Published in final edited form as:
Cell. 2009 September 18; 138(6): 1109–1121. doi:10.1016/j.cell.2009.07.013.

Recombinational repair within heterochromatin requires ATP-dependent chromatin remodeling

Manisha Sinha[1,2], **Shinya Watanabe**[1], **Aaron Johnson**[3], **Danesh Moazed**[3,4], and **Craig L. Peterson**[1,5]

[1]Program in Molecular Medicine, University of Massachusetts Medical School, 373 Plantation Street, Worcester, MA 01605

[2]Interdisciplinary Graduate program, University of Massachusetts Medical School, 373 Plantation Street, Worcester, MA 01605

[3]Howard Hughes Medical Institute, Harvard Medical School, 240 Longwood Ave., Boston, MA 02115

[4]Department of Cell Biology, Harvard Medical School, 240 Longwood Ave., Boston, MA 02115

Summary

Heterochromatin plays a key role in protection of chromosome integrity by suppressing homologous recombination. In *Saccharomyces cerevisiae*, Sir2p, Sir3p, and Sir4p are structural components of heterochromatin found at telomeres and the silent mating type loci. Here we have investigated whether incorporation of Sir proteins into minichromosomes regulates early steps of recombinational repair in vitro. We find that addition of Sir3p to a nucleosomal substrate is sufficient to eliminate yRad51p-catalyzed formation of joints, and that this repression is enhanced by Sir2p/Sir4p. Importantly, Sir-mediated repression requires histone residues that are critical for silencing in vivo. Moreover, we demonstrate that the SWI/SNF chromatin remodeling enzyme facilitates joint formation, thereby promoting subsequent Rad54p-dependent formation of a strand invasion product. These results suggest that recombinational repair in the context of heterochromatin presents additional constraints that can be overcome by ATP-dependent chromatin remodeling enzymes.

Keywords

DNA repair; heterochromatin; Sir3p; Sir2p/Sir4; SWI/SNF; Rad54p

Introduction

Eukaryotic genomes are organized into two structurally distinct chromatin regions, heterochromatin and euchromatin. Early cytological studies defined heterochromatin as the region of the genome that remained visibly condensed and deeply stained throughout the cell cycle whereas euchromatin underwent decondensation as the cells progressed from metaphase to interphase (Passarge, 1979). These regions that remain condensed throughout the cell cycle

5To whom correspondence should be addressed Ph# 508-856-5858, Fax# 508-856-5011, craig.peterson@umassmed.edu.

are mainly found at centromeres and telomeres and are referred to as constitutive heterochromatin. Heterochromatin-like structures are also found at developmentally regulated loci where the chromatin state can change in response to cellular signals and gene activity. Highly conserved features that characterize heterochromatin and distinguish it from euchromatin include a high percentage of repetitive DNA sequences, repressed RNA polymerase II transcription, low or absent gene density, late S phase replication timing, regular nucleosome spacing, less accessibility of chromatin to nucleases, and hypoacetylation of histones (Grewal and Jia, 2007; Henikoff, 2000; Richards and Elgin, 2002). Heterochromatin is required for the organization and function of centromeres (White and Allshire, 2008), protection of telomeres, and limitation of telomere length in Drosophila (Savitsky et al., 2002). In addition, heterochromatin protects genome integrity by repressing the transposition of abundant transposable elements and by preventing extensive or illicit recombination between dispersed repetitive DNA elements (Peng and Karpen, 2007, 2008). Heterochromatin also shows suppressed crossing-over frequencies during meiosis (Allshire et al., 1994; Barton et al., 2008; Westphal and Reuter, 2002), and repressed mitotic recombination (Jaco et al., 2008). However, recombination does occur at measurable frequencies within heterochromatin (Jaco et al., 2008; Paques and Haber, 1999), and in the case of the yeast transposon Ty5, transpositions into heterochromatin are actually preferred (Zou et al., 1996).

In *Saccharomyces cerevisiae*, heterochromatin-like domains are localized to the rDNA gene cluster, sub-telomeric regions, and the silent mating type loci, *HMLα* and *HMR**a***. At subtelomeric and silent mating type loci, heterochromatin assembly requires the Silent information regulators, Sir2p, Sir3p, and Sir4p. The assembly of Sir-dependent heterochromatin is a stepwise process in which DNA binding proteins like Rap1p or the ORC complex recruits Sir4p, which is required for subsequent recruitment of Sir2p and Sir3p (Rusche et al., 2003). All three Sir proteins are then required for spreading of the heterochromatic domain. Sir2p is a NAD^+-dependent histone deacetylase whose HDAC activity is required for heterochromatin assembly (Imai et al., 2000). Sir2p removes the acetyl group from histone H4 K16, thereby promoting the binding of Sir3p and Sir4p to the hypoacetylated histone H4 N-terminal domain. Multiple cycles of histone deacetylation and Sir3p/Sir4p binding thus control the spreading of the heterochromatic domain from the initial point of recruitment.

Several studies indicate that Sir3p is the dominant structural component of Sir-dependent heterochromatin. In vitro, Sir3p can bind to DNA (Georgel et al., 2001) and to nucleosomes, and nucleosome binding requires H4 K16 (Onishi et al., 2007). Binding of Sir3p to nucleosomes also requires histone H3 K79, as well as several other surrounding histone residues (Onishi et al., 2007). These and other studies have defined a putative nucleosome binding surface for Sir3p that includes the globular domain of H3 and the N-terminus of H4 (Hecht et al., 1996; Onishi et al., 2007). Within cells, overexpression of Sir3p extends the domain of transcriptional silencing (Hecht et al., 1996; Renauld et al., 1993; Strahl-Bolsinger et al., 1997). Within the extended domain, Sir3p spreads in the absence of Sir2p or Sir4p. Furthermore, inactivation of the Sas2p histone acetylase leads spreading of Sir3p at telomeres in the absence of Sir2p (Suka et al., 2002).

Mating type switching in *Saccharomyces cerevisiae* requires the recombinational repair of a DSB formed at the *MAT* locus on chromosome III with homologous sequences located at the heterochromatic HM donor loci (Haber, 1998). Previous studies have shown that five different ATP-dependent chromatin remodeling enzymes – Rad54p, SWI/SNF, RSC, Ino80.com, Swr1.com -- are recruited to an HO-induced DSB, and that each enzyme plays distinct roles during DSB repair (Osley et al., 2007). For instance, formation of the initial joint between the DSB at *MAT* and the heterochromatic *HMLα* donor requires the SWI/SNF chromatin remodeling enzyme (Chai et al., 2005), while Rad54p is required for subsequent extension of

the joint by DNA polymerases (Sugawara al., 2003; Wolner and Peterson, 2005). In contrast, RSC is involved in nucleosome re-arrangements during early steps of DSB processing and for the final ligation of the repair product (Chai et al., 2005). The role of Swr1.com is not yet clear, but Ino80.com may control nucleosome loss at the HM donor that occurs either during or after joint formation (Tsukuda et al., 2009).

In this study, we studied the mechanism for how heterochromatin-like structures suppress homologous recombination and tested the hypothesis that the ATP-dependent chromatin remodeling enzyme, SWI/SNF, can counteract such repression. To this goal, we reconstituted Sir3p-nucleosome and Sir2p/Sir3p/Sir4p-nucleosome complexes with purified components and used them in an assay that monitors the early steps of HR. We find that Sir3p-nucleosome complexes are sufficient to block formation of the initial, Rad51p-mediated joint, and that this repression requires the histone H4 N-terminal domain, and the key histone residues, H4 K16, H3 K79. In addition, Sir2p/Sir4p enhances repression by Sir3p, especially at lower ratios of Sir3p to nucleosomes. Furthermore, we demonstrate that the chromatin remodeling activity of SWI/SNF facilitates formation of a joint and a strand invasion product. Interestingly, the remodeling activities of RSC, Ino80.com, Swr1.com, or Rad54p can not substitute for SWI/SNF. Hence, our findings recapitulate the repression of recombination mediated by heterochromatin in vivo, and they provide insight into probable roles of chromatin remodeling enzymes during recombinational repair when the homologous target is buried in heterochromatin.

Results

Repair of a DSB by homologous recombination (HR) requires that the Rad51p recombinase assembles onto ssDNA to form a presynaptic filament. This filament can search and capture a homologous donor sequence that is encompassed in chromatin. Previously, we developed a biotin-streptavidin capture assay to study these early events of HR on nucleosomal substrates (Sinha and Peterson, 2008). The key feature of this assay is the use of recombinant histone octamers that contain a derivative of histone H2A that is site-specifically biotinylated at an engineered cysteine residue within its C-terminal domain. Nucleosomal donors are assembled by depositing these biotinylated octamers onto a circular plasmid that contains two head-to-tail arrays of five 5S rDNA nucleosome positioning sequences flanking a di-nucleosome length E4 promoter (Top panel, Figure 1A; see also Sinha and Peterson, 2008). The Rad51-presynaptic filament is then assembled with recombinant yRad51p and a radioactively end-labeled oligonucleotide that is homologous to DNA sequences encompassed within one of the E4 promoter nucleosomes (arrow, top panel, Figure 1A). A typical joint formation assay involves incubation of the presynaptic filament with the nucleosomal donor, and joints are captured with streptavidin magnetic beads and quantified by scintillation counting (Figure 1B). Using this assay, joints are formed on 20–30% of the nucleosomal donors in the absence of chromatin remodeling enzymes (Sinha and Peterson, 2008; see also Figure 1C).

Sir3p inhibits early steps of HR

To study the impact of heterochromatin on the early steps of HR, Sir3p was purified from yeast and bound to nucleosomal donors. We added Sir3p at ratios of 1.0, 2.0, or 4.0 monomers per nucleosome, yielding nucleosomal donors with different levels of bound Sir3p. These nucleosomal donors were first analyzed by *Eco*RI analysis to ensure that all donors had comparable nucleosome densities. *Eco*RI restriction sites flank each 5S rDNA repeat, and therefore extensive *Eco*RI digestion of the nucleosomal donor followed by native gel electrophoresis allows the quantification of nucleosomal occupancy. Similar to our previous results (Sinha and Peterson, 2008), when donors were assembled at a ratio of 0.7 histone octamers per 200 bp of DNA, *Eco*RI digestion released mononucleosomes and very little free,

5S DNA repeats (Figure S1). These results are indicative of a 5S nucleosomal array in which >90% of the 5S rDNA repeats are occupied by nucleosomes both in the presence or absence of Sir3p.

