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RECOMBINATIONAL REPAIR OF A CHROMOSOMAL DNA DOUBLE STRAND BREAK

A Dissertation Presented

By

Manisha Sinha

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences
Worcester
In partial Fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

March 16, 2009

Biomedical Sciences

RECOMBINATIONAL REPAIR OF A CHROMOSOMAL DNA DOUBLE STRAND BREAK

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Interdisciplinary Graduate Program

March 16, 2009

DEDICATION

I dedicate this thesis to:

My parents (Ma-Bapi: Neera & Asit Baran Das), for introducing me to the world of science.

My parents-in-law (Ma-Baba: Sipra & Dipak Kumar Sinha), for extending your kind and loving hands even to this part of the globe to help bring up my infant daughter to toddler hood;

And to my daughter, Risha for braving countless hours of separation from me during the most vulnerable years of your life; I want you to know that you are the reason science has become my religion.

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In my daily work, it was a pleasure to be a part of a wonderful group of fellow students and colleagues in the Peterson lab. All of you have contributed both personally and professionally to the work of this thesis with so much help and support. Special thanks to my office-mates, Manolis, Kim, Shinya and Pranav for the stimulating scientific and non-scientific discussions. I would also like to extend my thanks to a past Peterson lab member, Corey Smith, for the hands-on help and mentoring through the first year in the lab. As well as keeping the lab stocked with clean glasswares, Marty Van Auken has without fail, provided something much greater in all these years: the time to say hello, every time we met.

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I have been truly blessed with a great family and circle of friends. I thank every single one of you for your enormous support and encouragements in pursuing my dreams. I would not be here at this point of my career, without a few special people. First, my jyethu (Uncle), Mr. Prabhas Sen, whose positive attitude towards life continues to guide me through the dark times. Second, my jijia, my elder sister Manjula, who has been nothing less than a role-model in my life and whose opinion and thoughts I have been addicted to in every aspect of my life for as long as I can remember. Similarly, I will be ever grateful to my brother-in-law, Joy Kuri for inspiring me to join academic research at a critical crossroads of my career. Finally, my husband and best friend, Raktim, whose endurance, patience and understanding hold no match. He has been my partner in the truest sense, being there by my side to share the greatest excitements and disappointments of life and graduate career and complementing with innumerable qualities that I lack in myself; often making sure none of us starved or forgot to laugh and have a good time even in apparently overwhelming and difficult times.

ABSTRACT

Repairing a chromosomal DNA double strand break is essential for survival and maintenance of genomic integrity of a eukaryotic organism. The eukaryotic cell has therefore evolved intricate mechanisms to counteract all sorts of genomic insults in the context of chromatin structure. Modulating chromatin structure has been crucial and integral in regulating a number of conserved repair processes along with other fundamental genomic processes like replication and transcription.

The work in this dissertation has focused on understanding the role of chromatin remodeling enzymes in the repair of a chromosomal DNA double strand break by homologous recombination. This has been approached by recapitulating the biochemical formation of recombination intermediates on chromatin *in vitro*. In this study, we have demonstrated that the mere packaging of DNA into nucleosomal structure does not present a barrier for successful capture of homologous DNA sequences, a central step of the biochemical pathway of recombinational repair. It is only the assembly of heterochromatin-like more complex nucleo-protein structure that presents additional constraints to this key step. And, this additional constraint can be overcome by the activities of ATP-dependent chromatin remodeling enzymes. These findings have great implications for our perception of the mechanism of the recombinational repair process of a chromosomal DNA double strand break within the eukaryotic genome.

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LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
APY	apyrase
ATP	adenosine triphosphate
Bp	base pair
BSA	bovine serum albumin
C-terminal	carboxyl terminal
ChIP	chromatin immunoprecipitation
dCTP	deoxycytidine 5'-triphophate
DNA	deoxyribonucleic acid
DSB(s)	double strand break(s)
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol diamino ethylether tetraacetic acid
et al.	Et aibi (and others)
GST	glutathione S-transferase
HAT	histone acetyltransferase
HMG	high mobility group
HP1	heterochromatin protein 1
HR	homologous recombination
INO80	inositol requiring
IR	ionizing radiation
ISWI	imitation SWI
Kb	kilobase

kDa	kilodalton
Min	minutes
MNase	micrococcal nuclease
Mono	Mononucleosome
NHEJ	non-homologous end joining
nm	nanometer
nM	nanomoles
N-terminal	amino terminal
PAGE	polyacrylamide gel electrophoresis
PMSF	phenylmethylsulfonyl fluoride
rDNA	ribosomal DNA
RPA	replication protein A
rpm	revolutions per minute
RSC	remodels structure of chromatin
SANT	Swi3, Ada2, N-CoR, and TFiiiB
S.C.	synthetic complete
SDS	sodium docecyl sulfate
SF2	helicase super family 2
SIR	silencing information regulator
Snf	sucrose non-fermenting
ssDNA	single stranded DNA
SWR1	sick with rat8
TAP	tandem affinity purification
TAE	Tris-Acetic-EDTA
TBE	Tris-Boric acid-EDTA
TE	Tris-EDTA buffer

WB	western blot
WT	wild type
YEPD	Yeast Extract Peptone with 2% Dextrose

CHAPTER I

INTRODUCTION

Introduction

The genomic integrity of eukaryotic organisms is often challenged by double strand breaks (DSBs) in the DNA. Even a single unrepaired DSB can be lethal (Rich et al. 2000), and can result in chromosomal instability and loss of genetic information (Khanna and Jackson 2001). Therefore, efficient repair of chromosomal DNA DSBs is crucial in safeguarding the genomic integrity of organisms. Defects in the pathways that respond to and repair DSBs, can be the driving force for onset of several human pathologic disorders with pleiotropic clinical features including age-related diseases and cancer (O'Driscoll and Jeggo 2006). For decades, studies have been directed towards elucidating the enzymatic mechanisms involved in recognizing; signaling and repairing double strand breaks within the eukaryotic cells. A significant amount of knowledge has also been generated from biochemical studies of DNA repair that were carried out using not so physiologically relevant, naked DNA substrates. Only recently, efforts have been geared towards understanding how the repair machinery deals with breaks in the chromatin fiber, a nucleo-protein complex into which DNA is packaged within the eukaryotic nucleus thus becoming relatively inaccessible. This thesis chapter aims to discuss our understanding of the relationship of chromatin structure and DNA double strand break repair process, and how the potential barrier of chromatin structure is relieved to enable repair of DSBs.

Organization of the eukaryotic genome into chromatin

The basic unit of chromatin is the nucleosome core particle, which consists of ~146 base-pairs of DNA wrapped in left-handed superhelical turns ~1.7 times around an octamer of histone proteins (Luger et al. 1997). Two heterodimers of histones H3 and H4 interact to form a tetramer, which in turn binds heterodimers of H2A and H2B on either side, thus organizing a canonical octamer of histone proteins (Figure 1).

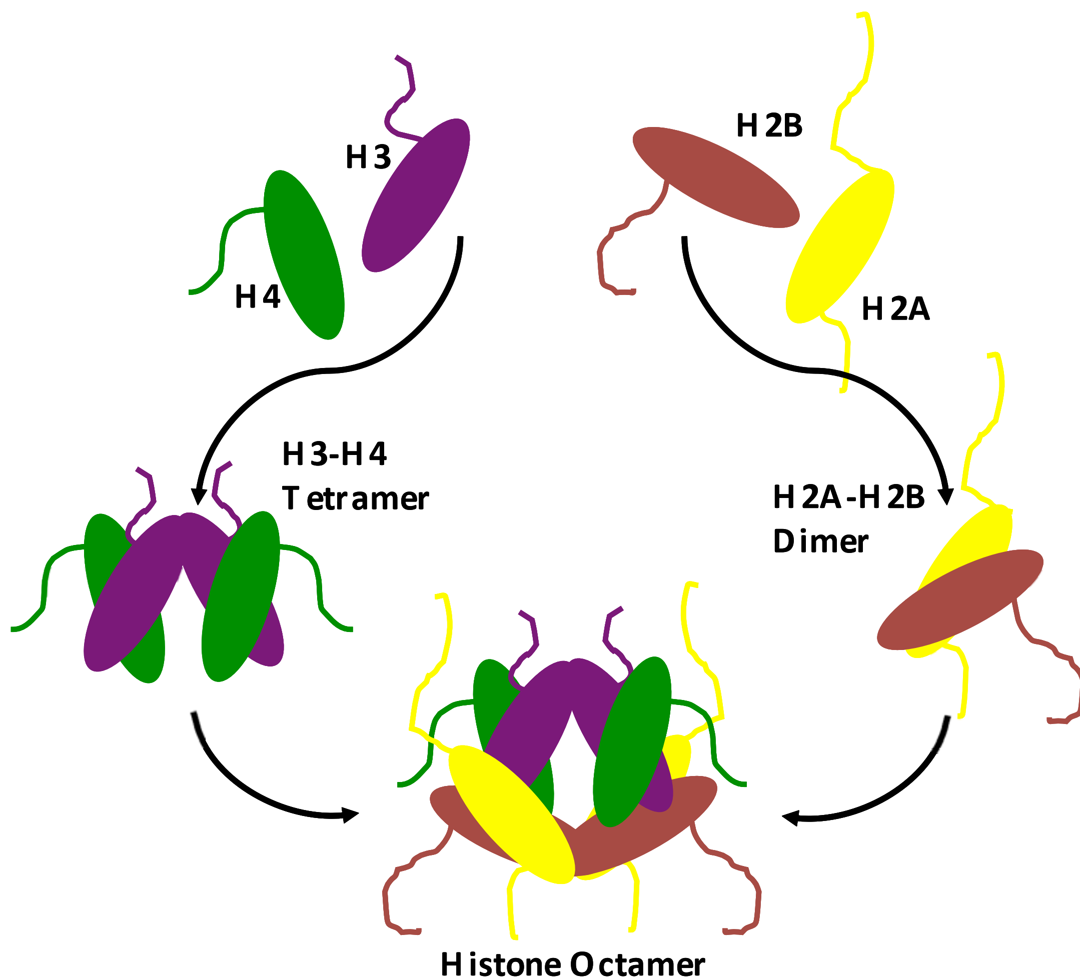

Figure 1. Schematic of histone octamer assembly. The various steps of assembly of a canonical histone octamer are illustrated here.

The histone proteins are of low molecular weight, very basic in nature and, highly conserved across all eukaryotes. These proteins harbor a structured, three-helix bundle called a histone fold motif, which mediates the histone–histone and histone–DNA interactions. So, these motifs are important for the formation of the central structure of the nucleosome core particle. The structured histone fold domains are flanked by unstructured flexible domains or tails, which protrude from the nucleosome core particle (Luger et al. 1997). Although the histone tails are not required for assembly of the octamer or nucleosomes, they are essential for regulation of many biological processes. Numerous post-translational modifications occur at different amino acid residues of these tails (Gelato and Fischle 2008), regulating key biological processes. The tails are also important for both intramolecular and intermolecular folding of nucleosomal arrays *in vitro* to mediate different levels of compaction (Fletcher and Hansen 1996; Carruthers and Hansen 2000; Horn and Peterson 2002).

