Figure 4. Analysis of *HML* circle topology in various yeast mutants. (**A**) Re-expression of Rad4p in *rad4*Δ cells partially corrected the altered heterochromatin structure observed in *rad4*Δ cells. Shown is a Southern blot using an *HML*-specific probe to label the *HML* topoisomers. DNA was isolated from yeast strains indicated on top of the gel. The density profile for each lane was shown for comparison. (**B–D**) Analysis of *HML* topology in various yeast mutants. DNA was isolated from yeast strains indicated on top of the gel. Shown are Southern blots using an *HML*-specific probe to label the *HML* topoisomers. The density profiles for *HML* topoisomers are also shown. Gel exposures were adjusted to show the subtle changes.

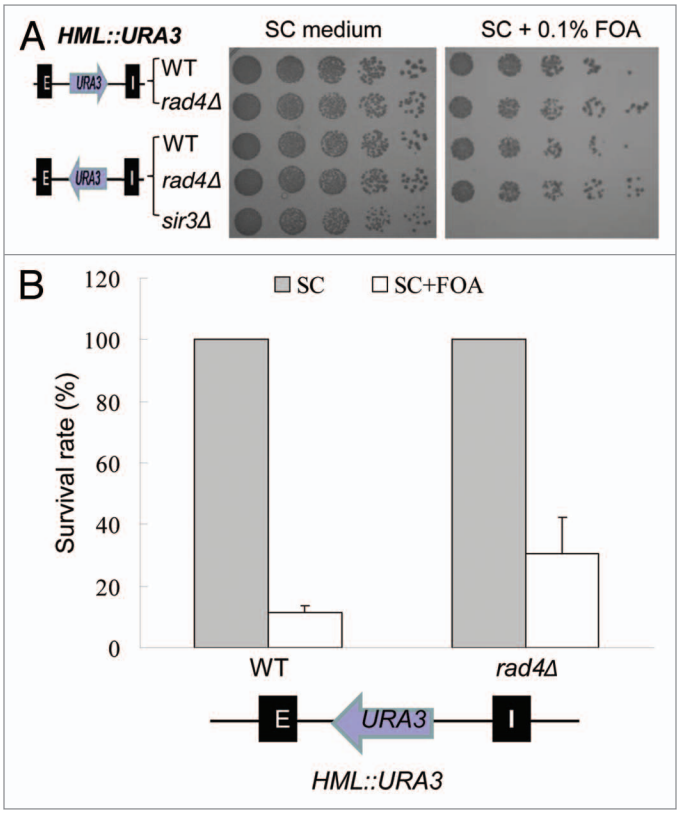

Figure 5. Deletion of *RAD4* gene leads to enhanced gene silencing at the *HML* locus. (**A**) Yeast strains YXB61-I and YXB61-II with the *URA3* gene inserted at the *HML* locus were used to analyze the effect of *RAD4* deletion on *HML* gene silencing. (**B**) Quantitation of cell survival in synthetic complete (SC) medium containing FOA. Data are shown as mean ± s.d. for three biological replicates.

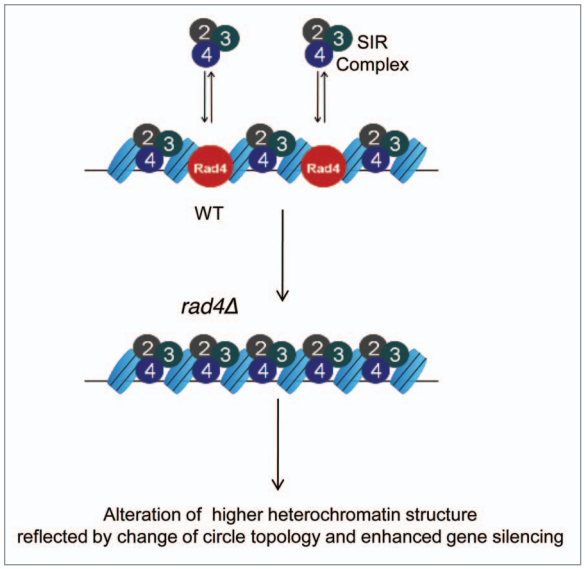

Figure 6. A model depicting the role of Rad4p in the control of heterochromatin conformation by competing with the SIR complex for *HML* binding.

strand. Instead, it binds to the undamaged DNA strand distorted by the DNA lesion located on the opposite strand.[6] This may shed light on how Rad4p binds to the *HML* silent chromatin. Future work will need to elucidate how Rad4p binds to the *HML* locus to modulate chromatin structure. Together with the study by Le May and coworkers,[7] our study highlights novel roles of NER proteins in the regulation of transcription and chromatin structure.