One hallmark of heterochromatin is a diminished accessibility to nucleases and restriction enzymes (Loo and Rine, 1994). To probe the accessibility of Sir3p-nucleosomal donors, minichromosomes were digested with increasing concentrations of micrococcal nuclease (Mnase) which preferentially cleaves DNA within the linker between nucleosomes. Mnase digests were electrophoresed on agarose gels and Southern blots were probed with a ^{32}P-labelled E4 oligonucleotide (arrow, top panel, Figure 1A). When this analysis was performed on minichromosomes that lacked Sir3p, the E4 oligonucleotide hybridized to mononucleosomal DNA and to an extensive nucleosomal ladder (Figure 1A, lane1–3), confirming that this E4 promoter sequence is fully encompassed by nucleosomes (Sinha and Peterson, 2008). Strikingly, minichromosomes assembled with 4 Sir3p monomers per nucleosome were more resistant to Mnase digestion, requiring >10-fold higher concentrations of Mnase before significant digestion products were released (Figure 1A, compare lanes 2 and 5). However, at these higher Mnase concentrations, the E4 oligonucleotide also detected an extensive nucleosomal ladder (Figure 1A, lane 6). Interestingly, the nucleosomal ladder was more distinct in the presence of Sir3p, suggesting that the binding of Sir3p may lead to more homogeneous positioning of the E4 promoter and/or 5S nucleosomes. Thus, the binding of Sir3p to nucleosomal donors is sufficient to create a chromatin structure that is less accessible to nuclease digestion.

Biotin-streptavidin capture assays were performed with yRad51p presynaptic filaments and nucleosomal donors that contain different amounts of Sir3p. Markedly, Sir3p inhibited yRad51p-mediated joint formation in a concentration dependent manner (Figure 1C). At a ratio of 1.0 Sir3p monomer per nucleosome, joint formation was reduced 2-fold, but addition of Sir3p at a ratio of 4 monomers per nucleosome decreased joint formation by at least 20-fold (Figure 1C). Importantly, incorporation of Sir3p into minichromosomes had no influence on the capture efficiency of minichromosomes by streptavidin beads (M.S. and C.L.P., data not shown). Since Sir3p has been shown to bind cooperatively and nonspecifically to DNA (Georgel et al., 2001), we investigated whether Sir3p would also inhibit joint formation in assays where the joint was formed on a nucleosome-free region. To this end, nucleosomal donors were assembled at a ratio of 0.35 octamers per 200 bp of DNA, conditions that yield a donor in which the E4 promoter is nucleosome-free (Sinha and Peterson, 2008). In this case, the presence or absence of Sir3p yielded a similar level of joints, even at a ratio of 4 monomers of Sir3p per 200 bp of DNA (Fig. 1C). Importantly, the binding of Sir3p to saturated and subsaturated nucleosomal donors was equivalent (Figure S2). Thus, Sir3p-dependent inhibition of joint formation requires that the homologous donor sequences be encompassed by nucleosomes.

Sir3p-dependent inhibition requires key histone residues, the H4 N-terminal domain, and the Sir3p BAH domain

In vivo studies have demonstrated that assembly and function of Sir3p-containing heterochromatin requires an intact histone H4 N-terminal domain, and histone residues, H4 K16 and H3 K79 (Hecht et al., 1995; Hecht et al., 1996; Ng et al., 2002). To investigate whether Sir3p-dependent inhibition of joint formation might reflect assembly of heterochromatin-like structures, nucleosomes were reconstituted with histone octamers that either lacked the H4 N-terminal domain (H4ΔN), or contained H4K16Q or H3K79E derivatives. First, the ability of Sir3p to bind to mononucleosomes that harbor each of these derivatives was tested by gel shift assay. Each of these histone alterations decreased the binding affinity of Sir3p for these mononucleosome substrates (Figure 2A).

H4 ΔN, H4 K16Q, and H3 K79E histone octamers were next assembled into nucleosomal donors that were used in biotin-streptavidin capture assays. In each case, Sir3p-dependent inhibition of joint formation was strongly alleviated (Figure 2B). Specifically, donors assembled with nucleosomes that lacked the H4 N-terminal domain or contained H4K16Q were remarkably resistant to Sir3p-dependent inhibition compared to wildtype nucleosomal donors, whereas donors assembled with H3 K79E responded weakly to Sir3p-dependent repression (Figure 2B). Thus, Sir3p-dependent inhibition of joint formation not only requires nucleosomes, but inhibition requires key histone residues that are known to regulate heterochromatin assembly and function in vivo.

Previous studies have shown that the conserved bromo-adjacent homology (BAH) domain within Sir3p is essential for heterochromatin function in vivo and that this domain binds to nucleosomes in vitro (Onishi et al., 2007). As expected, a derivative of Sir3p that lacks the BAH domain (Sir3ΔBAHp) does not bind well to nucleosomes (Figure 2A, right panel; see also Onishi et al., 2007). We added Sir3ΔBAHp to nucleosomal donors and performed biotin-streptavidin capture assays and found that the BAH domain is essential for Sir3p-dependent inhibition of Rad51p-mediated joints. Addition of 4 Sir3ΔBAHp monomers per nucleosome had no affect on the ability of Rad51p presynaptic filaments to capture the homologous duplex (Figure 2C). Taken together, these results indicate that the Sir3p-dependent inhibition of Rad51p-catalyzed joints has many of the hallmarks of functional Sir3p-containing heterochromatin.

ATP-dependent chromatin remodeling disrupts Sir3p-containing heterochromatin

Mating type switching in *Saccharomyces cerevisiae* requires the recombinational repair of a DSB with a heterochromatic HM donor, and formation of the joint between the DSB at *MAT* and the heterochromatic donor requires the ATP-dependent chromatin remodeling enzyme, SWI/SNF (Chai et al., 2005). As an initial test for whether SWI/SNF might be required for HR only when the homologous donor was assembled into heterochromatin, we deleted the *SWI2* gene, which encodes the catalytic subunit of SWI/SNF, in a yeast strain that contains a *MAT**a**$_{inc}$* donor locus at an ectopic site on chromosome V. In this strain, a single DSB can be created within the *MAT* locus on chromosome III by expression of the HO endonuclease from the galactose-inducible *GAL10* promoter. This strain also lacks both *HMLα* and *HMR**a***, and thus the DSB is repaired using the euchromatic *MAT**a**$_{inc}$* donor. Both DSB induction and the formation and extension of the initial strand invasion product at the *MAT**a***$_{\text{inc}}$ locus are monitored by PCR (See Figure 3A schematic). In a *SWI2*+ strain, DSB induction occurred within 1.5 hours, and appearance of the initial strand invasion/extension product at the *MAT**a***$_{\text{inc}}$ donor occurred by 5 hours (Figure 3A, left panel). In contrast, and as expected, no strand invasion/extension product was detected in a *rad54Δ* strain (data not shown). Inactivation of SWI/SNF led to slower kinetics of DSB induction (7–8 hours; Figure 3A, right panel), presumably due to decreased expression of the HO endonuclease. However, following formation of the DSB, appearance of the initial, strand invasion/extension product was efficient, reaching 70% wildtype levels by 12 hours (Figure 3A, right panel). Although we cannot rule out the possibility that SWI/SNF is required for optimal kinetics of joint formation, these data confirm that SWI/SNF is not essential for recombinational repair, and furthermore that SWI/SNF may only be required for early steps of HR when the homologous donor is assembled into heterochromatin.

To directly test whether SWI/SNF might antagonize Sir3p-dependent inhibition of HR, we employed biotin-streptavidin capture assays (Figure 3B). As shown previously, SWI/SNF does not enhance joint formation with nucleosomal donors (Figure 3B; Sinha and Peterson, 2008). However, addition of a low concentration of SWI/SNF (1 SWI/SNF per 15 nucleosomes) restored joint formation on the Sir3p-nucleosomal donors to ~50% of the level observed for

the nucleosomal donor (Figure 3B). Importantly, the stimulation by SWI/SNF was not observed in the absence of ATP, indicating that the catalytic activity of SWI/SNF is required (Figure 3B). Furthermore, the effect of SWI/SNF was dependent on both the concentration of enzyme and time of remodeling (Figure 3C). Interestingly, Rad54p was not able to substitute for SWI/SNF, but addition of both SWI/SNF and Rad54p further stimulated joint formation (Figure 3B). Thus, these data indicate that SWI/SNF is essential for early steps of HR with a heterochromatic donor, and that the ATPase activity of Rad54p can augment SWI/SNF function.

In vivo studies have demonstrated that SWI/SNF, RSC, Ino80.com, and Swr1.com remodeling enzymes are all recruited to a DSB, but only SWI/SNF is required for synapsis of the Rad51p filament with the *HM* donor (Chai et al., 2005; Papamichos-Chronakis et al., 2006). These results suggest that SWI/SNF may be uniquely capable of antagonizing Sir3p-dependent inhibition of joint formation. To directly address this hypothesis, we purified RSC, Ino80.com, and Swr1.com and tested whether these enzymes could restore joint formation with the Sir3p-nucleosomal donor. When equal amounts (20 nM) of each remodeling enzyme were added, only SWI/SNF showed robust stimulation of joint formation with Sir3p-nucleosomal donors. RSC and Ino80.com provided a ~2-fold stimulation of joint formation, but addition of even 5-fold higher levels of RSC was not sufficient to restore joint formation to the level obtained with 20 nM SWI/SNF (M.S. and C.L.P., data not shown). Thus, these in vitro reactions recapitulate the unique requirement for the SWI/SNF remodeling enzyme for early steps of HR on a heterochromatic donor.

During a mating type switching event, it is essential that disruption of heterochromatin is reversible so that both sets of mating type information are not expressed. Therefore, we tested whether SWI/SNF action on the Sir3p-nucleosomal donor was reversible in vitro (Figure 4A). Joint formation assays were assembled that contained yRad51p presynaptic filament, Sir3p-nucleosomal donor, SWI/SNF, and ATP. After 5’ of incubation, half of the reaction was captured on magnetic beads to quantify joint formation, while the second half received either buffer or 1.0 units of apyrase to remove ATP and eliminate SWI/SNF activity (Figure S3). Reactions were further incubated, aliquots removed, and joints captured on magnetic beads at time intervals. As shown, inactivation of SWI/SNF led to the time-dependent decrease in joint formation, reaching near background levels by 60 minutes of apyrase treatment. Notably, this decrease in joint formation in the presence of apyrase was not observed with donors that lacked Sir3p (M.S. and C.L.P., data not shown). These data indicate that once SWI/SNF is inactivated, Sir3p can re-establish nucleosomal structures that block joint formation.

The biotin-streptavidin capture assay measures formation of the initial, unstable joint and the stable plectonemic joint (Riddles and Lehman, 1985). Whereas the initial joint is stabilized by protein-DNA interactions, the plectonemic joint or D-loop is stable even in the absence of protein (e.g. yRad51p). To monitor formation of stable D-loop products, reactions were de-proteinized and DNA products separated on agarose gels. As we showed previously, Rad54p is essential for formation of stable D-loops on nucleosomal donors, whereas SWI/SNF could not substitute for Rad54p nor did it stimulate Rad54p activity (Figure 4B, lanes 2 and 3; see also Jaskelioff et al., 2003; Sinha and Peterson, 2008). In contrast to nucleosomal donors, Rad54p did not promote formation of a D-loop product on the Sir3p-nucleosomal donor (Figure 4B, lane 8). Strikingly, addition of SWI/SNF and ATP led to significant levels of D-loop product (33 +/− 7.5% of D-loop product formed in absence of Sir3p; Figure 4B, lane 6), and further addition of Rad54p led to levels of D-loops that paralleled the amount of stable joints formed in the absence of Sir3p (88 +/− 3.7% of D-loop; Figure 4B, lane 9). Since SWI/SNF does not promote D-loops on a nucleosomal donor that lacks Sir3p, these data indicate that assembly of a Sir3p-nucleosome complex can facilitate the ability of SWI/SNF to convert unstable joints to stable D-loops.