The primary structure of chromatin is the linear array of nucleosomes forming the 10nm fiber or “beads on a string” as seen by cryo-electron microscopy (Woodcock 2006). This linear array is folded into a 3-dimensional secondary structure, called a 30nm fiber via internucleosomal interactions and stabilized by the association of linker histones H1, H5 and non-histone proteins like HMG proteins (Woodcock and Dimitrov 2001; Horn and Peterson 2002). The linker histones bind the nucleosomes at the DNA entry/exit point with the stoichiometry of one linker histone per nucleosome (Hansen 2002), forming a particle called chromatosome and stabilizing an additional 20bp of DNA with the

nucleosome. Binding of linker histones to the nucleosomes rearranges the amino-terminal tails of the histones in conformations that can favor further folding (Wolffe and Hayes 1999). Enormous tertiary structures of chromatin are formed by self-association of the 30nm fibers into 100-400nm thick filaments also called the chromonema filaments, as visualized by electron microscopy in interphase cells and detected by biophysical methods (Fletcher and Hansen 1996; Carruthers et al. 1998).

Chromatin also contains a variety of histone variants apart from the canonical histones H2A, H2B, H3 and H4, which are expressed during S-phase and incorporated into chromatin at the replication fork. In contrast, the histone variants are expressed throughout the cell cycle, incorporated into chromatin independent of replication and play specific roles in addition to those of canonical histones (Ahmad and Henikoff 2002). Specifically, histone H2A has many variants, including H2A.X, H2A.Z, macroH2A and H2ABbd. Amongst these variants, H2A.X and H2A.Z have been well characterized and linked to repair of DNA DSBs and regulation of transcription (Horn and Peterson 2002; Downs et al. 2007). Other well-studied histone variants are variants of histone H3. For instance, Cse4/CENP-A, which is essential for centromere function or assembly and found in yeast and mammalian centromeres respectively (Basrai and Hieter 1995; Durand-Dubief and Ekwall 2008).

In general, the eukaryotic genome is organized into specialized domains of chromatin that can regulate distinct functions and attain specialized structures by incorporating

different histone variants or by associating with different non-histone proteins. Early cytological studies defined the region of the genome that underwent decondensation as the cells progressed from metaphase to interphase, as euchromatin (Passarge 1979). On the other hand, the regions that remained visibly condensed and deeply stained throughout the cell cycle were defined as heterochromatin. These regions that are mainly found at centromeres, telomeres and appear to be maintaining chromosome structures and hence chromosomal stability and integrity, are referred to as constitutive heterochromatin. The state of heterochromatin can change in response to cellular signals and reverted back to euchromatin at developmentally regulated loci (Grewal and Jia 2007). The reversible heterochromatin is termed facultative heterochromatin, which provides a means of controlling DNA metabolism, such as replication and transcription. Likewise, it is also crucial for regulating V(D)J recombination during mammalian immune-system development (Yancopoulos and Alt 1985). Structural features that characterize heterochromatin include the presence of predominantly repetitive DNA sequences, low or absent gene density, late S phase replication timing, frequent position effect variegation, regular nucleosome spacing, less accessibility to nucleases, loss of hyper-sensitive sites and hypoacetylation of histones (Henikoff 2000; Richards and Elgin 2002; Grewal and Jia 2007). These structural features distinguish heterochromatin from euchromatin and are conserved throughout eukaryotes. In addition, except for budding yeast, methylation of histone H3 at position K9 and its associated chromo-domain containing protein, HP1 are also hallmarks of heterochromatin, conserved from fission yeast to mammals. Moreover, in vertebrates and plants, heterochromatin is supplemented

with cytosine hypermethylation and associated proteins.

Chromatin remodeling enzymes

In order to carry out basic DNA-mediated processes like replication, transcription, DNA-damage repair or recombination, eukaryotic cells have to contend with the chromatin fiber. Two types of highly conserved chromatin remodeling enzymes have been discovered that can modulate the architecture of chromatin. The first group is comprised of histone modifying enzymes, which can covalently add or remove post-translational modifications to amino acids in the various histone proteins (Strahl and Allis 2000). Most of the well-characterized histone modifications occur at the tail regions of the histones including acetylation, phosphorylation, methylation, ubiquitination, sumoylation and ADP-ribosylation. In addition to the histone tail modifications, a few post-translational modifications within the nucleosome core also appear to be controlling recruitment of regulatory proteins to chromatin, alteration of chromatin structure and architecture, and also alteration of histone-DNA contacts (Khorasanizadeh 2004).

The second group of chromatin remodeling enzymes consists of multi-subunit complexes that can use energy from ATP hydrolysis to disrupt the histone-DNA contacts actively by changing nucleosome positioning, exchanging or displacing histone-dimers or completely evicting the histone octamers (Vignali et al. 2000). The hallmark of the ATP-dependent remodeling enzymes is the presence of a catalytic ATPase subunit that is related to the

SWI2/SNF2 subfamily of the DEAD/H superfamily of nucleic-acid stimulated ATPases (Eisen et al. 1995).

These ATPases from different organisms have been sub-divided into groups based on sequence homology within the catalytic subunit and the presence of unique sequence motifs such as bromodomain (in SWI2/SNF2 family), SANT domain (in ISWI family) and chromodomain (in Mi-2 Family) (Boyer et al. 2000). So far, six distinct

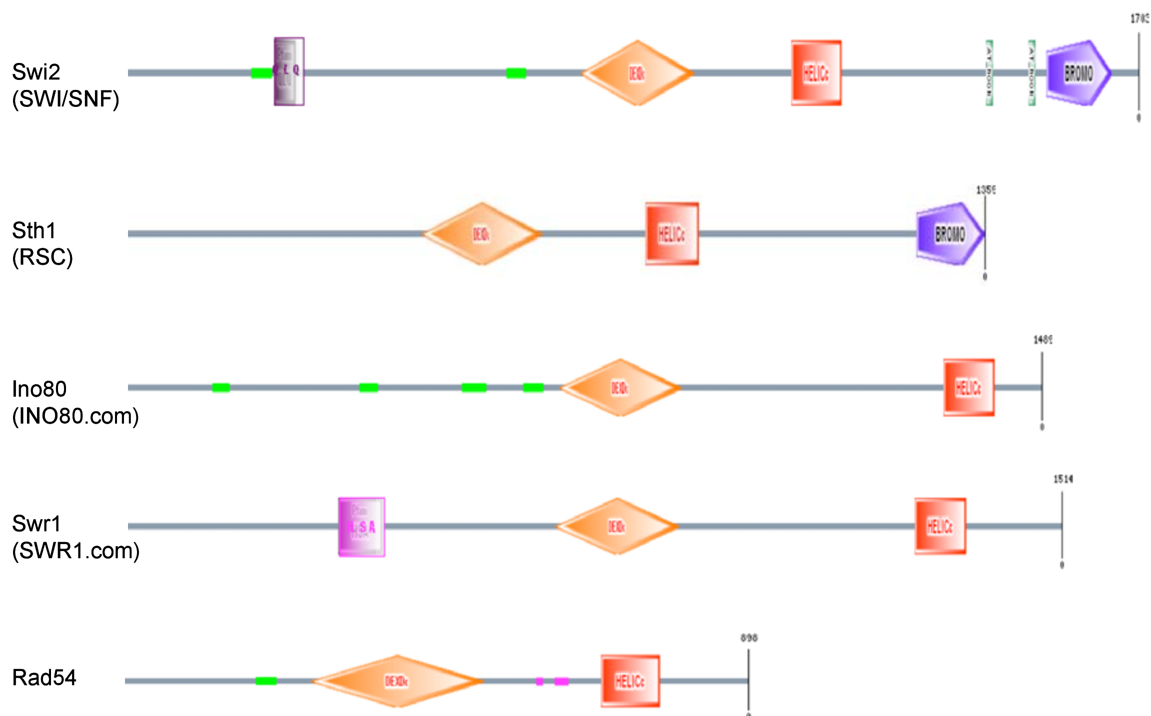

Figure 2. Schematic of the catalytic subunits of the SWI2/SNF2 family of proteins. The hallmark of the multisubunit ATP-dependent chromatin remodeling enzymes is the presence of a Swi2/Snf2p homologue subunit. All homologues contain the conserved SWI2/SNF2-like ATPase domain, composed of a DEXH box (orange) and a HELICc3 motif (red). This domain contains the nucleotide binding and ATP hydrolysis sites. Specific features outside the ATPase domain, like bromodomains (blue), QLQ (purple) or HSA (pink) are also shown here. This figure was generated using the Simple Modular Architecture Research Tool (SMART; http://smart.embl-heidelberg.de).

remodeling complexes have been purified from *S.cerevisiae*: SWI/SNF (Peterson et al. 1994), RSC (Cairns et al. 1996), ISW1, ISW2 (Tsukiyama et al. 1999), INO80.com (Shen et al. 2000) and SWR1.com (Mizuguchi et al. 2004).

An exciting theme has emerged that histone modifying enzymes and ATP-dependent chromatin remodeling enzymes do not necessarily function independently of each other in regulating basic chromatin-mediated processes like replication, transcription, DNA damage repair and chromatin compaction. There is a lot of evidence that modified histones can recruit ATP-dependent chromatin remodeling enzymes to target sites (Hassan et al. 2002). For instance, bromodomains bind acetylated lysine residues, whereas, chromodomains, tudor domains and plant-homeodomain-finger domains bind distinct methylated residues (Jacobs and Khorasanizadeh 2002; Nielsen et al. 2002; Kim et al. 2006; Shi et al. 2007). On the other hand, ATP-dependent chromatin remodeling is also a prerequisite for recruitment of histone-modifying enzymes at certain target sites (Fry and Peterson 2001). Moreover, histone modifications can also cross-talk with each other in a way that one modification recruits or activates chromatin-modifying complexes to generate a different histone modification or chromatin remodeling (Berger 2007; Suganuma et al. 2008).

In this chapter, I will focus on how certain constitutive and break-induced covalent histone modifications in union with specific chromatin remodeling enzymes function in the process of repair of chromosomal DNA DSBs.

DNA double strand break repair and chromatin

Cell viability and genomic stability are frequently threatened by chromosomal DNA double strand breaks. DSBs are generated endogenously as metabolic byproducts when replication forks encounter blocking lesions leading to fork collapse; during programmed genome rearrangements induced by nucleases such as in mating-type switching of *S. cerevisiae* (Paques and Haber 1999), V(D)J recombination (Franco et al. 2006), class-switch recombination (Chaudhuri et al. 2007), and meiosis (Keeney and Neale 2006). In addition, physical stress can give rise to DSBs when dicentric or catenated chromosomes are pulled to opposite poles during mitosis (Acilan et al. 2007). DSBs are also produced exogenously when cells are exposed to DNA damaging agents including ionizing radiation (IR), chemical agents and UV light that create alkyl adducts, pyrimidine dimers, and crosslinks (Limoli et al. 2002; Bosco et al. 2004); and chemotherapeutics that poison topoisomerase I or topoisomerase II (Degrassi et al. 2004).