Gratuitous NER in undamaged DNA

It has long been realized that NER can attack undamaged DNA,[28,29] and this was thought to be a source of spontaneous mutations.[29] However, other studies have shown that that NER operates to reduce the spontaneous mutation frequency in *S. cerevisiae*,[30,31] indicating a role for NER in limiting the extent of spontaneous damage to DNA. Therefore, it is possible that the subtle changes in the *HML* heterochromatin structure in the absence of Rad4p may be due to lack of functional NER that may operate on low levels of spontaneous damage at *HML*. However, we did not detect the subtle changes in a *rad16*Δ mutant, which is deficient in GG-NER at *HML*.[32-34] Since Rad4p is essential for both GG-NER and transcription coupled repair (TC-NER),[5] our data cannot completely rule out the possibility that the changes in chromatin structure at *HML* are NER-related. Our findings suggest that the detected role of Rad4p in heterochromatin is independent of Rad16p-mediated GG-NER.

Implications for DNA repair in heterochromatin

Our findings indicate that the degree of heterochromatin compaction is probably regulated extensively to allow orderly access for proper DNA metabolism, such as DNA replication and repair. Repair of UV damage at the heterochromatic *HML* locus is surprisingly very efficient in yeast. Approximately 80% of CPDs are removed within 3 h after UV irradiation.[15] Pre-existing Rad4p bound at *HML* prior to exogenous DNA insults may facilitate DNA repair in heterochromatic regions.

Materials and Methods

Yeast strains

Yeast strains used in this study are listed in **Table S1**. *RAD4* gene deletion was confirmed by PCR and the UV sensitivity (Fig. S1).

Chromatin immunoprecipitation (ChIP), antibodies and primers used for real-time PCR

ChIP was performed as described.[35] Quantitation of ChIP signals was performed by real-time PCR. SYBR Green Supermix (Bio-Rad) and a Bio-Rad iCycler were used. Quantitative PCR data are shown as mean ± s.d. for 4 replicates (2 biological replicates).

Mid-log phase yeast cells were treated with 1% formaldehyde for 15 min at room temperature, pelleted, and washed twice with TBS (25 mM Tris, pH 7.5, 137 mM NaCl, 2.7 mM KCl). Cross-linked cells were suspended in a lysis buffer (50 mM HEPES–KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 1 mM PMSF, 1 mg/ml leupeptin, 1 mg/ml pepstatin A) and disrupted using glass beads (425–600 mm, Sigma), followed by sonication to yield DNA fragments with an average size of 300 bp. Protein levels in the extract were estimated using the Bradford assay. Equal amounts of protein

from each sample were used for immunoprecipitation with antibodies specific for Sir2p and Sir3p (Santa Cruz Biotechnology). The reaction mixture was incubated overnight at 4 °C, and the immunocomplex was precipitated using Protein A sepharose beads. The beads were consecutively washed with the lysis buffer, wash buffer 1 (lysis buffer containing 500 mM NaCl), wash buffer 2 (10 mM Tris-HCl, pH 8, 250 mM LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA) and TE buffer and then treated with RNase A in TE at 37 °C for 30 min. Chromatin was then eluted from the beads using elution buffer (1% SDS, 0.1 M NaHCO3) and the cross-link reversed by incubation at 65 °C overnight. DNA was then deproteinized by the addition of 4 µl of a solution of 10 mg/ml proteinase K and incubation at 37 °C for 2 h. After phenol–chloroform extraction and ethanol precipitation, DNA was resuspended in 20 µl of TE. *HML*-specific primers were used for PCR. PCR products were resolved on 1.5% agarose gels.

The antibodies used for the ChIP analysis are all rabbit polyclonal IgG: α-Sir2p (Santa Cruz Biotechnology, sc-25753), α-Sir3p (Santa Cruz Biotechnology, sc-28552), α-Rad4p antibody, and pre-immune rabbit IgG (custom antibody produced by Sigma, pre-immune IgG was collected from the same rabbit before immunization). The *HML* locus was amplified using the following primer sets: *HMLα1*-Nuc8-F: 5′AATCATACAGAAACACAGC-3′ and *HMLα1*-Nuc8-R: 5′-AAATCGAGAGGAAGGAAC-3′. The repressed (cells grown in dextrose glucose) *GAL10* promoter region was amplified as a negative control using the following primer sets: Gal10 NucB-F: 5′-TGACAGCTCAGTTACAAAGTG-3′ and Gal10 NucB-R: 5′-CACAGTCATATCCATTCTCA-3′

Topological analysis

The strategy to analyze DNA topology at the *HML* and *HMR* loci was developed prebiously.[19,20] Yeast cells were grown at 30 °C in YPR medium (1% yeast extract, 2% peptone, and 2% raffinose) to early log phase (OD600 = 0.6). Cells were grown for 2.5 h in the presence of 2% galactose to induce expression FLP1 recombinase under the control of the *GAL10* promoter. DNA isolated from yeast cells was fractionated on 1.2% agarose gels in the presence of 30 µg/ml chloroquine. *HML* circles were revealed by Southern blot using an *HML*-specific probe that hybridizes to the α1 gene coding region.[15] The Gaussian center of topoisomer distributions from each strain was determined as described previously[18,21] and indicated in the figure by a dot.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Supplemental Materials

Supplemental materials may be found here:
http://www.landesbioscience.com/journals/cc/article/25457

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