SWI/SNF catalyzes the ATP-dependent displacement of Sir3p

To investigate how SWI/SNF antagonizes the repressive effects of Sir3p on HR, we tested whether SWI/SNF can remodel Sir3p-nucleosomal substrates. One hallmark of ATP-dependent chromatin remodeling enzymes is their ability to mobilize ("slide") nucleosomes in cis (Cairns, 2007). Centrally-positioned mononucleosomes were assembled on a 343 bp DNA fragment that contains a'601' nucleosome positioning element. Mononucleosomes or Sir3p-mononucleosome complexes (see Figure 2A) were incubated with SWI/SNF and ATP, and aliquots were removed at several timepoints and electrophoresed on a native polyacrylamide gel. (Note that excess DNA is added to each reaction just prior to gel loading to remove SWI/SNF from the substrate; DNA addition also removes Sir3p.) As shown in Figure 5A, SWI/SNF action mobilizes the nucleosome, leading to formation of faster migrating species and the appearance of a free DNA product. Notably, addition of 4 Sir3p monomers per nucleosome had no obvious effect on the kinetics or extent of SWI/SNF remodeling.

As a second measure of SWI/SNF remodeling activity, we monitored kinetics of *Hha*I digestion of the 601-mononucleosome substrate (Figure 5B) and the kinetics of *Sal*I digestion of a nucleosomal array substrate (Figure 5C). For both substrates, the restriction enzyme site is located near the center of a positioned nucleosome, and thus little digestion occurs in the absence of SWI/SNF activity (Figure 5B, C). However, SWI/SNF-dependent remodeling leads to greatly enhanced digestion rates for both mononucleosomal and array substrates (Figure 5B,C). Furthermore, incorporation of 2–4 monomers of Sir3p per nucleosome had very little impact on the apparent rates of SWI/SNF remodeling activity (Figure 5B, C). Taken together, these data indicate that SWI/SNF is effective at remodeling Sir3p-nucleosome substrates.

We tested whether SWI/SNF action might lead to the displacement of Sir3p from the minichromosome donors. The Sir3p-nucleosomal donor was incubated for 15' with SWI/SNF, and the donor was captured on magnetic beads. Supernatant and bead-bound fractions were analyzed by western blot with antibodies to Sir3p. As shown, Sir3p is exclusively found in the bound (B) fraction in the absence of SWI/SNF or when ATP is omitted from reactions that contain SWI/SNF. However, addition of SWI/SNF and ATP catalyzed the eviction of the Sir3p into the supernatant (U) fraction (Figure 5D and data not shown). SWI/SNF-mediated eviction of Sir3p was concentration dependent and increased with time of incubation, though we observe a maximum displacement of ~40% of Sir3p even at higher concentrations of SWI/SNF (20 nM; 1 SWI/SNF per 10 nucleosomes; Figure 5D, lower panel). Interestingly, the ATPase activity of Rad54p cannot evict Sir3p, even when Sir3p is present at a low ratio of Sir3p per nucleosome (Figure 5E, bottom panel). Likewise, Sir3p was not evicted by the remodeling activities of RSC or Ino80.com (Figure 5E, top two panels). Thus, SWI/SNF seems uniquely able to evict Sir3p from nucleosomal templates, and this activity correlates with the stimulation of joint formation.

SWI/SNF facilitates joint formation with a Sir2p/Sir3p/Sir4p heterochromatin donor

Although Sir3p-nucleosome complexes exhibit many of the properties of native, Sir-dependent heterochromatin, it remained a possibility that a complete heterochromatic structure, containing Sir2p, Sir3p, and Sir4p, might be a more potent repressor of recombination. We purified Sir2p/Sir4p from yeast cells and added increasing amounts of Sir2p/Sir4p to biotin-streptavidin capture assays. Due to an inability to extensively concentrate Sir2p/Sir4p, these assays included only 4 nM donor minichromosome (100 nM nucleosomes), rather than the 17 nM (400 nM nucleosomes) used in other assays. Importantly, the overall efficiency of joint formation in the absence of Sir2p/Sir4p was not greatly affected by this change in donor minichromosome concentration (Figure 6A). When Sir2p/Sir4p was added to assays at a ratio of 1 Sir2p/Sir4p heterodimer per nucleosome, there was only a minor repressive effect, whereas higher concentrations led to a 2-fold repression (Figure 6A and data not shown). Unlike

repression by Sir3p, the weak repression due to Sir2p/Sir4p was largely insensitive to substitution of H4 K16 (Figure 6A).

To investigate whether Sir2p/Sir4p might enhance the repressive effects of Sir3p, we added Sir2p/Sir4p to biotin-streptavidin capture assays that contained different amounts of Sir3p (Figure 6A and data not shown). At 4 nM minichromosome donor, we found that higher concentrations of Sir3p monomers were required to observe repression of joint formation (compare Figure 1C and 6A). For instance, addition of 2 Sir3p monomers per nucleosome yielded only ~ 2-fold repression, 4 monomers of Sir3p per nucleosome led to ~3-fold repression, and 8 Sir3p monomers per nucleosome was required for complete repression (Figure 6A and data not shown). Strikingly, addition of 1 Sir2p/Sir4p heterodimer per nucleosome led to complete and synergistic repression of joint formation at 4 monomers of Sir3p per nucleosome (Figure 6A). In contrast, 1 Sir2p/Sir4p heterodimer and 2 Sir3p monomers per nucleosome yielded very little repression of joint formation (data not shown). Thus, Sir2p/Sir4p can enhance repression of recombination by Sir3p, but this role for Sir2p/Sir4p requires a threshold concentration of Sir3p. Importantly, repression by Sir2p/Sir3p/Sir4p was alleviated by the H4 K16Q substitution, consistent with a physiologically relevant heterochromatic structure. These results are consistent with a recent study showing that Sir2p/Sir4p enhances the affinity of Sir3p for a trinucleosomal substrate (Martino et al., 2009).

We then tested whether SWI/SNF action could restore joint formation with the Sir2p/Sir3p/Sir4p-nucleosomal donors. SWI/SNF was added to biotin-streptavidin capture assays in which the nucleosomal donor contained 1 Sir2p/Sir4p heterodimer and 4 Sir3p monomers per nucleosome. Similar to the results with Sir3p-nucleosomal donors, SWI/SNF remodeling restored joint formation to nearly 50% of the level observed in the absence of Sir proteins (Figure 6B and C). Likewise, SWI/SNF could efficiently remodel mononucleosomes that contained Sir2p/Sir3p/Sir4p (data not shown). The Rad54p, RSC, Ino80.com, and Swr1.com remodeling enzymes could not substitute for SWI/SNF (Figure 6C). Thus, although addition of Sir2p/Sir4p enhanced Sir3p repression, SWI/SNF was still able to promote efficient joint formation.

Discussion

In vivo, assembly of Sir-dependent heterochromatin requires Sir2p, Sir3p, and Sir4p, and these three proteins form a complex in vitro (Rusche et al., 2003). Moreover, an intact Sir2/3/4 complex is required to form extended filaments on yeast nucleosomal arrays in vitro (Onishi et al., 2007), and the intact complex is apparently required to observe transcriptional silencing on nucleosomal templates in vitro (A.J. and D.M., submitted). These data are consistent with a model in which all three Sir proteins contribute to the structural and functional properties of Sir-dependent heterochromatin. Although we find that Sir2p/Sir4p enhances the repressive effects of Sir3p, Sir3p is sufficient to assemble an Mnase resistant nucleosomal structure and to repress early steps of recombination. This may be due in part to the fact that our in vitro study employed histones that lack posttranslational modifications, obviating the need for the HDAC activity of Sir2p. In addition, Sir3p is bound to all nucleosomes within the minichromosome substrate, eliminating the need for the known targeting activity of Sir4p (Rusche et al., 2003).

What is the stoichiometry of Sir proteins within heterochromatin? In vivo chromatin immunoprecipitation studies do not yield insights into this question, although the purification of Sir complexes from yeast cells indicates that Sir2p and Sir4p form a heterodimer. Recently, Gasser and colleagues have monitored the binding of Sir2p, Sir3p, and Sir4p to a trinucleosome in vitro (Martino et al., 2009). Their data indicate that one Sir2p/Sir3p/Sir4p heterotrimer may bind to each linker between two nucleosomes, although they also showed that nucleosomes

are competent to bind additional Sir complexes. Our joint formation assays monitor the amount of Sir proteins required to assemble heterochromatin that represses joint formation, and under our assay conditions, Sir3p must be in excess to the Sir2/Sir4 heterodimer. Our results suggest that functional heterochromatin may require a minimum of one heterodimer of Sir2/Sir4 and four monomers (or two dimers) of Sir3p per nucleosome.

We were surprised to find that SWI/SNF was able to displace Sir3p from minichromosomes, even when SWI/SNF was present at a low ratio of enzyme to nucleosome. Furthermore, the extent of displacement correlated with joint formation, suggesting that these two activities are mechanistically linked. How does SWI/SNF action evict Sir3p? ATP-dependent remodeling enzymes are able to use the energy from ATP hydrolysis to translocate DNA, and in the case of SWI/SNF-like enzymes, DNA translocation is key for mobilizing nucleosomes (Cairns, 2007). Thus, SWI/SNF might displace Sir3p simply by its DNA translocation activity. However, Rad54p and RSC also have potent DNA translocase activities (Cairns, 2007; Jaskelioff et al., 2003), but none of these enzymes can evict Sir3p from nucleosomal substrates. Furthermore, the RSC remodeling enzyme is highly related to SWI/SNF, and RSC typically performs identically, if not better than SWI/SNF, in other remodeling assays (Carey et al., 2006; Zhang et al., 2006). Recent studies indicate that a nucleosome may bind to SWI/SNF within a shallow cleft, whereas RSC may completely envelope the nucleosome (Chaban et al., 2008; Dechassa et al., 2008). Once a nucleosome is bound, both SWI/SNF and RSC initiate DNA translocation ~2 DNA turns from the dyad axis (Dechassa et al., 2008). Based on these structural studies, we favor a model in which RSC is unable to envelop a Sir3p-nucleosome complex and thus is unable to initiate DNA translocation, whereas SWI/SNF is competent to bind such a structure, initiate DNA translocation and displace Sir3p. Displacement could be the result of altered histone-DNA interactions or destabilization of histone-histone interactions, which also occurs during SWI/SNF remodeling (Bruno et al., 2003).