The failure or improper repair of DSBs can result in cell death or gross chromosomal changes including deletions, translocations, and fusions that promote genome instability and tumorigenesis. Cells have developed signaling networks to sense DSBs, arrest the cell cycle, and activate repair pathways. These cellular responses can activate DNA damage checkpoints throughout the cell cycle to minimize genomic instability. Moreover, in the case of too much damage, overlapping signaling pathways can trigger apoptosis to prevent propagation of cells with highly unstable genomes (Su 2006).

All these molecular responses to DSBs must occur in the context of chromatin. How cells contend with the apparently repressive nature of the chromatin fiber at the very basic nucleosomal organization and/or higher order compaction levels has been a recent focus of research in the field. The "access-repair-restore" model has emerged to predict that chromatin structure is altered to expose damaged DNA to repair factors at sites of DNA damage, and once repair has taken place, chromatin structure is restored to its original state (Peterson and Cote 2004). In accordance with the model, a number of studies have implicated both classes of chromatin remodeling factors, histone modifying enzymes and ATP-dependent chromatin remodeling enzymes in DNA damage repair. In the rest of this chapter, I have reviewed the relationships between chromatin modifications and remodeling during recombinational repair of DSBs.

Eukaryotic cells have evolved two major mechanisms to repair chromosomal DNA DSBs: nonhomologous end-joining (NHEJ) and homologous recombination (HR). NHEJ can take place throughout the cell cycle as it obviates the need of a repair template and is the predominant DSB repair mechanism in G1 phase. In this pathway, the broken DNA ends are recognized and bound by the Ku70–Ku80 heterodimers, which are conserved throughout the eukaryotes. In *S. cerevisiae*, the Mre11/Rad50/Xrs2 (MRX) complex has also been shown to be involved in processing of DNA broken ends for NHEJ. However, in mammals, NHEJ is associated with a complex of DNA-dependent protein kinase (DNA-PK) and nuclease, Artemis (Lieber et al. 2003). Finally, the ligation of the broken DNA ends is accomplished by the Lig4–Lif1 complex in *S. cerevisiae* and Lig4–XRCC4

in mammals (Daley et al. 2005; Cahill et al. 2006). Due to nucleolytic processing of DNA ends to make them compatible for subsequent ligation, NHEJ may result in short deletions and so is typically error-prone (Lieber et al. 2003).

Homologous recombinational repair functions in late S–G2 phase and relies on sequence homology for copying the missing information from an undamaged sister chromatid or homologous chromosome (Jackson 2002). Therefore, this pathway is essentially error free, unlike NHEJ. Genetic analyses in S. cerevisiae revealed that the proteins coded by the RAD52 epistasis group of genes – RAD50, RAD51, RAD52, RAD54, RAD55, RAD57, RAD59, MRE11 and XRS2 - mediate HR (Paques and Haber 1999). The basic HR pathway is highly conserved throughout eukaryotes as all known gene products involved in yeast HR, have functional homologs in higher eukaryotes (Sonoda et al. 2001; Thompson and Schild 2001). During HR, 5'-ends of the DNA duplex flanking the break are first nucleolytically processed to a certain extent by MRX (in S. cerevisiae; MRN in mammals) in union with Sae2 (Clerici et al. 2005). Following the initial and limited end processing, the 3'-tailed DNA ends are rapidly processed by either the Sgs1–Dna2 complex or Exo1, to generate long, 3' single-stranded tails to which RPA binds (Mimitou and Symington 2008; Raynard et al. 2008; Zhu et al. 2008). Subsequently, RPA is replaced by the key recombinase enzyme, Rad51 to form nucleoprotein filaments. The nucleoprotein filaments of Rad51 and ssDNA, also known as the presynaptic filaments are capable of searching for sequence homology followed by invading and transferring strands with the homologous duplex (donor locus).

Notably, the polymeric Rad51 protein has a highly conserved structure among eukaryotes and can assemble onto ssDNA as well as dsDNA to form a right handed helical filament in which, ~6 protein monomers span ~18-19 bases or bps of DNA per helical turn with a pitch of ~10 nm (Yu and Egelman 1993; Sung and Robberson 1995; Conway et al. 2004; Yu et al. 2004). Although Rad51 is an ATPase enzyme, ATP-hydrolysis is not required for its assembly onto the presynaptic filament (Benson et al. 1994; Sung 1994; Gupta et al. 1997). Instead, ATP hydrolysis promotes dissociation of Rad51 from DNA resulting in high turnover and recycling of the recombinase (Bugreev and Mazin 2004; Bugreev et al. 2005; Ristic et al. 2005; Chi et al. 2006). Formation of Rad51-presynaptic filaments is facilitated by many “mediator” proteins including Rad55 and Rad57 in *S. cerevisiae* (Gasior et al. 2001), and five others in higher eukaryotes (Rad51B, Rad51C, Rad51D, XRCC2, and XRCC3). In addition, Rad52 and Rad54 also interact with Rad51 and facilitate formation of Rad51-presynaptic filament as well as stimulate the catalytic activity of Rad51. Likewise, in higher eukaryotes, the BRCA1 and BRCA2 tumor suppressors interact and mediate the function of Rad51 in HR (West 2003; San Filippo et al. 2008). After the initial synapsis of the RAD51 nucleoprotein filament with the donor locus, an initial, transient intermediate joint molecule, termed a paranemic joint is formed whose stability depends on DNA-protein interaction and the 3’ end of the invading presynaptic filament is not engaged within the joint intermediate. Subsequently, the paranemic joint gets converted into a plectonemic joint (commonly known as Displacement-loop or D-loop), whose stability no longer depends on DNA-protein interaction and the 3’end of the invading presynaptic filament also gets involved. The

3'end of the presynaptic filament within the stable, plectonemic joint intermediate serves as the primer for beginning of new DNA synthesis by DNA polymerase. Repair is completed when HR intermediates are further extended, Holliday junctions are formed

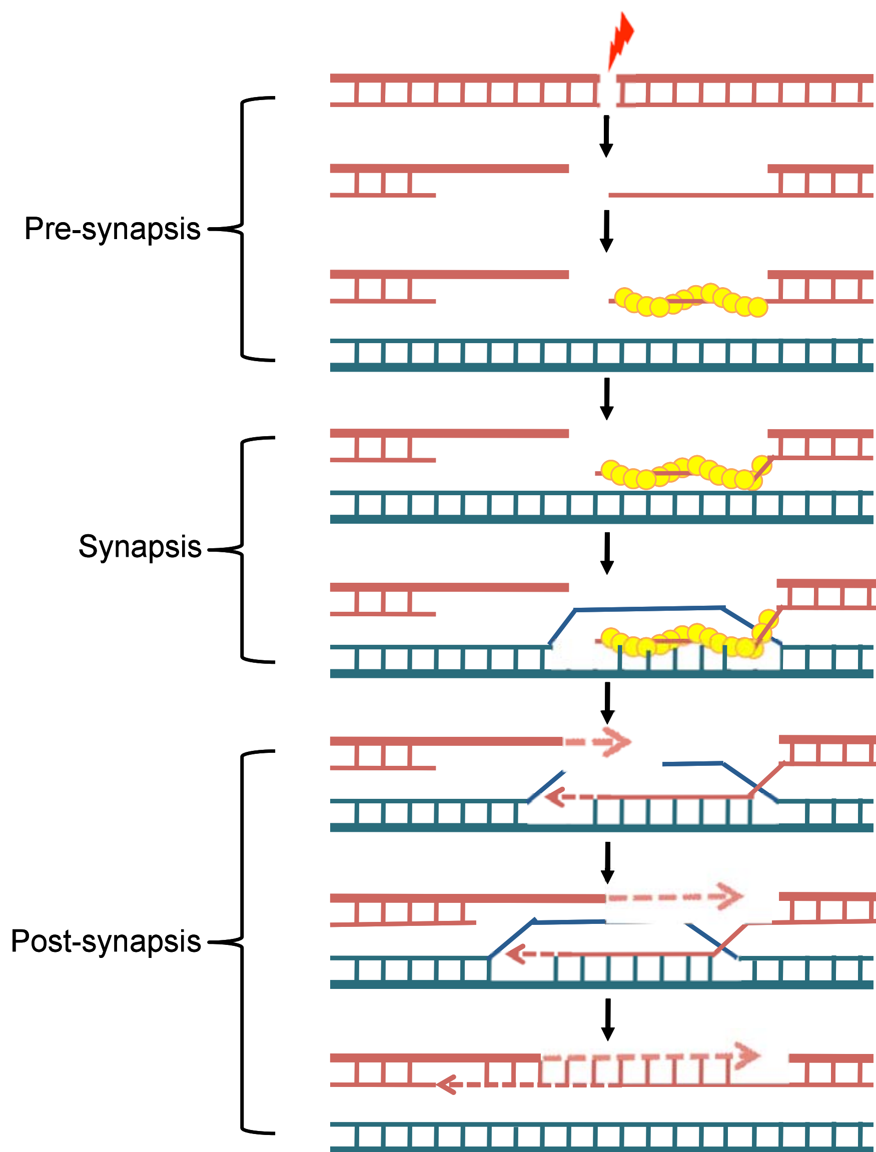

Figure 3. Schematic of the biochemical steps of the recombinational repair process of a DNA DSB. The Pre-synapsis includes nucleolytic processing of the 5'ends of DNA flanking a break to generate long 3' ssDNA tails and assembly of the presynaptic filament. The Synapsis includes homology search and formation of paranemic followed by plectonemic joints. The Post-synapsis involves extension of the joints, new DNA synthesis, formation of Holliday junctions, resolution of joints and ligation of broken DNA ends. 5'-3' orientation of DNA strands in the duplexes are marked by bold lines.

and resolved by cleavage and ligation, or by dissociation of the extended strand from the donor and re-annealing to ssDNA on the opposite side of the DSB (termed synthesis-dependent strand annealing) (San Filippo et al. 2008).

The study of homologous recombinational repair of a single DSB *in vivo* has utilized the mating type switching event of *S. cerevisiae*, well established as a paradigm by the Haber group (Haber 1998b). Numerous genetic, cytological and chromatin immunoprecipitation (ChIP) assays have shed light on the order of recruitment of repair proteins, histone modifications and chromatin remodeling enzymes to the site of the break. Haploid cells of *S. cerevisiae* harbor the mating type information at the *MAT* locus on chromosome III. Depending on what genes are located within the *MAT* locus, the mating type of one haploid cell can be either a, or α. On opposite arms of chromosome III, there exist two silent donor loci, *HML*α and *HMR*a, which contain the genes of two mating types. Owing to the packaging in heterochromatin, both the HM loci genes are silenced and only the genes at the *MAT* locus are expressed for a given haplotype. In wild type yeast cells, a highly cell-cycle regulated expression of the HO, a site-specific endonuclease during G1 phase of the cell-cycle initiates the mating type switching event. The HO endonuclease recognizes and cleaves a single site at the *MAT* locus (heterochromatic state inhibits HO recognition and cleavage at HM). After the cleavage, only one side of the break gets involved in searching and finding homology on only one arm of the chromosome in the majority of times, via a phenomenon known as “donor preference” (Haber 1998a). Upon finding homology, the genetic information is copied and replaced at the *MAT* locus,

keeping the silent donor locus unchanged. Strains having the HO endonuclease under regulation of *GAL*1-10 promoter (Stern et al. 1984) as well as strains that lack both the donor loci (Moore and Haber 1996) have been proven as powerful tools for dissecting initial steps of recombinational repair of an inducible DSB *in vivo*.