Numerous studies have demonstrated that in vitro formation of a stable D-loop requires the combined actions of Rad51p and the Rad54p ATPase, even when naked DNA is used as the donor molecule. Sung and colleagues have shown that Rad54p can alter DNA topology, presumably through its DNA translocase activity, and changes in topology are likely to drive DNA duplex opening and strand invasion (Van Komen et al., 2000). SWI/SNF can not substitute for Rad54p with either a DNA or nucleosomal donor, even though SWI/SNF can also function as a translocase (Jaskelioff et al., 2003; Sinha and Peterson, 2008; Cairns, 2007). Based on these previous results, it was unexpected that SWI/SNF activity was able to drive formation of D-loops on the Sir3p-nucleosomal donor in the absence of Rad54p. This suggests that the Sir3p that is not evicted from the donor plays an active role in facilitating SWI/SNF action. We envision that Sir-nucleosomes that surround the initial joint molecule might constrain SWI/SNF-dependent changes in DNA topology that drive D-loop formation; topology changes that would normally be insufficient or rapidly dissipated in the absence of Sir3-nucleosomal structures. This novel activity of SWI/SNF on a heterochromatic donor may provide an explanation for why a surprisingly large percentage of yeast cells can switch mating type in the absence of Rad54p (Schmuckli-Maurer and Heyer, 1999).

Our data suggest a model that explains the sequence of events for recombinational repair in the context of euchromatic and heterochromatic donor loci (Figure 7). After formation and exonucleolytic processing of a DSB, Rad51p polymerizes onto the resulting ssDNA with the help of mediators such as Rad52p. The Rad51p presynaptic filament then searches for a homologous duplex, and this filament is sufficient to capture homology within euchromatin. However, if the donor is located within a heterochromatic domain, our data suggest that the Rad51p presynaptic filament is no longer sufficient to locate homology and form an initial joint. In this case, the ATP-dependent remodeling enzyme, SWI/SNF, plays an essential role in the initial capture event, disrupting heterochromatin. Once the initial joint is formed, the

combination of SWI/SNF and the Rad54p ATPase converts this metastable joint into a stable D-loop in which the 3' end of the presynaptic filament is engaged in base-pairing interactions with the donor and is thus competent for subsequent events of recombinational repair.

Heterochromatin is generally associated with telomeric and centromeric regions of the genome that are characterized by a high percentage of repetitive elements. Typically these genomic regions are characterized by a general suppression of mitotic and meiotic cross-over events, and many studies indicate that the suppression of recombination requires heterochromatin components (Westphal and Reuter, 2002; Peng and Karpen, 2007; Jaco et al., 2008; Allshire et al., 1994). However, DSBs are certainly formed and repaired within heterochromatin, so it is not too surprising that recombination within heterochromatin does occur (Jaco et al., 2008; Peng and Karpen, 2008).Presumably heterochromatin functions as a brake on the recombination machinery, such that DSBs can be repaired but promiscuous recombination is suppressed. Our data suggest that the recombination machinery that functions within heterochromatin must acquire additional chromatin remodeling activities in order to repair DSBs in this repressive environment. The mechanisms that control recruitment of chromatin remodeling enzymes like SWI/SNF to sites of DNA damage are still being elucidated, but it seems likely that the recruitment of such enzymes will be strictly controlled in order to preserve genome integrity.

Experimental Procedures

Reagent Preparation

DNA—Oligonucleotide E4 (5'CAAAATAGCACCCTCCCGCTCCAGAACAACATACAGCGCTTCCACAGCGGCAGCCATAACAGTCAGCCTTACCAGTAAAAAAGAAAA) was obtained from Integrated DNA Technologies, Inc. (Coralville, IA) and was 5' end labeled with ^{32}P using γ-^{32}P -ATP and T4 polynucleotide kinase (New England Biolabs, Inc.; Beverly, MA). Plasmid CP943 (p2085S-G5E4) (Ikeda et al., 1999), CP1024 (601-Mono) and CP589 (208-11array) were prepared by standard alkaline lysis method.

Proteins—Recombinant yRad51 was over expressed in *E.coli* and purified as described (Zaitseva et al., 1999). GST-yRad54, Swi2-TAP and Sir3-FLAG were purified from yeast as described (Buchberger et al., 2008; Smith et al., 2003; Solinger et al., 2001). Sir2-HA/Sir4-TAP (Liou et al., 2005), RSC2-TAP, Ino80 -TAP, and Swr1 -TAP were purified essentially as described for Swi2-TAP. All remodeling enzymes were analyzed by SDS-PAGE and silver-staining (Figure S4). Subunit compositions were confirmed by mass spectrometry. To ensure that equal amounts of active enzyme were added to all assays, ATPase activity was determined for each enzyme and equal ATPase units were used in assays. Notably, equal ATPase units also yielded equivalent levels of mononucleosome sliding activity, with the exception of Swr1.com which does not mobilize nucleosomes. Concentrations of GST-yRAD54, yRAD51, Sir3-FLAG and Sir2-HA/Sir4-TAP heterodimer were estimated by Bradford assay and comparing to known concentrations of BSA on the same coomassie-stained gel. Recombinant H2B, H3, H4, H2AS113C, H4K16Q, H3K79E and globular domain of H4 (H4ΔN) Xenopus histones were purified and octamers containing different modified histones were reconstituted as described (Luger et al., 1999). Biotinylated octamers were reconstituted as described (Sinha and Peterson, 2008).

Nucleosome assembly—To assemble all nucleosomal templates, salt step dialysis of supercoiled or linear DNA and recombinant octamers were used as described (Logie and Peterson, 1999). Nucleosomes were reconstituted at different ratios of histone octamer per 200 bp of donor DNA (R value). To prepare nucleosomal array template for the restriction enzyme

accessibility assay, plasmid CP589 was digested with NotI and HindIII generating a 2.3kb DNA fragment that contains 11 5S repeats (208-11). To reconstitute mononucleosomes, a 343-bp DNA fragment (601) was generated by digesting CP1024 with EcoRI and HindIII. Following digestion, 208-11 and 601 templates were purified from agarose gels and end labeled with [alpha-^{32}P] dCTP by Klenow fill-in at 37 °C and purified through a Sephadex G-25 column after phenol-chloroform extraction. Reconstituted mononucleosomes were analyzed by native polyacrylamide gel electrophoresis in 1xTBE buffer. In reactions containing Sir3p/Sir2p/Sir4p, the minichromosomes, mononucleosomes or linear nucleosomal arrays were incubated with Sir proteins at different molar ratios (M/N: monomer per nucleosome) in a buffer containing 35mM Tris-HCl, pH 7.5, 2.5mM $MgCl_2$, 30mM KCl, 1mM DTT at room temperature for 30 min. Binding of Sir3p to mononucleosomes was analyzed by subjecting the products to 4% native polyacrylamide gel electrophoresis in 0.5× TBE.

Mnase assay—Minichromosomes (200ng DNA equivalent), bound or unbound to Sir3 were subjected to Mnase analysis as described (Sinha and Peterson, 2008).

Joint capture assays—Joints were captured essentially as described with some minor modifications (Sinha and Peterson, 2008). To form the presynaptic filaments, 3μM of yRad51p was incubated with radio-labeled oligonucleotide (3μM nucleotides) in a buffer containing 35mM Tris-HCl, pH 7.5, 2.5mM $MgCl_2$, 30mM KCl, 1mM DTT at 30°C for 5 min in a 10μl reaction volume. As a nucleotide cofactor, 0.3mM AMP-PNP was used. Then, 3μl of minichromosome donor (pre-bound to Sir proteins at indicated ratios or unbound) at a final concentration of 17nM DNA (~400nM nucleosomes) was added to the reaction and allowed to form joints at 30°C for 15 min. For Sir2/Sir3/Sir4 reactions, minichromosome donor was added at ~4nM DNA / 100nM nucleosomes, and the Rad51 preynaptic filament was present at 0.75 μM. In reactions containing yRad54p, 200nM yRad54p and 2mM ATP were added during formation of presynaptic filaments after incubating the oligonucleotide with yRad51 for 2 min, and then reactions were incubated for an additional 3 min. In reactions containing remodeling enzymes, 20nM of each of the remodeling enzymes (unless indicated otherwise) was preincubated with minichromosome donors in presence of 2mM Mg-ATP at 30°C

Remodeling assays—Remodeling assays were performed as described (Logie and Peterson, 1999).

Sir3p displacement assay—Standard Sir3p-containing heterochromatin was assembled in 3μl with 17nM DNA equivalent of biotinylated minichromosomes and Sir3p at a molar ratio of 2–4 monomers per nucleosome at room temperature for 30 minutes in the same reaction buffer as in joint-capture assay. Subsequently, 200nM yRad54p or 20nM of remodeling enzymes and 2mM ATP were added, allowing a minimal volume expansion and incubated for another 15 minutes at 30°C. Finally, the minichromosomes were captured on magnetic beads, and the supernatant and bead-bound fractions were analyzed by 8% SDS-PAGE and immunoblotting with anti-FLAG antibody (Courtesy of Tony Imbalzano, UMMS).

Yeast strains and DSB/SI monitor assay—The *MATα* ectopic recombination strain (yJK17) lacks *HMLα* or *HMR**a*** on Chr III and the donor sequence is a *MAT**a*** locus containing an incleavable HO site (*MAT***a**-inc) on Chr V (Vaze et al., 2002). The *SWI2* and *RAD54* genes were disrupted in this strain background by replacing the coding regions with *KAN-MX6* (Longtine et al., 1998). Kinetics of DSB formation and strand invasion by PCR as described (Keogh et al., 2006). Specifically, cultures were grown in media containing 2% glucose until an OD_{600} of ≈ 0.6–0.8, then expression of HO endonuclease was induced by 2% galactose. Cells were harvested at the times indicated followed by genomic DNA isolation, which was used in PCR reactions containing primers previously described (Keogh et al., 2006). Efficiency

of DSB formation and strand invasion was arbitrarily set to 100% for the WT DSB product i.e. at 0 timepoint. All values were normalized to a *PHO5* internal control.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Anthony Imbalzano (UMMS) for antibody to the FLAG epitope, Blaine Bartholomew (Univ. S. Illinois) for providing the strain harboring the RSC2-TAP allele. We apologize to those colleagues whose work we were unable to cite due to space restrictions. This work was supported by grants from the National Institutes of Health to C.L.P. (GM54096) and to D.M. (GM061641), and by a Ruth L. Kirschstein National Research Service Award Individual Postdoctoral Fellowship through the National Institute of General Medical Sciences to A. J. (F32 GM078799). D.M. is a Howard Hughes Medical Institute investigator.

References

Allshire RC, Javerzat JP, Redhead NJ, Cranston G. Position effect variegation at fission yeast centromeres. Cell 1994;76:157–169. [PubMed: 8287474]

Barton AB, Pekosz MR, Kurvathi RS, Kaback DB. Meiotic recombination at the ends of chromosomes in Saccharomyces cerevisiae. Genetics 2008;179:1221–1235. [PubMed: 18562657]

Bruno M, Flaus A, Stockdale C, Rencurel C, Ferreira H, Owen-Hughes T. Histone H2A/H2B dimer exchange by ATP-dependent chromatin remodeling activities. Mol Cell 2003;12:1599–1606. [PubMed: 14690611]

Buchberger JR, Onishi M, Li G, Seebacher J, Rudner AD, Gygi SP, Moazed D. Sir3-nucleosome interactions in spreading of silent chromatin in Saccharomyces cerevisiae. Molecular and cellular biology 2008;28:6903–6918. [PubMed: 18794362]

Cairns BR. Chromatin remodeling: insights and intrigue from single-molecule studies. Nat Struct Mol Biol 2007;14:989–996. [PubMed: 17984961]

Carey M, Li B, Workman JL. RSC exploits histone acetylation to abrogate the nucleosomal block to RNA polymerase II elongation. Molecular cell 2006;24:481–487. [PubMed: 17081996]

Chaban Y, Ezeokonkwo C, Chung WH, Zhang F, Kornberg RD, Maier-Davis B, Lorch Y, Asturias FJ. Structure of a RSC-nucleosome complex and insights into chromatin remodeling. Nature structural & molecular biology 2008;15:1272–1277.

Chai B, Huang J, Cairns BR, Laurent BC. Distinct roles for the RSC and Swi/Snf ATP-dependent chromatin remodelers in DNA double-strand break repair. Genes & development 2005;19:1656–1661. [PubMed: 16024655]

Dechassa ML, Zhang B, Horowitz-Scherer R, Persinger J, Woodcock CL, Peterson CL, Bartholomew B. Architecture of the SWI/SNF-nucleosome complex. Molecular and cellular biology 2008;28:6010–6021. [PubMed: 18644858]

Georgel PT, Palacios DeBeer MA, Pietz G, Fox CA, Hansen JC. Sir3-dependent assembly of supramolecular chromatin structures in vitro. Proceedings of the National Academy of Sciences of the United States of America 2001;98:8584–8589. [PubMed: 11447281]

Grewal SI, Jia S. Heterochromatin revisited. Nature reviews 2007;8:35–46.

Haber JE. Mating-type gene switching in Saccharomyces cerevisiae. Annual review of genetics 1998;32:561–599.

Hecht A, Laroche T, Strahl-Bolsinger S, Gasser SM, Grunstein M. Histone H3 and H4 N-termini interact with SIR3 and SIR4 proteins: a molecular model for the formation of heterochromatin in yeast. Cell 1995;80:583–592. [PubMed: 7867066]

Hecht A, Strahl-Bolsinger S, Grunstein M. Spreading of transcriptional repressor SIR3 from telomeric heterochromatin. Nature 1996;383:92–96. [PubMed: 8779721]

Henikoff S. Heterochromatin function in complex genomes. Biochimica et biophysica acta 2000;1470:O1–O8. [PubMed: 10656988]

Ikeda K, Steger DJ, Eberharter A, Workman JL. Activation domain-specific and general transcription stimulation by native histone acetyltransferase complexes. Molecular and cellular biology 1999;19:855–863. [PubMed: 9858608]

Imai S, Armstrong CM, Kaeberlein M, Guarente L. Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. Nature 2000;403:795–800. [PubMed: 10693811]

Jaco I, Canela A, Vera E, Blasco MA. Centromere mitotic recombination in mammalian cells. The Journal of cell biology 2008;181:885–892. [PubMed: 18541703]

Jaskelioff M, Van Komen S, Krebs JE, Sung P, Peterson CL. Rad54p is a chromatin remodeling enzyme required for heteroduplex DNA joint formation with chromatin. The Journal of biological chemistry 2003;278:9212–9218. [PubMed: 12514177]

Keogh MC, Kim JA, Downey M, Fillingham J, Chowdhury D, Harrison JC, Onishi M, Datta N, Galicia S, Emili A, et al. A phosphatase complex that dephosphorylates gammaH2AX regulates DNA damage checkpoint recovery. Nature 2006;439:497–501. [PubMed: 16299494]

Liou GG, Tanny JC, Kruger RG, Walz T, Moazed D. Assembly of the SIR complex and its regulation by O-acetyl-ADP-ribose, a product of NAD-dependent histone deacetylation. Cell 2005;121:515–527. [PubMed: 15907466]

Logie C, Peterson CL. Purification and biochemical properties of yeast SWI/SNF complex. Methods in Enzymology 1999;304:726–741. [PubMed: 10372393]

Longtine MS, McKenzie A 3rd, Demarini DJ, Shah NG, Wach A, Brachat A, Philippsen P, Pringle JR. Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. Yeast (Chichester, England) 1998;14:953–961.

Loo S, Rine J. Silencers and domains of generalized repression. Science 1994;264:1768–1771. [PubMed: 8209257]

Luger K, Rechsteiner TJ, Richmond TJ. Expression and purification of recombinant histones and nucleosome reconstitution. Methods in molecular biology (Clifton, N.J 1999;119:1–16.

Martino F, Kueng S, Robinson P, Tsai-Pflugfelder M, van Leeuwen F, Ziegler M, Cubizolles F, Cockell MM, Rhodes D, Gasser SM. Reconstitution of yeast silent chromatin: multiple contact sites and O-AADPR binding load SIR complexes onto nucleosomes in vitro. Molecular cell 2009;33:323–334. [PubMed: 19217406]

Ng HH, Feng Q, Wang H, Erdjument-Bromage H, Tempst P, Zhang Y, Struhl K. Lysine methylation within the globular domain of histone H3 by Dot1 is important for telomeric silencing and Sir protein association. Genes Dev 2002;16:1518–1527. [PubMed: 12080090]

Onishi M, Liou GG, Buchberger JR, Walz T, Moazed D. Role of the conserved Sir3-BAH domain in nucleosome binding and silent chromatin assembly. Molecular cell 2007;28:1015–1028. [PubMed: 18158899]

Osley MA, Tsukuda T, Nickoloff JA. ATP-dependent chromatin remodeling factors and DNA damage repair. Mutation research 2007;618:65–80. [PubMed: 17291544]

Papamichos-Chronakis M, Krebs JE, Peterson CL. Interplay between Ino80 and Swr1 chromatin remodeling enzymes regulates cell cycle checkpoint adaptation in response to DNA damage. Genes Dev 2006;20:2437–2449. [PubMed: 16951256]

Paques F, Haber JE. Multiple pathways of recombination induced by double-strand breaks in Saccharomyces cerevisiae. Microbiol Mol Biol Rev 1999;63:349–404. [PubMed: 10357855]

Passarge E. Emil Heitz and the concept of heterochromatin: longitudinal chromosome differentiation was recognized fifty years ago. American journal of human genetics 1979;31:106–115. [PubMed: 377956]

Peng JC, Karpen GH. H3K9 methylation and RNA interference regulate nucleolar organization and repeated DNA stability. Nature cell biology 2007;9:25–35.

Peng JC, Karpen GH. Epigenetic regulation of heterochromatic DNA stability. Current opinion in genetics & development 2008;18:204–211. [PubMed: 18372168]

Renauld H, Aparicio OM, Zierath PD, Billington BL, Chhablani SK, Gottschling DE. Silent domains are assembled continuously from the telomere and are defined by promoter distance and strength, and by SIR3 dosage. Genes & development 1993;7:1133–1145. [PubMed: 8319906]

Richards EJ, Elgin SC. Epigenetic codes for heterochromatin formation and silencing: rounding up the usual suspects. Cell 2002;108:489–500. [PubMed: 11909520]

Riddles PW, Lehman IR. The formation of paranemic and plectonemic joints between DNA molecules by the recA and single-stranded DNA-binding proteins of Escherichia coli. The Journal of biological chemistry 1985;260:165–169. [PubMed: 2981202]

Rusche LN, Kirchmaier AL, Rine J. The establishment, inheritance,and function of silenced chromatin in Saccharomyces cerevisiae. Annual review of biochemistry 2003;72:481–516.

Savitsky M, Kravchuk O, Melnikova L, Georgiev P. Heterochromatin protein 1 is involved in control of telomere elongation in Drosophila melanogaster. Molecular and cellular biology 2002;22:3204–3218. [PubMed: 11940677]

Schmuckli-Maurer J, Heyer WD. The Saccharomyces cerevisiae RAD54 gene is important but not essential for natural homothallic mating-type switching. Mol Gen Genet 1999;260:551–558. [PubMed: 9928934]

Sinha M, Peterson CL. A Rad51 presynaptic filament is sufficient to capture nucleosomal homology during recombinational repair of a DNA double-strand break. Molecular cell 2008;30:803–810. [PubMed: 18570881]

Smith CL, Horowitz-Scherer R, Flanagan JF, Woodcock CL, Peterson CL. Structural analysis of the yeast SWI/SNF chromatin remodeling complex. Nature structural biology 2003;10:141–145.

Solinger JA, Lutz G, Sugiyama T, Kowalczykowski SC, Heyer WD. Rad54 protein stimulates heteroduplex DNA formation in the synaptic phase of DNA strand exchange via specific interactions with the presynaptic Rad51 nucleoprotein filament. Journal of molecular biology 2001;307:1207–1221. [PubMed: 11292336]

Strahl-Bolsinger S, Hecht A, Luo K, Grunstein M. SIR2 and SIR4 interactions differ in core and extended telomeric heterochromatin in yeast. Genes & development 1997;11:83–93. [PubMed: 9000052]

Sugawara N, Wang X, Haber JE. In vivo roles of Rad52, Rad54, and Rad55 proteins in Rad51-mediated recombination. Molecular cell 2003;12:209–219. [PubMed: 12887906]

Suka N, Luo K, Grunstein M. Sir2p and Sas2p opposingly regulate acetylation of yeast histone H4 lysine16 and spreading of heterochromatin. Nature genetics 2002;32:378–383. [PubMed: 12379856]

Tsukuda T, Lo YC, Krishna S, Sterk R, Osley MA, Nickoloff JA. INO80-dependent chromatin remodeling regulates early and late stages of mitotic homologous recombination. DNA repair 2009;8:360–369. [PubMed: 19095087]

Van Komen S, Petukhova G, Sigurdsson S, Stratton S, Sung P. Superhelicity-driven homologous DNA pairing by yeast recombination factors Rad51 and Rad54. Molecular cell 2000;6:563–572. [PubMed: 11030336]

Vaze MB, Pellicioli A, Lee SE, Ira G, Liberi G, Arbel-Eden A, Foiani M, Haber JE. Recovery from checkpoint-mediated arrest after repair of a double-strand break requires Srs2 helicase. Molecular cell 2002;10:373–385. [PubMed: 12191482]

Westphal T, Reuter G. Recombinogenic effects of suppressors of position-effect variegation in Drosophila. Genetics 2002;160:609–621. [PubMed: 11861565]