The cellular response to DSBs is mediated by several different classes of proteins, which are commonly known as sensors, mediators and effectors of checkpoint control pathways. The cellular response to DSBs is initiated by rapid recruitment of a complex of Mre11, Rad50 and Xrs2 (MRX in *S. cerevisiae*) or Nbs1 (MRN in mammals), which has an integral role in initial recognition of the DSB, checkpoint activation, signal amplification as well as bridging and nucleolytically processing the two broken DNA ends at the site of the break (Williams et al. 2007). MRN also activates ATM (Ataxia Telengiectasia Mutated; Tel1 in *S. cerevisiae*) *in vivo* and *in vitro*, which is one of the key checkpoint cascade kinases. Once recruited to the site of break, MRN and ATM can in turn activate another kinase ATR (ATM-Related; Mec1 in *S. cerevisiae*) (Jazayeri et al. 2006). Long stretches of ssDNA, which are bound by the single stranded binding protein RPA and resulted from nucelolytic processing of broken DNA ends, are also a critical signal in the damage repair signaling cascade. RPA recruitment at the site of break is necessary for recruitment of sensor kinases like ATR and ATRIP (ATR interacting partner; Ddc2 in *S. cerevisiae*) (Zou and Elledge 2003). The subclass of downstream substrates that get phosphorylated directly by ATM and ATR sensor kinases, and act as either recruiters or activators of a plethora of additional complexes ("effectors") or scaffolds on which

complexes can be assembled (Harper and Elledge 2007) are known as mediators. Finally in *S. cerevisiae*, arrest of cell cycle progression through anaphase takes place via inhibition of cohesin degradation, which is necessary for separation of sister chromatids (Heideker et al. 2007).

Histone modifications and histone modifying enzymes in repair of DSBs

The first chromatin modifying event that occurs in response to a DSB is the rapid and extensive phosphorylation of a serine residue (S129 in histone H2A in *S.cerevisiae* and S139 in H2AX in mammals) in the SQE motif of C-terminal domain of histone H2AX, conventionally referred to as γ-H2AX. This phosphorylation event was first identified in the histone H2AX variant, covering mega bases on both sides of DSBs in vertebrate cells (Rogakou et al. 1998). Later, it was also shown to spread over 50Kb domains flanking a DSB in *S.cerevisiae* (Shroff et al. 2004). The ATM, ATR and DNA-PK in mammals and Mec1, Tel1 in *S.cerevisiae* have been detected as the responsible kinases for this rapid response (Altaf et al. 2007). The formation of γ-H2AX nuclear foci, containing DNA repair factors such as the MRN complex, 53BP1, BRCA1 and MDC1, which are easily detectable by immunofluorescence microscopy, has proven to be a useful experimental marker for DSB induction. Although, γ-H2AX is not essential for the initial recruitment of DSB response factors and checkpoint signaling, the phosphorylation event is required for effective repair of DSBs in multiple ways (Celeste et al. 2002; Celeste et al. 2003) contributing to both NHEJ and HR pathways (Karagiannis et al. 2007).

Recent studies have suggested that γ-H2AX leads to recruitment of proteins that are necessary to ubiquitinate γ-H2AX, which in turn can recruit downstream players of the DNA damage response (Huen et al. 2007; Kolas et al. 2007; Mailand et al. 2007). Another established role of γ-H2AX is the recruitment of different subunits of cohesin complex onto chromatin in response to DSBs specifically in S and G2 phases, promoting HR pathway of repair using the sister chromatids (Strom et al. 2004; Unal et al. 2004). Importantly, the other major role of γ-H2AX is to interact with chromatin remodeling and modification enzymes, like INO80. com, SWR1.com and NuA4 complexes and thus to recruit and retain these complexes at the site of a DSB (Downs et al. 2004; Morrison et al. 2004; van Attikum et al. 2004).

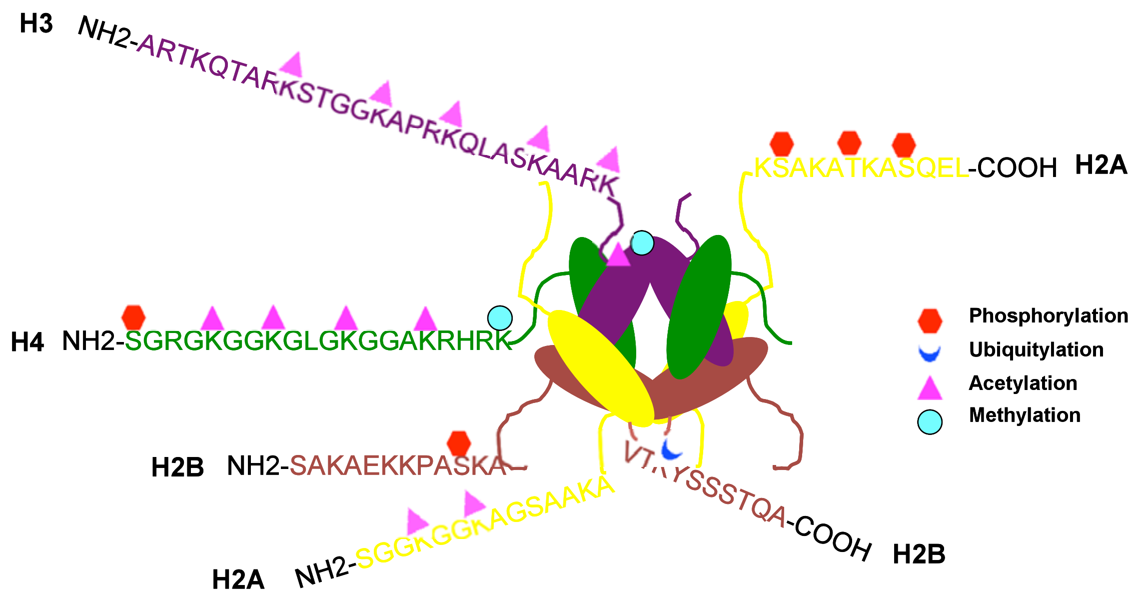

Figure 4. Schematic of histone modifications implicated in DSB-repair processes. The various post-translational modifications of the histone N-terminal and C-terminal domains and also the histone fold domain of histone H3, which have been described in the literature so far, are illustrated here.

In addition, recent studies show that dephosphorylation of γ-H2AX following DNA

repair is necessary for efficient recovery from the DNA damage checkpoint (Keogh et al. 2006a). These studies indicate that a three-protein complex, HTP-C (histone phosphatase H2A complex) containing the phosphoserine-specific phosphatase Pph3 (in *S. cerevisiae*) and protein phosphatase 2A (in mammalian cells) regulate the dephosphorylation of H2AX (Chowdhury et al. 2005). Furthermore, it has been hypothesized in this study that γ-H2AX may be dephosphorylated in *S.cerevisiae* and mammalian cells only after its displacement from chromatin.

Other phosphorylatable residues in the C-terminal domain of H2A, S122 (Harvey et al. 2005) and T126 (Wyatt et al. 2003) have been reported to be essential for survival of *S.cerevisiae* upon occurrence of different types of DNA damages (Moore et al. 2007). However, precise roles of these residues are yet to be determined. Likewise, acetylation of H2A at its N-terminal tail by NuA4 seems to be important for cell survival after DNA damage (Wyatt et al. 2003).

Phosphorylation of S14 (in mammals) or S10 (in S. cerevisiae) at the N-terminal tail of H2B has also been implicated in DSBs (Fernandez-Capetillo et al. 2004). Immunofluorescence detects that this phosphorylation of H2B colocalizes with γ-H2AX foci in response to DNA DSBs. Later, it was also detected during meiosis of S. cerevisiae (Ahn et al. 2005) and antibody class-switching in B cells (Odegard et al. 2005). Another study identified ubiquitination on lysine 123 of H2B by the Rad-BreI complex to be necessary for activation of DNA-damage checkpoint in budding yeast (Giannattasio et al.

2005).

In addition, methylation of lysine 79 by Dot1 in the histone fold domain of histone H3 was also found to be involved in checkpoint activation after DNA damage, in a Rad9 dependent manner (Giannattasio et al. 2005). In mammalian cells, 53BP1, which co-localizes rapidly with γ-H2AX upon onset of DSB and functions in checkpoint activation, has conserved residues in its tudor domains, common with yeast Rad9. Both 53BP1 and Rad9 have been found to bind methylated H3K79 *in vitro* (Daniel et al. 2005). *In vivo*, it is hypothesized that constitutive methylation of H3K79, that gets exposed due to changes in chromatin conformation upon induction of break, recruits 53BP1 at the site of break and thus activates the checkpoint signal (Huyen et al. 2004).

Recently, acetylation of H3K56 mediated by the HAT Rtt109 and chaperone Asf1 has been linked with turning off the DNA damage checkpoint recovery following a DSB (Chen et al. 2008). However, a more recent study pointed out that deactivation of the checkpoint signal is redundantly mediated by Asf1 and Caf-1 (Kim and Haber 2009). Whether acetylation of H3K56 by Rtt109 really plays a role by affecting Caf-1 and/or Asf-1 has yet to be unequivocally determined from future studies.

The question of how DNA damage signals are turned off has revealed another DSB dependent modification of histone residue, i.e. phosphorylation of serine 1 of H4. H4S1 is phosphorylated by Casein Kinase II (CK2), at a 10kb domain on both sides of a DNA

DSB after 2 hours of γ-H2AX (Cheung et al. 2005). It has been proposed that to restore the chromatin state and turn off damage signal after DSB repair, CK2 phosphorylates H4S1 to inhibit further rounds of acetylation of N-terminal tail of H4, which occurs during early remodeling of chromatin after DSB formation (Utley et al. 2005).

Acetylation of conserved lysine residues in the N-terminal domains of H3 and H4 mediated by a number of histone acetyl transferases (HATs) like NuA4, GCN5 and Hat1 is important for repair of DSBs by both NHEJ and HR (Bird et al. 2002; Tamburini and Tyler 2005; Mersfelder and Parthun 2006). Acetylation of all acetylable residues in the H3 (K9, 14, 18, 23 & 27) and H4 (K5, 8, 12 and 16) N-terminal tails increases at 600 bp from the site of the DSB and decreases as the break gets repaired. In mammalian ES cells, the HAT complex, TIP60 and the cofactor, TRRAP are recruited to DSBs, where they induce acetylation of H4 and facilitate HR (Murr et al. 2006). Differential acetylation of lysine 16 residue of histone H4 was earlier identified in budding yeast following induction of DSB (Jazayeri et al. 2004) and in human cells, MOF/MYST1 mediate irradiation induced acetylation of H4K16 (Gupta et al. 2005). In mammals, trimethylation of H4K20 in the pericentric region is the target for 53BP1 recruitment at a DSB located in heterochromatin, just like H3K79 is for a DSB in euchromatic region (Lachner et al. 2004). Moreover, di-methylated H4K20 may also play a role in recruiting 53BP1 to DSBs (Botuyan et al. 2006).