White SA, Allshire RC. RNAi-mediated chromatin silencing in fission yeast. Current topics in microbiology and immunology 2008;320:157–183. [PubMed: 18268844]

Wolner B, Peterson CL. ATP-dependent and ATP-independent roles for the Rad54 chromatin remodeling enzyme during recombinational repair of a DNA double strand break. The Journal of biological chemistry 2005;280:10855–10860. [PubMed: 15653683]

Zaitseva EM, Zaitsev EN, Kowalczykowski SC. The DNA binding properties of Saccharomyces cerevisiae Rad51 protein. The Journal of biological chemistry 1999;274:2907–2915. [PubMed: 9915828]

Zhang Y, Smith CL, Saha A, Grill SW, Mihardja S, Smith SB, Cairns BR, Peterson CL, Bustamante C. DNA translocation and loop formation mechanism of chromatin remodeling by SWI/SNF and RSC. Molecular cell 2006;24:559–568. [PubMed: 17188033]

Zou S, Ke N, Kim JM, Voytas DF. The Saccharomyces retrotransposon Ty5 integrates preferentially into regions of silent chromatin at the telomeres and mating loci. Genes Dev 1996;10:634–645. [PubMed: 8598292]

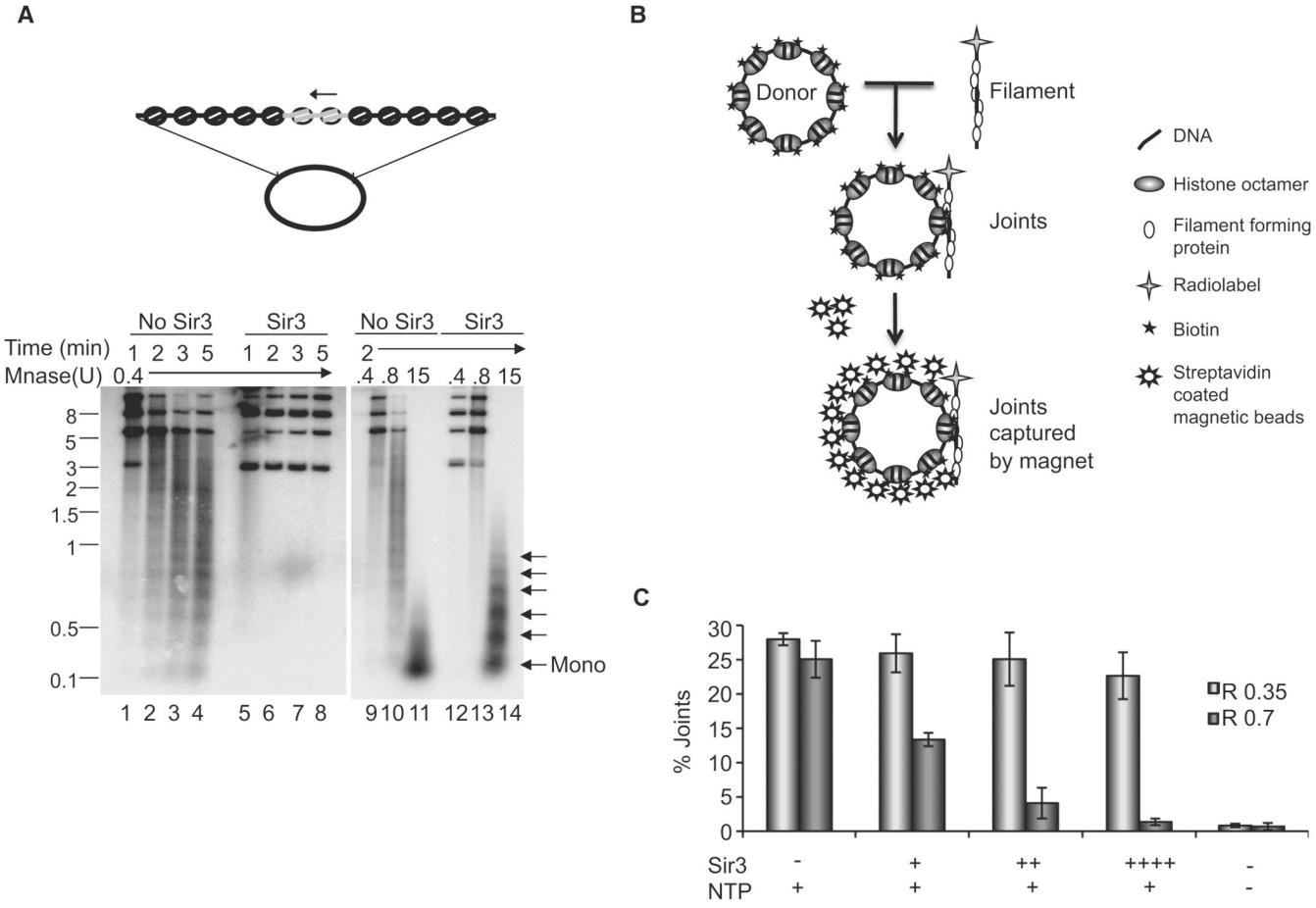

Figure 1. Sir3p-nucleosomes pose a barrier to nucleosome accessibility and Rad51p-dependent joint formation

(A) Top: Schematic of the minichromosome donor. Black ovals; nucleosomes on 5S repeats; grey ovals, nucleosomes on E4 sequences; black arrow, 87-nucleotide E4 oligonucleotide. Bottom: Minichromosomes (R=0.7; 0.7 octamers per 200bp of DNA) were analyzed in absence (lanes 1–4, 9–11) or presence (lanes 5–8, 12–14) of Sir3p (4 monomers per 200 bp) by MNase digestion. Units of MNase enzyme and time of incubation at 23°C are indicated at top. DNA size markers (kb) are shown on left. Black arrows on the right denote MNase cleavages. (B) Schematic of biotin-streptavidin joint capture assay. (C) Results of joint capture assays, with minichromosome donors subsaturated (R=0.35) or saturated (R=0.7) with nucleosomes. Sir3p was bound to minichromosomes at 1 (+), 2 (++), or 4 (++++) monomers of Sir3p per 200 bp of DNA. AMP-PNP was the NTP cofactor. Results are from at least three independent experiments; error bars indicate standard deviations.

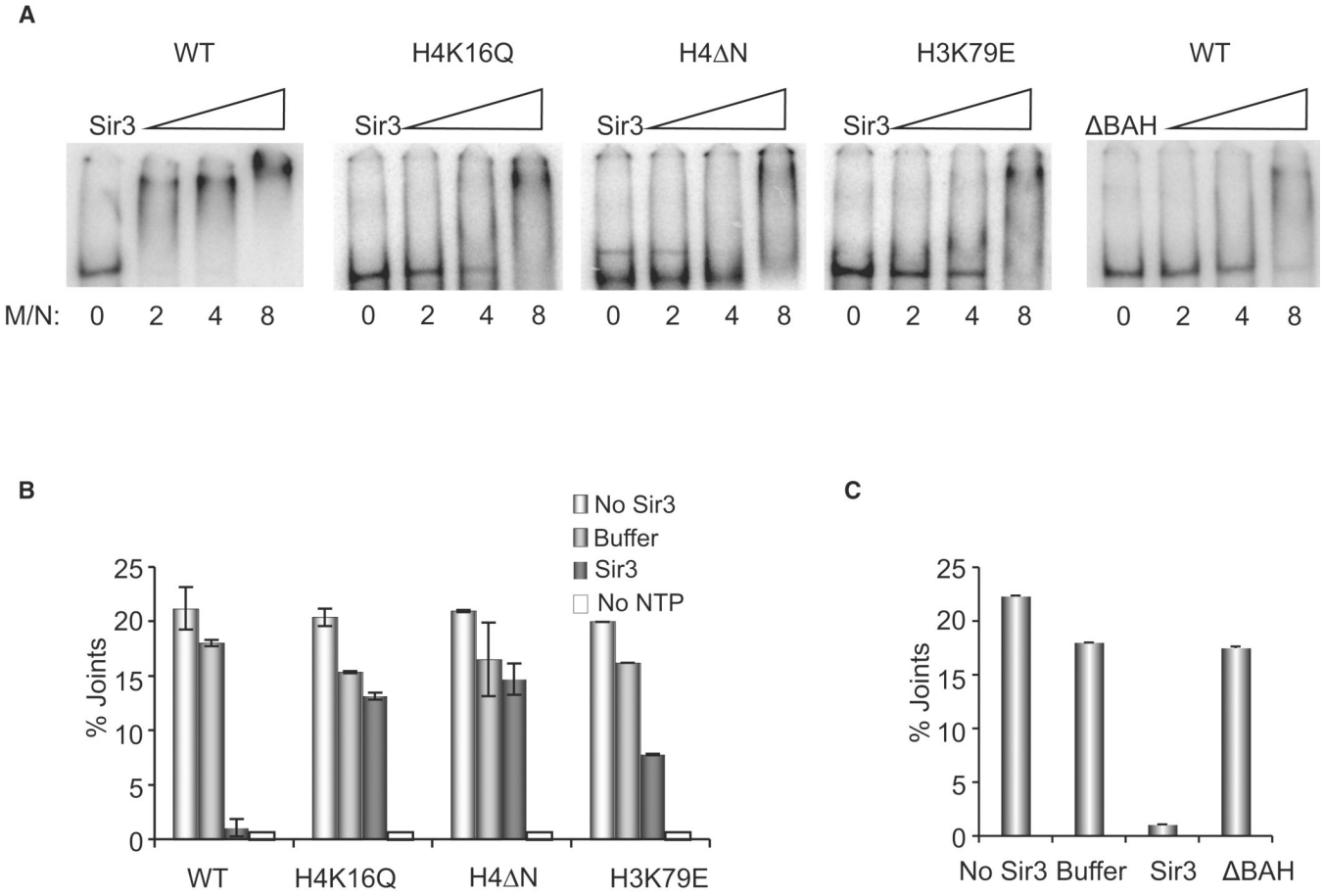

Figure 2. Histone residues and the Sir3p BAH-domain regulate Sir3p-dependent inhibition of yRad51p-mediated joints

(A) Mononucleosome binding assays. Mononucleosomes were reconstituted on a '601' positioning sequence, incubated with increasing amounts of Sir3p or Sir3pΔBAH, and electrophoresed on native PAGE. M/N: Monomers of Sir3p or Sir3pΔBAH per nucleosome. Mononucleosomes carried either wild type (WT), truncated histone H4 (H4 ΔN), H4 K16Q, or H3 K79E. (B–C) Results of joint capture assays with saturated (R=0.7) minichromosomes. AMP-PNP was the NTP cofactor. Results are from at least three independent experiments; error bars indicate standard deviations. (B) Donors contained either wild type histones (WT), H4 ΔN, H4 K16Q, or H3 K79E as indicated. Where indicated, Sir3p was present at 4 monomers per nucleosome. (C) Minichromsomes contained WT histones, buffer, Sir3p, or Sir3p (ΔBAH) at molar ratios of 4 monomers per nucleosome.

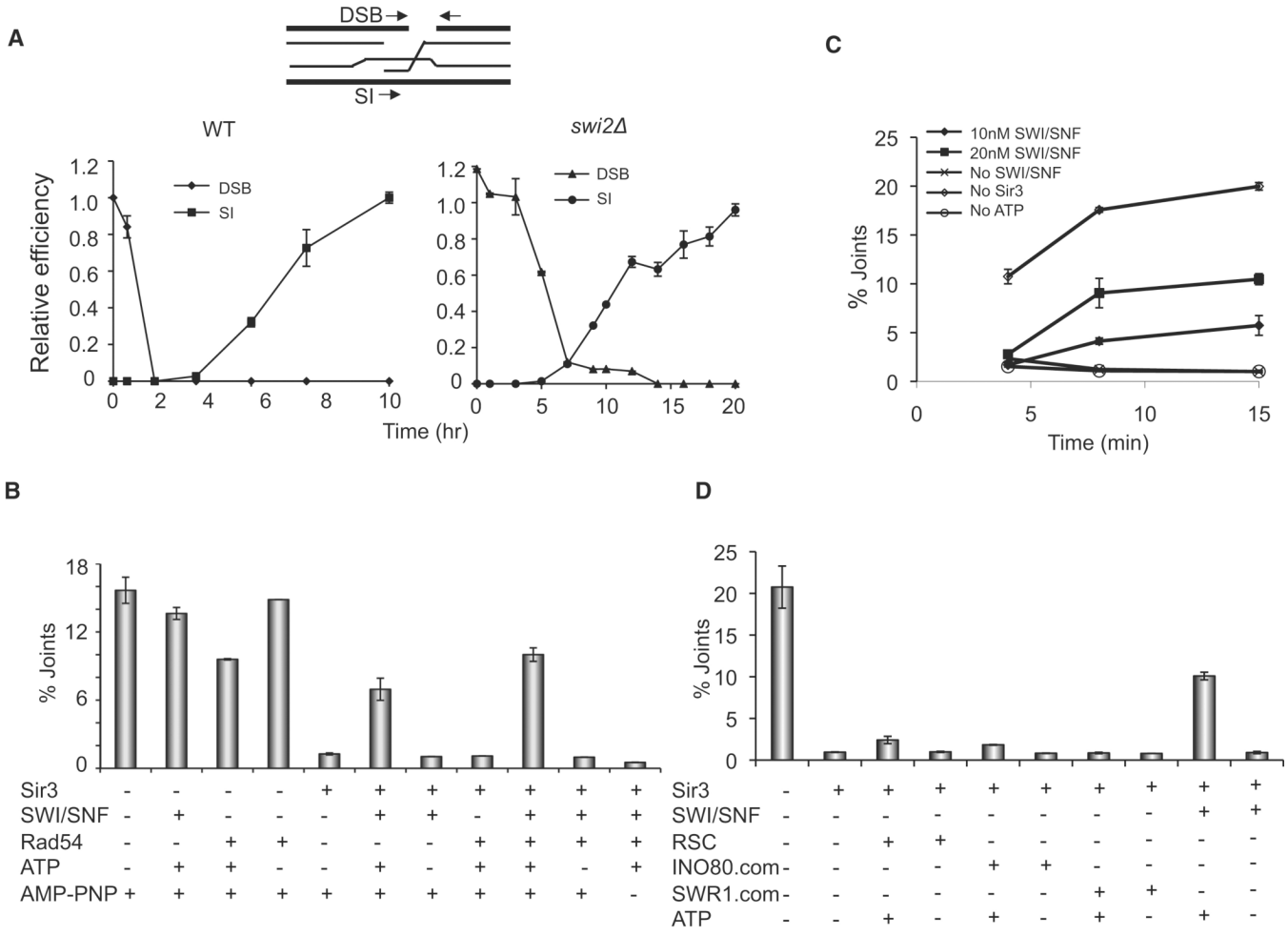

Figure 3. SWI/SNF antagonizes Sir3p-dependent inhibition of HR

(A) Top Panel: Schematic of the strand invasion intermediate of the HR pathway. Black arrows indicate primers used for monitoring double strand break (DSB) and strand invasion/extension (SI) product formation. Bottom panels: Graphs show kinetics of DSB formation and SI product formation in wild type (WT) or *swi2*Δ strains that contain a *MAT**a***$_{inc}$ donor locus at an ectopic site on chromosome V. Efficiency of DSB formation and strand invasion was set to 100% for the WT DSB product at 0 timepoint. All values were normalized to a *PHO5* internal control. (B) Joint capture assays with saturated (R=0.7) minichromosomes in absence or presence of Sir3p at a molar ratio of four monomers per nucleosome. Indicated reactions were supplemented with 30nM SWI/SNF or 200nM Rad54p. (C) Joint capture assays as in B, showing kinetics of joint formation in presence or absence of different concentrations of SWI/SNF. (D) Joint capture assays as in B; reactions were supplemented with 20nM of the indicated remodeling enzyme. Results are from at least three independent experiments; error bars indicate standard deviations.

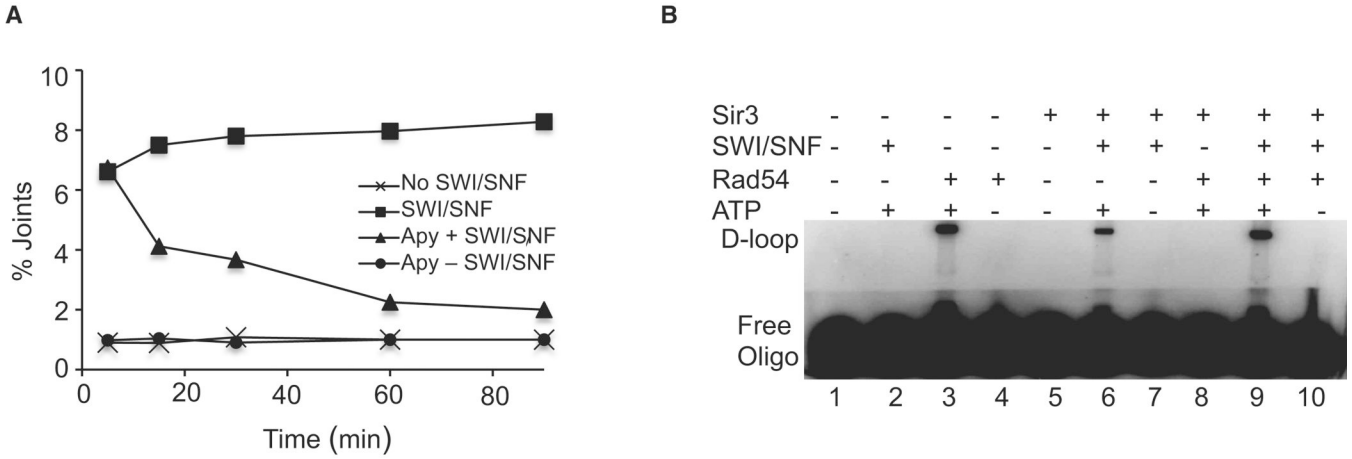

Figure 4. SWI/SNF action on the Sir3p-nucleosomal donor is reversible and stimulates D-loop formation

(A) Joint capture assays with saturated (R=0.7) minichromosomes and Sir3p at 4 monomers per nucleosome. yRad51p-presynaptic filament was assembled in presence of 0.3mM AMP-PNP and allowed to form joints on Sir3p-minichromosomes pre-incubated with 30nM SWI/SNF and 2mM ATP, unless indicated otherwise. After 5 minutes, half of the reaction received 1U of Apyrase (Apy) and kinetics of joint formation was assayed for 90 min. (B) Representative autoradiograph shows yRad51p-catalyzed D-loop formation on minichromosomes in presence or absence of Sir3p (4 monomers per nucleosome). Reactions contained 2mM ATP as nucleotide cofactor unless indicated and either 30nM SWI/SNF and/or 200nM Rad54 as indicated.