In general, going with the “access-repair-restore” model, the current notion in the field

remains that acetylation of histones close to the site of break by different HATs facilitates access of repair machinery and then deacetylation by different HDACs may restore the chromatin to its original sate and deactivate the damage checkpoint signal at the end of the repair process.

ATP-dependent chromatin remodeling enzymes in repair of DSBs

In addition to histone modifying enzymes, numerous ATP-dependent chromatin remodeling enzymes have been implicated in many aspects of repair of a chromosomal DNA DSB. Notably, it is believed that the coordinated and interdependent actions of histone modifying and chromatin remodeling enzymes and their interaction with the repair machinery make the repair of a DSB successful.

The most extensively studied multi-subunit, ATP-dependent, chromatin remodeling enzyme complex in repair of DSBs by both NHEJ and HR has been INO80.com (Osley et al. 2007). The catalytic subunit of this complex, Ino80 as well as Arp5 and Arp8 are recruited to the break over a region of 1.6Kb within an hour of break induction (Morrison et al. 2004; van Attikum et al. 2004). The complex is recruited to the break via the interaction of its subunit Nhp10 with γ-H2AX and is reported to play a role in DSB processing and ssDNA formation (van Attikum et al. 2004) and most importantly, nucleosome eviction and chromatin structure disruption at the site of the break (Tsukuda et al. 2005). However, it is not unequivocally established that complete nucleosome

eviction happens around a DSB that is dependent on complexes like MRX and INO80.com as claimed by this study (Chen et al. 2008). In *S. cerevisiae*, a phenomenon called adaptation has been characterized, that allows cells to turn off checkpoint and resume cell cycle even when a DSB persists (Toczyski et al. 1997). The INO80.com complex has been found to be required for checkpoint adaptation in budding yeast (Papamichos-Chronakis et al. 2006).

The SWR1.com complex is another ATP-dependent remodeling enzyme that is recruited to the DSB in γ-H2AX dependent manner (van Attikum et al. 2007a). Recruitment increases over 4 hours and spreads almost 10kb from the break. SWR1.com remodeling enzyme has a unique property of exchanging the H2A-H2B dimers of the nucleosomes with H2B-H2AZ (Htz1 in budding yeast) dimers *in vitro* (Mizuguchi et al. 2004). Hence, it is proposed that SWR1.com may remove γ-H2AX from the site of the break and deposit Htz1, and thus may play a role in turning off the repair signal. However, this phenomenon is yet to be established experimentally.

In higher eukaryotes, homologs of Swr1, the catalytic subunit of yeast SWR1.com complex is found in TIP60 complex. Interestingly, the TIP60 complex seems to be a fusion of budding yeast SWR1.com and NuA4 complexes (Doyon et al. 2004; Raisner and Madhani 2006). The human TIP60 acetylates histone H4 at DSBs and stimulates HR by recruiting repair machinery (Ikura et al. 2000; Murr et al. 2006). The drosophila TIP60 acetylates (Kusch et al. 2004) and then exchanges the phosphorylated histone H2Av

(equivalent of γ-H2AX) for unmodified H2Av (equivalent of H2AZ). It has also been found that deposited H2AZ is acetylated by NuA4 predominantly at lysine 14 (Keogh et al. 2006b; Millar et al. 2006). All these data indicate that NuA4 and SWR1.com may function together to regulate the H2AZ deposition at the DSBs. Moreover, data from our lab suggest an antagonistic interplay of INO80.com and SWR1.com in regulation of H2Az and γ-H2AX at the site of DSBs during cell-cycle checkpoint adaptation (Papamichos-Chronakis et al. 2006). All these mechanistic possibilities await further investigations in future.

A third remodeling complex implicated in DSB repair is RSC. RSC is recruited to broken DNA ends, depending on MRX (Shim et al. 2005) and like MRX, has been implicated in both NHEJ and HR pathways. Two functional roles have been attributed to the remodeling activities of RSC complex, one being early and one being late during the repair process. Right after recruitment at the site of the break, it is reported that RSC in turn influences the level of H2AX phosphorylation and accessibility of nucleases like restriction enzymes and Micrococcus Nuclease (MNase) to sites immediately surrounding the break. This implies that RSC may mobilize a few nucleosomes flanking the break to enhance recruitment of repair machinery to the broken chromatin. In addition, RSC is also required for proper loading of cohesins to hold sister chromatids together and promote HR (Shim et al. 2005; Shim et al. 2007). Another contribution of RSC complex to the HR pathway of DSB repair is during post-synaptic “restoration” of chromatin state. RSC regulates the dissociation of the invading presynaptic filament from

the HM loci and ligation of the presynaptic filament with the DNA on the other side of the break (Chai et al. 2005).

Another multifunctional chromatin remodeling enzyme implicated in DSB repair by HR pathway has been Rad54 (Heyer et al. 2006; San Filippo et al. 2008). This evolutionarily conserved, dsDNA activated ATPase acts as a mediator during presynaptic phase of HR by helping Rad51 to from the presynaptic filament (Mazin et al. 2003; Wolner et al. 2003). During synapsis, Rad54 promotes Rad51 filament mediated strand exchange (Petukhova et al. 1999) and formation of stable joint intermediates (Solinger et al. 2001; Jaskelioff et al. 2003), that are substrates for DNA polymerases (Solinger and Heyer 2001; Sugawara et al. 2003). Both genetic and *in vitro* studies have shown that Rad54's roles in HR differentially include ATP-dependent and –independent functions (Kiianitsa et al. 2002; Wolner and Peterson 2005). The ATPase domain of Rad54 seems to be dispensable for the presynaptic activity, but it is required to facilitate formation of stable, plectonemic DNA joints, suggesting that chromatin may be a substrate for Rad54 only during post-synaptic stages of HR.

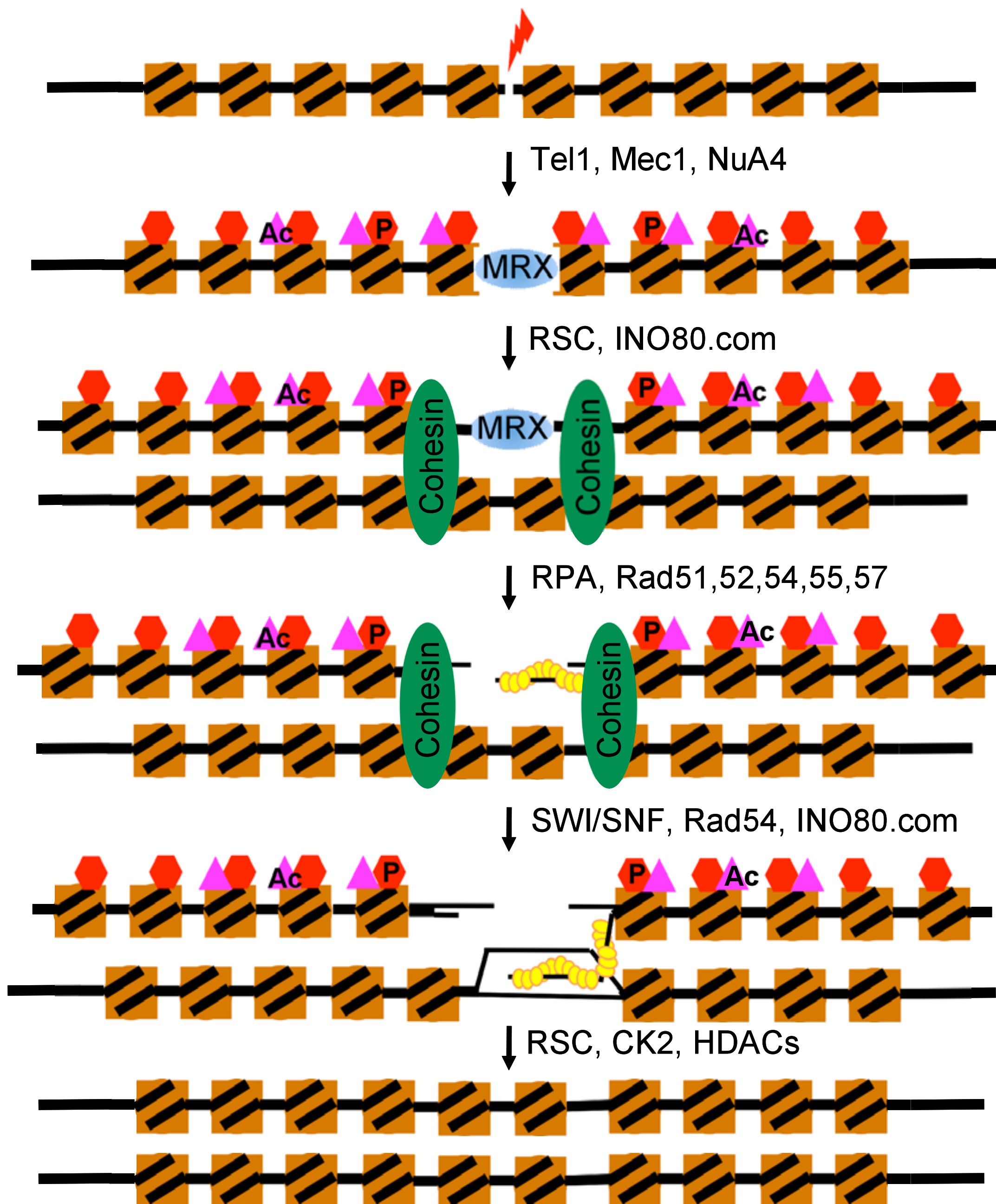

Figure 5. Schematic of established roles of ATP-remodeling enzymes implicated in different steps of the recombinational repair process of a chromosomal DNA DSB. Following the rapid phosphorylation of H2AX, RSC remodeling enzyme is recruited to the break. RSC mobilizes a few nucleosomes flanking the break. This in turn helps to load subunits of cohesin complex. INO80.com is involved in MRX-dependent nucleolytic processing of the DNA ends at the break. Next, Rad51 assembles onto ssDNA tails to generate functional presynaptic filament. ATP-dependent remodeling activity of SWI/SNF, Rad54 and INO80.com are involved in formation of stable recombination intermediates during strand invasion event by remodeling the donor nucleosomes. Finally, RSC comes back during the ligation of the newly synthesized DNA strand to the other end of the break, and hence may restore the remodeled nucleosomes to their original state.

.Along this line, we have shown that indeed homology search and formation of initial paranemic joint formation on nucleosomal donor is not facilitated by Rad54 (Sinha and Peterson 2008). Rad54 is postulated to convert the outcome of a successful homology search by Rad51 filament, the initial joint into a stable, intertwined plectonemic joint that can entail further events during HR. This is corroborated by the fact that *in vivo*, Rad54 remodels a single positioned nucleosome that occludes the HO cut site at the *HML*α donor locus during repair of an HO-induced DSB at *MAT* (Wolner and Peterson 2005). Thus, during HR between *MATa* and *HMLα*, Rad54 remodels chromatin without large-scale rearrangement of nucleosomes. Finally, in the post-synaptic phase of HR, Rad54 promotes branch migration of Holliday junctions and destabilizes the joint intermediates by removing Rad51 from dsDNA and thus recycling Rad51 (Bugreev et al. 2006; Kiianitsa et al. 2006).

Lastly, the founding member of the class of ATP-dependent chromatin remodeling proteins conserved throughout eukaryotes, the SWI/SNF complex has been recently linked to DSB repair (Chai et al. 2005). SWI/SNF may not play a role in the NHEJ pathway; however, more studies need to be carried out to rule out the possibility. During HR, SWI/SNF was found to be recruited both at the break site and the donor locus and absence of the catalytic subunit of SWI/SNF blocked the synapsis between the invading *MATa* presynaptic filament and HMLα donor locus. All the data so far, predict a role of SWI/SNF in remodeling the chromatin at the donor locus. Paradoxically, strains lacking functional SWI/SNF complex are not robustly sensitive to DSB-inducing agents,

questioning its requirement for general DSB repair by HR. Moreover, we have previously showed using in vitro biochemical studies that the chromatin remodeling activity of SWI/SNF does not stimulate Rad51-mediated homology search on chromatin (Sinha and Peterson 2008). Combined with the data from our unpublished studies, we believe that the role of SWI/SNF in HR is unique at donor sequences that are assembled into some heterochromatic chromatin structures, such as the SIR-dependent chromatin at HM loci.

Concluding Remarks

In summary, a number of chromatin modifying and ATP-dependent remodeling enzymes function in concert in a number of ways to counteract the inherent repressive nature of chromatin and grant the repair machinery access to the damaged DNA. This is true for any other DNA-mediated basic processes like transcription or replication. Although there has been a rapid increase in our knowledge of different chromatin modifications and the recruitment of chromatin remodeling complexes that can be functionally linked to the DNA damage response and repair processes, further research is needed to attribute specific roles of these modifications and factors and their complex intricate relationships.

Preface to Chapter II

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CHAPTER II

A RAD51 PRESYNAPTIC FILAMENT IS SUFFICIENT TO CAPTURE NUCLEOSOMAL HOMOLOGY DURING RECOMBINATIONAL REPAIR OF A DNA DOUBLE STRAND BREAK.

CHAPTER II

A Rad51 presynaptic filament is sufficient to capture nucleosomal homology during recombinational repair of a DNA double strand break.

SUMMARY

Repair of chromosomal DNA double strand breaks by homologous recombination is essential for cell survival and genome stability. Within eukaryotic cells this repair pathway requires a search for a homologous donor sequence and a subsequent strand invasion event on chromatin fibers. We employ a novel biotin-streptavidin minichromosome capture assay to show that yRad51 or hRad51 presynaptic filaments are sufficient to locate a homologous sequence and form initial joints even on the surface of a nucleosome. Furthermore, we present evidence that the Rad54 chromatin remodeling enzyme functions to convert these initial, metastable products of the homology search to a stable joint molecule that is competent for subsequent steps of the repair process. Thus, contrary to popular belief, nucleosomes do not pose a potent barrier for successful recognition and capture of homology by an invading presynaptic filament.

Introduction

Cells suffer from DNA damage due to exogenous stresses that are present in their environment and endogenous stresses that are generated by metabolism (Allard et al. 2004). Chromosomal DNA double strand breaks (DSBs) are one of the most severe types of DNA damage with respect to preservation of genomic integrity. If left unrepaired, DSBs can cause genomic instability in the form of chromosome loss or rearrangements, compromising faithful transmission of genetic information and eventually leading to apoptosis or carcinogenesis.

Eukaryotic cells have developed several conserved mechanisms for repair of DSBs, including homologous recombinational repair (HRR) or non-homologous end joining (NHEJ) (Paques and Haber 1999; Allard et al. 2004; Shin et al. 2004). NHEJ is the predominant form of DSB repair in G0/G1 phase of the cell-cycle, whereas HRR is prevalent during S and G2 phases. HRR is inherently error free because it repairs DSBs by using either the sister chromatid or other homologous DNA sequences as templates to repair the missing genetic information.

The *RAD52* epistasis group of genes, encoding Rad50, Rad51, Rad52, Rad55, Rad57, Rad59, Mre11 and Xrs2, mediates HRR in organisms ranging from yeast to human (Paques and Haber 1999). These genes are highly conserved among all eukaryotes and alterations in any one of these proteins render the cells sensitive to DSB causing agents.

The biochemical steps of HR have been well established in both prokaryotic and eukaryotic systems (Bianco et al. 1998; Sung et al. 2003). First, the 5' ends of DNA that flank the break are processed by an exonuclease to create single stranded DNA tails. Next, a recombinase (RecA in prokaryotes and Rad51 in eukaryotes) polymerizes onto these DNA tails to form a presynaptic, nucleoprotein filament that can search for a homologous duplex DNA. In some cases this homology search can occur on a genome wide level (Haber et al. 1991; Richardson et al. 1998; Inbar and Kupiec 1999). The initial detection of homology is thought to result in formation of transient, metastable DNA joints in which the 3' end of the presynaptic filament is not engaged in base-pairing interactions. These metastable joints are considered paranemic in nature as they are stabilized by, and require, protein-DNA interactions (e.g. RecA-DNA or Rad51-DNA) (Bianco et al. 1998; Sung et al. 2003). Such paranemic joints are believed to be the precursor for a stable plectonemic joint (Riddles and Lehman 1985) in which the presynaptic filament fully base pairs with its complementary strand, displacing the non-complementary strand and forming what is known as a D-loop. The stability of the plectonemic joint does not require protein-DNA interactions and the 3' end of the presynaptic filament is engaged in Watson-Crick base-pairing. Subsequent steps of HRR entail DNA synthesis to replace the missing information, followed by resolution of DNA intermediates to yield two intact duplex DNA molecules.

Although studies in yeast and mammals have provided a wealth of information on the detection, response, and repair of DSBs, study of DSB repair in the context of chromatin

is still rather nascent (Peterson and Cote 2004). It is well known that histone and non-histone proteins package the eukaryotic genome into a condensed structure termed chromatin (Kornberg 1974). The first level of chromatin organization is the nucleosome, which is formed by wrapping ~147 basepairs of DNA around a histone octamer. Each histone octamer is composed of two H2A-H2B dimers and one H3-H4 tetramer. Nucleosomes are organized as long, linear arrays that fold into condensed fibers, and are stabilized by linker histones and intra- and inter-nucleosomal interactions (Hansen 2002). In the case of DSB repair, the wrapping of DNA on the surface of the histone octamer and the formation of chromatin fibers are likely to constitute potent barriers both for the homology search and strand invasion steps of HRR. Consistent with this view, numerous studies have demonstrated that ATP-dependent chromatin remodeling enzymes are recruited to DSBs *in vivo* (Chai et al. 2005; van Attikum et al. 2007b). However, it is not yet clear if chromatin remodeling is required for a presynaptic filament to find homology in the context of nucleosomes. In this study, we directly investigate whether the search for homology and the initial capture of the homologous sequence can take place on the surface of nucleosomes in the absence or presence of remodeling enzymes *in vitro*. We report that yeast and human Rad51 presynaptic filaments are sufficient to locate the homologous sequence and form initial joints even on the surface of a nucleosome. On the other hand, formation of stable plectonemic joints requires Rad54, an ATP-dependent chromatin remodeling enzyme.

Results

The initial product of a successful homology search is believed to be a transient, protein-stabilized joint. Previous studies have used filter binding assays (Riddles and Lehman 1985) or electron microscopy (Christiansen and Griffith 1986) to visualize these metastable joints with DNA substrates. Filter binding assays have also been used to follow RecA-catalyzed formation of metastable joints on a nucleosomal donor (Ramdas et al. 1991). However, a drawback of such filter binding assays is that the synapsis reactions must be treated with high salt (1.5M NaCl) so that nucleosomes are displaced and joints can be quantified. In addition, these previous studies used presynaptic filaments assembled on very large ssDNA molecules (>6 Kb), and thus it is not clear whether the filter binding assay can detect synapsis between smaller presynaptic filaments that might be targeted to more well-defined and positioned nucleosomes. To circumvent the problems inherent to the filter binding assay, and to study the initial homology capture on a well-defined nucleosomal donor, we developed a novel biotin-streptavidin capture assay (Figure 6A). In this assay, a nucleosomal donor is assembled with recombinant histone octamers that are deposited onto a plasmid that harbors nucleosome positioning sequences. The recombinant histone octamers are engineered to contain a site-specific biotin group on the C-terminal domain of histone H2A. Presynaptic filaments are then assembled with purified recombinases (RecA, yeast or