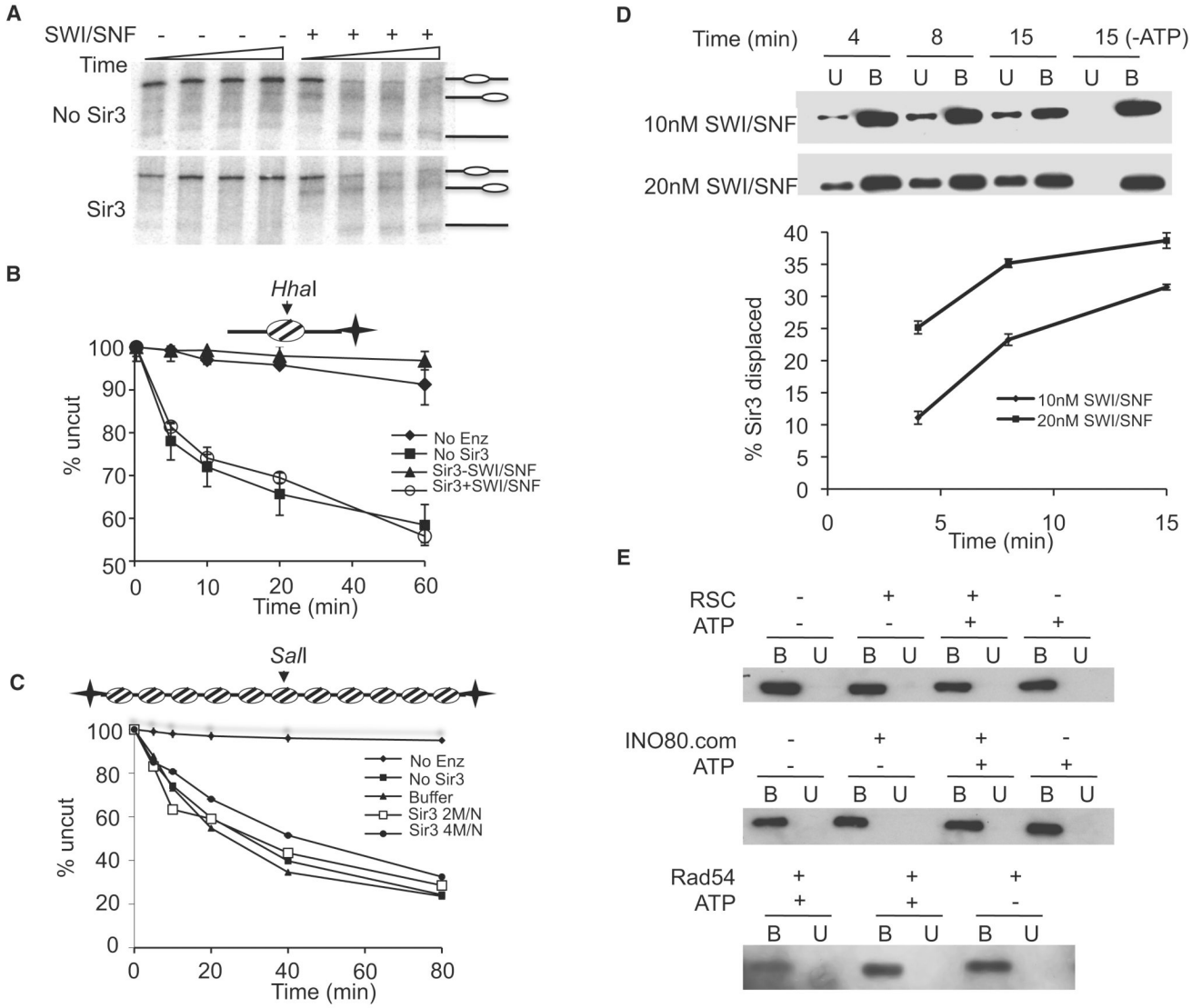

Figure 5. SWI/SNF remodels Sir3p-heterochromatin by evicting Sir3p

(A) Representative native PAGE analysis of a 343-bp mononucleosome (2nM) with (Bottom) or without (Top) 4 monomers of Sir3p, incubated with 1nM SWI/SNF or buffer as indicated. Predicted nucleosome positions are shown at right. (B) HhaI accessibility assay on the 601-mononucleosome (2nM) in absence or presence of 4 monomers of Sir3p and 1nM SWI/SNF as indicated. (C) *Sal*I accessibility assay on 208-11 nucleosomal array (2nM). Reactions contained 2nM SWI/SNF and Sir3p at a molar ratio of 2 or 4 monomers per nucleosome, unless indicated otherwise (D, E) Representative immunoblot analysis of Sir3p, probed with anti-FLAG antibody. Reactions contained biotinylated minichromosomes (17nM), 10nM or 20nM SWI/SNF (D) or 20nM RSC, 20 nM Ino80.com, or 200nM yRad54 (E) and 2 mM ATP where indicated. B, bead-bound fractions; U, unbound supernatants. Kinetics of Sir3p displacement was monitored in at least 3 independent experiments and % Sir3p displaced was calculated as U/(B+U) for each time point (Lower panel, D). Error bars indicate standard deviations. Molar ratio of Sir3p was 4 monomers per nucleosomes in all reactions (D–E) except for left two lanes of Rad54p reactions (E), in which Sir3p was reduced to 2 monomers per nucleosome.

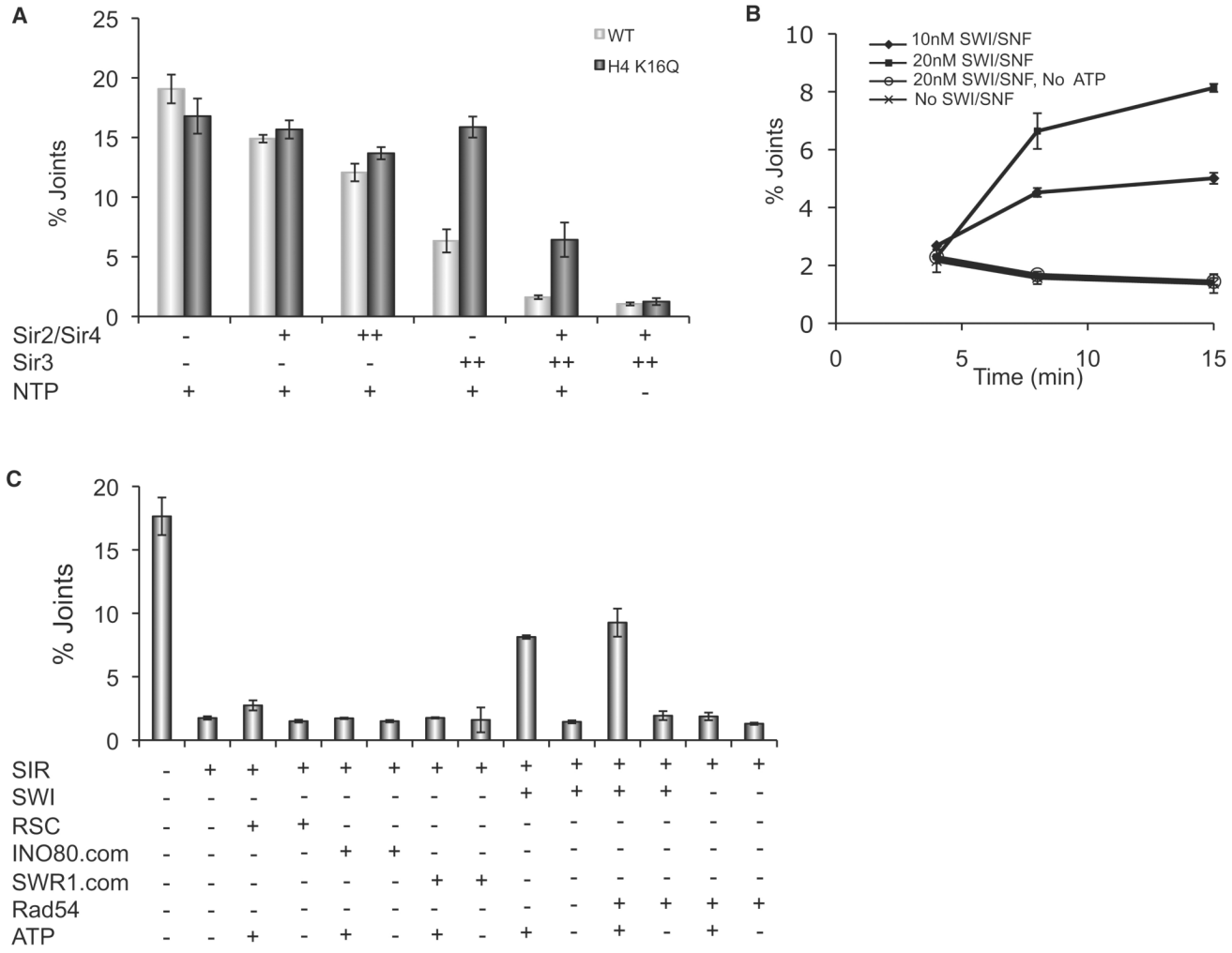

Figure 6. SWI/SNF restores joint formation on SIR-heterochromatin

(A) Joint capture assays with saturated (R=0.7) minichromosomes in absence or presence of Sir3p, Sir2p/Sir4p, or Sir2p/Sir3p/Sir4p. Sir3p was present in all reactions at a ratio of 4 monomers (++) per nucleosome. Where indicated, Sir2p/Sir4p was present at either 1 (+) or 2 (++) heterodimer(s) per nucleosome. (B) Joint capture assays with R=0.7 minichromosomes in absence or presence of 4 monomers Sir3p and 1 heterodimer Sir2p/Sir4p per nucleosome. Kinetics of joint formation was measured in presence or absence of different concentrations of SWI/SNF remodeling enzymes as indicated. (C) Joint capture assays as in B. Indicated reactions were supplemented with 20nM remodeling enzymes. Results are from at least three independent experiments; error bars indicate standard deviations.

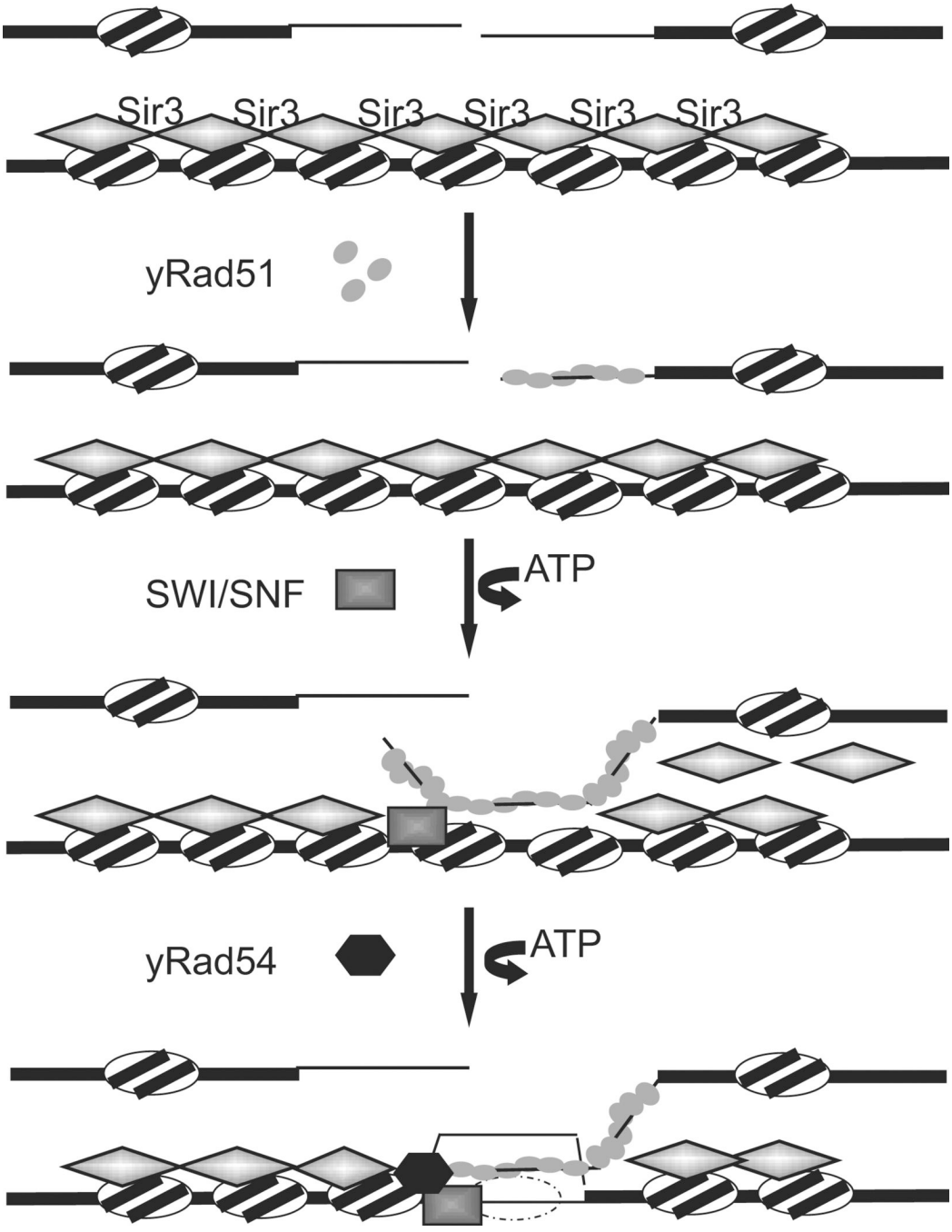

Figure 7. Model depicting early events of recombinational repair on a Sir3p-heterochromatic donor
See text for details. Note that cartoons are not drawn to scale, nor do they implicate specific stoichiometry or structures.