human Rad51) on ^{32}P-labeled 50-90 base oligonucleotides

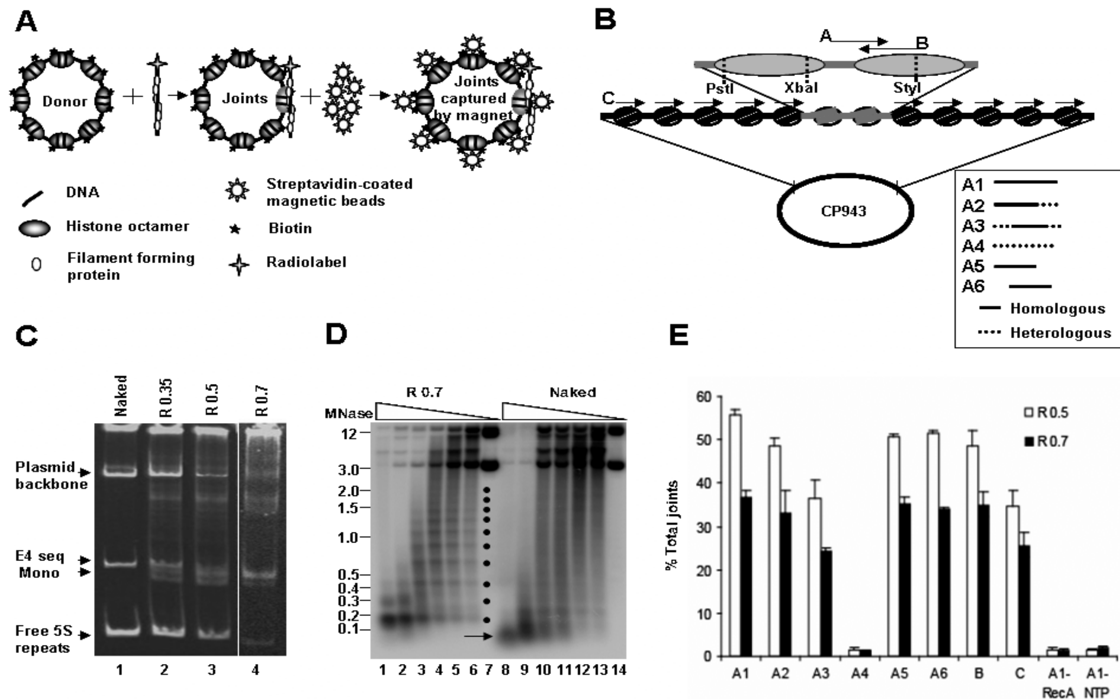


Figure 6. The biotin-streptavidin capture assay. (A.) Schematic of the strategy for capturing initial joint molecules. (B.) Schematic of the nucleosomal, minichromosome donor. Blank ovals, 5S-rDNA nucleosome positioning elements; grey ovals, putative positions of nucleosomes over central 400bp G5E4 sequence; solid grey line, G5E4 sequence; broken black lines, DNA restriction sites for *Pst*I, *Xba*I and *Sty*I; black arrows, oligonucleotides designed at positions A, B and C. Inset Panel: Oligonucleotides at Position A with varying amounts of homology to the donor. A1: 80 bases of homology, A2: 50 bases of homology followed by 30 bases of heterology, A3: 50 bases of central homology flanked by 25 bases of heterology at 5'end and 29 bases of heterology at 3'end, A4: 84 bases of heterology, A5: 50 bases of homologous sequence of A2 and A6: 50 bases of homologous sequence of A3. All oligonucleotides are depicted 5' to 3'; B and C oligonucleotides have 87 and 80 bases of homology respectively. (C.) *Eco*RI restriction enzyme analysis of nucleosomal donors. Reconstituted nucleosome (R=0.35, R=0.5 or R=0.7: lanes 2-4) or naked plasmid CP943 (lane 1) was digested with *Eco*RI and products were separated on a 4% native polyacrylamide gel. Ethidium stained gels are shown; lane 4 is from a separate gel. Mono, migration of 5S mononucleosome. (D.) Analysis of nucleosomal occupancy at position B of the donor. Reconstituted nucleosome (R=0.7: lanes 1-7) or naked plasmid CP943 (lanes 8-14) was digested with increasing amounts of MNase and purified products were separated by agarose gel electrophoresis followed by Southern hybridization using radiolabeled oligonucleotide B. Lanes 7 and 14 contain uncut purified DNA. DNA size markers in kilobases are shown at the left of the panel. Black arrowhead indicates free DNA fragments smaller than 100 bases in lanes 8-11. Black dots indicate the banding pattern of MNase sensitive sites due to nucleosome occupancy in lanes 1-6. (E.) Results of biotin-streptavidin capture assays using different RecA presynaptic filaments (see Figure 6B) with donors that are either subsaturated (R=0.5) or saturated (R=0.7) with nucleosomes. Reactions were incubated for 30 minutes and contained ATP-ϒ-S as nucleotide cofactor. % Total joints were calculated as the proportion of bead-bound radioactivity to total (bound+unbound) radioactivity. Results are from at least three independent experiments; error bars indicate standard deviations.

that are homologous to target sequences in the nucleosomal donor. After incubation of the presynaptic filaments with the biotinylated nucleosomal donor, products of a successful homology search are captured on streptavidin-coated magnetic beads and quantified by scintillation counting (Figure 6A). Importantly, to maintain the nucleosomal state of the donor, the capture step is performed in the identical buffer as in the initial synapsis reaction. Because the biotin-streptavidin capture assay measures total DNA joints (i.e. stable and metastable joints), one half of all reactions are also deproteinized so that stable plectonemic joints can be quantified by gel electrophoresis and autoradiography.

The donor plasmid contains a head-to-tail array of ten 5S rDNA nucleosome positioning sequences that flank a di-nucleosome length E4 promoter (Figure 6B) (Ikeda et al. 1999). *Eco*RI restriction sites flank each 5S rDNA repeat, and thus *Eco*RI digestion and native gel electrophoresis allow the quantification of nucleosomal occupancy (Figure 6C); (Carruthers et al. 1999). When chromatin reconstitutions were performed at a ratio of 0.35 histone octamers per 200 bp of plasmid DNA (R=0.35), very few of the 5S repeats were nucleosomal. At a ratio of 0.5 histone octamers per 200 bp of plasmid DNA (R=0.5), 50-60% of the 5S repeats were nucleosomal (Figure 6C, compare lanes 1 and 2, 3). And finally at a ratio of R=0.7, >90% of the 5S repeats were nucleosomal (Figure 6C, compare lanes 1 and 4). We confirmed the predicted locations of the two, centrally located E4 promoter-containing nucleosomes by two independent methods. First, *Sty*I (Figure 6B) restriction enzyme digestion was used to probe nucleosomal occupancy. As

predicted, *Sty*I site was occluded in the nucleosomal plasmid assembled at an R=0.7 (Figure 7).

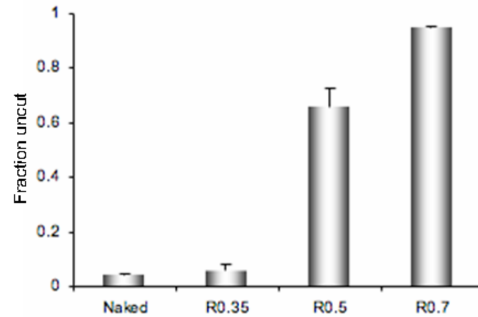
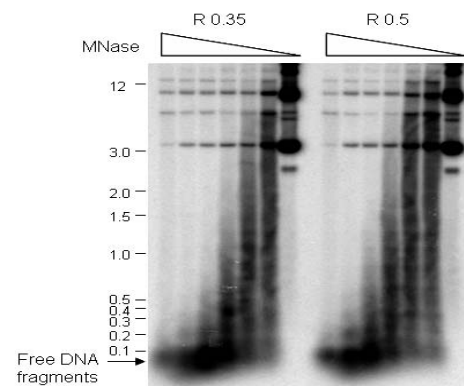


Figure 7. Restriction enzyme accessibility assay of nucleosomal donors. Nucleosome reconstituted (R= 0.35, R=0.5 and R=0.7) or naked plasmid CP943 (800ng of DNA equivalent) was digested with *Sty*I and products were separated by 1% agarose gel electrophoresis and visualized by ethidium bromide staining. Fraction uncut was calculated by quantifying the bands using ImageJ and normalizing to the same amount of undigested supercoiled plasmid.

Additionally, we digested nucleosomal donors with micrococcal nuclease (MNase), which cleaves nucleosomal DNA preferentially within the linker DNA between nucleosomes. MNase digests were electrophoresed on agarose gels and Southern blots were probed with ^{32}P-labelled oligonucleotide B which hybridizes to E4 sequences that are predicted to be encompassed by a positioned nucleosome (Figure 6B). When this analysis was performed on donors assembled at an R=0.7, oligonucleotide B hybridized exclusively to mononucleosomal DNA (Figure 6D, lanes 1-3) and to an extensive nucleosomal ladder (Figure 6D, lanes 3-6). Importantly, no free DNA fragments smaller than 100 bases were detected (Figure 6D; compare lanes 1-6 with 8-11). In contrast, MNase analysis of donors assembled at R=0.35 or R=0.5 allowed detection of smaller DNA fragments that are diagnostic of free DNA (Figure 8). These data confirm that the position B on the E4 promoter sequence (Figure 6B) is fully encompassed by a nucleosome in donors assembled at R=0.7, but this region is only partially occupied in donors assembled at R=0.35 and R=0.5.

Figure 8. Analysis of nucleosomal occupancy at position B of the donor. Reconstituted nucleosomal (R= 0. 35 or R=0.7) donor was digested with increasing amounts of MNase and purified products were separated by agarose gel electrophoresis followed by Southern hybridization using radiolabeled oligonucleotide B. DNA size markers in kilobases are shown at the left of the panel. Black arrowhead indicates free DNA fragments smaller than 100 bases.



Based on previous in vitro studies, we assembled presynaptic filaments on four different oligonucleotides that should allow discrimination between stable versus metastable joints (Christiansen and Griffith 1986; Konforti and Davis 1987). For instance, a presynaptic filament assembled on an oligonucleotide that is fully homologous to the donor (Figure 6B-inset, oligo A1) should lead to formation of stable, plectonemic joints. However, presynaptic filaments assembled on oligonucleotides lacking homology to the donor at the 3'-end (Figure 6B-inset, oligos A2 and A3) are expected to form primarily metastable joints, whose stability require protein-DNA interactions. On the other hand, a presynaptic filament that lacks homology to the donor (Figure 6B-inset, oligo A4) should not form joints, and it serves as a negative control. In addition, to determine if joints are more likely to form between nucleosomes rather than on the nucleosomal surface, we also assembled presynaptic filaments on oligonucleotides that are homologous to different positions on the chromatin donor (Figure 6B, arrows labeled A, B and C). Specifically, at position A, much of the presynaptic filament has homology to the linker region between nucleosomes and only the 3'end of the filament is predicted to lie within a nucleosome. On the contrary, at position B, most of the filament has homology to a sequence that is predicted to be buried within a nucleosome and only the 3' end of the filament lies at the

nucleosomal edge. At position C, the entire presynaptic filament has homology to sequences that are encompassed by each of the 5S positioning elements and thus should be entirely nucleosomal.

To validate the biotin-streptavidin capture assay, presynaptic filaments were first assembled using the bacterial RecA recombinase and the panel of ^{32}P-labelled oligonucleotides. When the biotin-streptavidin capture assay was applied to reactions using the donor that had few nucleosomes (R=0.5), 35% to nearly 60% of the presynaptic filaments were captured in a 30 minute reaction (Figure 6E and Figure 9). Formation of joints required nucleotide cofactor, RecA, and homology between the presynaptic filament and nucleosomal donor (Figure 6E, compare reactions with oligo A4 to other oligos; and Figure 9).

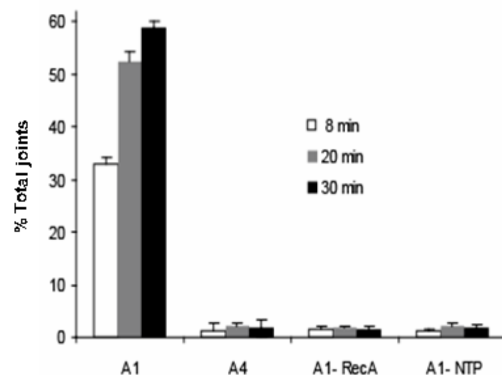
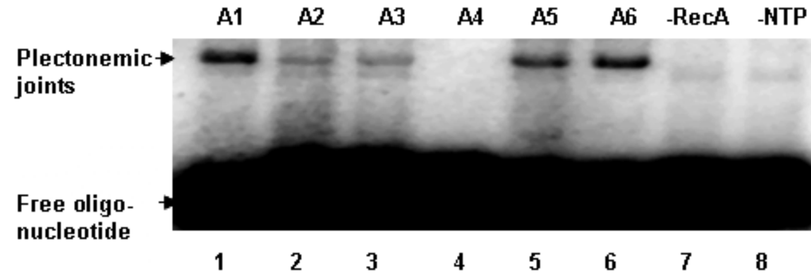


Figure 9. Validation of the biotin-streptavidin capture assay. Graph depicts timecourse of biotin-streptavidin capture assay used on subsaturated (R=0.5) nucleosomal donor. Note lack of joints with the nonhomologous presynaptic filament (oligo A4) or when reactions lack either RecA or nucleotide triphosphate (NTP) Reactions contained ATP-ϒ-S as nucleotide cofactor. Results are from at least three independent experiments; error bars indicate standard deviations.

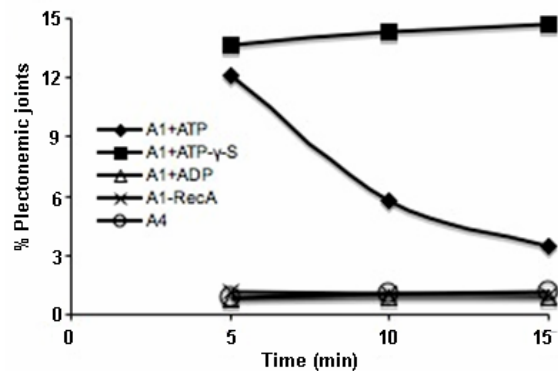
Moreover, joints were efficiently formed with all types of RecA presynaptic filaments; including those that lack 3’ homology and which produce mainly metastable, protein-dependent joints (Figure 6E and Figure 10).

Figure 10. RecA filaments need homology at 3'-end to form stable joints. Representative autoradiograph shows plectonemic joints formed by different RecA presynaptic filaments on the naked DNA donor. Reactions were incubated for 30 minutes in the presence of ATP-ϒ-S. Long exposure of the gel is shown to illustrate the poor formation of plectonemic joints with oligos that lack 3' homology.



In addition, joint formation was ~5-fold more efficient in the presence of a nonhydrolyzable analog of ATP, ATP-γS, which also enhanced formation of stable plectonemic joints as expected (Figure 11), (Menetski et al. 1990; Hsieh et al. 1992).

Figure 11. RecA-mediated plectonemic joints require RecA, nucleotide cofactor (NTP), and homology. Kinetics of RecA-mediated formation of plectonemic joints on a naked DNA donor as detected by agarose gel electrophoresis and autoradiography of the deproteinized joints. Average of two independent experiments is plotted.



Strikingly, RecA catalyzed formation of joints even when the nucleosomal donor was fully loaded with nucleosomes (R=0.7; Figure 6E) and the presynaptic filament was homologous to sequences located on the nucleosomal surfaces (Figure 6E, oligos B and C). However, consistent with our previous study (Jaskelioff et al. 2003), we observed that nucleosome-assembly blocked formation of stable, plectonemic joints by RecA filaments (Figure 12).

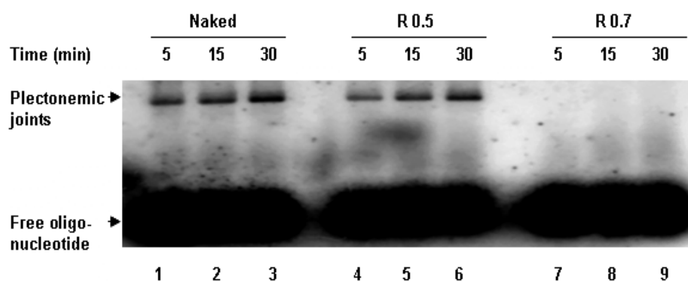


Figure 12. RecA-mediated formation of plectonemic joints is inhibited by nucleosomes. Representative autoradiograph shows RecA-mediated plectonemic joints (oligo A1) with either a naked DNA donor or on nucleosomal donors that have low (R=0.5) or high (R=0.7) density of nucleosomes. Reactions contained ATP-g-S.

Thus, as predicted by earlier filter binding studies (Ramdas et al., 1991), nucleosomes are not potent inhibitors of RecA-mediated homology search, but nucleosomes do block formation of stable, protein-independent plectonemic joints.

Eukaryotic recombinases are sufficient to mediate a homology search on chromatin

In contrast to RecA, previous studies have demonstrated that yeast or human Rad51 is not sufficient to catalyze significant levels of stable plectonemic joints on either a naked DNA template or on chromatin (Van Komen et al. 2002; Jaskelioff et al. 2003; Raschle et al. 2004). To determine whether yeast Rad51 is able to catalyze formation of protein-stabilized joints on nucleosomal donors, we performed biotin-streptavidin capture assays with donors that contained three levels of nucleosomal density (R=0.35, R=0.5, R=0.7). In addition, assays were performed with the entire panel of presynaptic filaments, including positions B and C which are located on the nucleosomal surface when donors are assembled at an R=0.7 (Figures 6C and D). Surprisingly, yeast Rad51 was sufficient to catalyze efficient formation of protein-stabilized joints on each of the nucleosomal donors and with all presynaptic filaments (Figures 13D and E). Like the case for RecA,

higher levels of joints were detected in the capture assay when a nonhydrolyzable analog

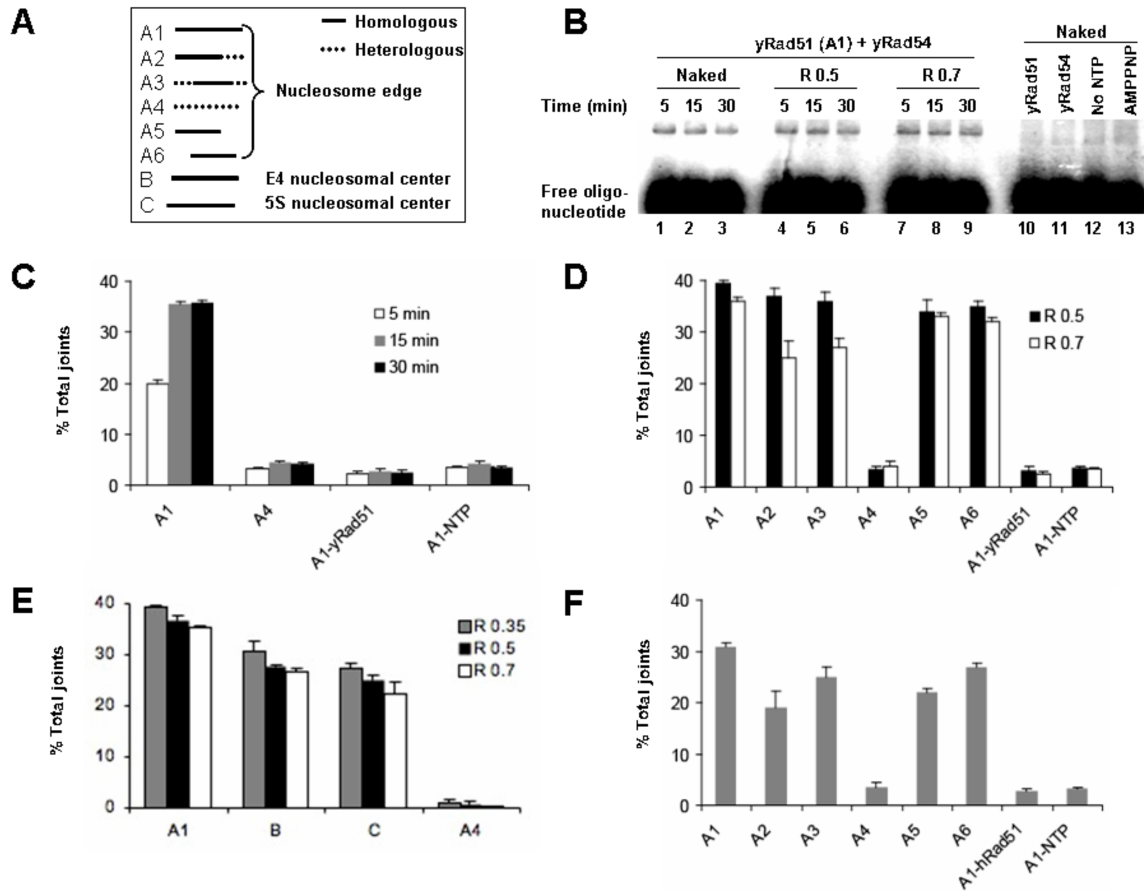


Figure 13. Yeast and human Rad51 are sufficient to capture homology on nucleosomes. (A.) Schematic of oligonucleotides indicates different positions and different amounts of homology to the donor. (B.) Representative autoradiograph shows yRad51-mediated formation of plectonemic joints on naked (lanes 1-3) or nucleosomal donors (R=0.5 or R=0.7; lanes 4-9) as detected by agarose gel electrophoresis of deproteinized reaction products. yRad54 was added where indicated. Lanes 10-13 show formation of stable joints after 30 min. Lanes 12-13 contained both yRad51 and yRad54. All reactions contained A1 oligonucleotide and ATP as nucleotide cofactor, except where indicated. (C.) Biotin-Streptavidin capture assays. Kinetics of yRad51-mediated formation of joints on the nucleosomal donor (R=0.7). (D.) Biotin-Streptavidin capture assays. Bar graph represents yRad51-mediated joint formation on nucleosomal donor with high (R=0.7) and low (R=0.5) nucleosomal occupancy. Oligonucleotides used for formation of presynaptic filaments are depicted on the x-axis. (E.) Biotin-Streptavidin capture assays. Bar graph represents yRad51 mediated joint formation at different positions on the nucleosomal donors with varying levels of nucleosomal saturation. (F.) Bar graph shows results from biotin-streptavidin assays with hRad51 presynaptic filaments and the R=0.7 nucleosomal donor. Reactions in C-F were incubated for 20 minutes and contained AMP-PNP as nucleotide cofactor. Results in C-F are from at least three independent experiments; error bars indicate standard deviations.

of ATP, AMP-PNP, was used as the nucleotide cofactor (compare Figure 13C to Figures 14A and B). Notably, joints were homology, recombinase, and nucleotide cofactor dependent (Figures 13C-E), and formation of joints reached a maximum by 15 minutes (Figure 13C). Strikingly, the efficiency of joint formation was insensitive to the level of nucleosome density, and moreover, all types of yRad51 presynaptic filaments, including the ones lacking homology at the 3'-end, formed joints with similar efficiencies (Figures 13D and E, compare oligos A2, A3 with A1). Likewise, presynaptic filaments assembled with human Rad51 were also sufficient to capture homology with nucleosomal donors (Figure 13F). Thus, eukaryotic recombinases are able to carry out a successful homology search even when synapsis occurs on the nucleosomal surface.

Chromatin remodeling enzymes do not stimulate the homology search

Rad54 belongs to the SWI2/SNF2 subfamily of DNA-dependent ATPases, and biochemical studies indicate that Rad54 can function as ATP-dependent chromatin remodeling enzyme (Alexiadis and Kadonaga 2002; Alexeev et al. 2003; Jaskelioff et al. 2003). As expected, addition of yRad54 facilitated yRad51-mediated formation of plectonemic joints on nucleosomal donors (Lanes 4-9, Figure 13B). Likewise, and consistent with previous studies (Van Komen et al. 2002; Jaskelioff et al. 2003; Raschle et al. 2004), yRad54 was also essential for formation of plectonemic joints on the naked DNA donor (Lanes 1-3, Figure 13B). Surprisingly, addition of yRad54 did not enhance formation of yRad51-dependent joints in the biotin-streptavidin capture assays (Figures

14A, B; note that the inclusion of ATP in these reactions leads to levels of joint formation that are much lower than in reactions using AMP-PNP). In fact, addition of yRad54 decreased the overall efficiency of joint formation by as much as 2-fold, and this inhibitory effect was most apparent when presynaptic filaments were assembled with oligonucleotide A1 (Figure 14B).

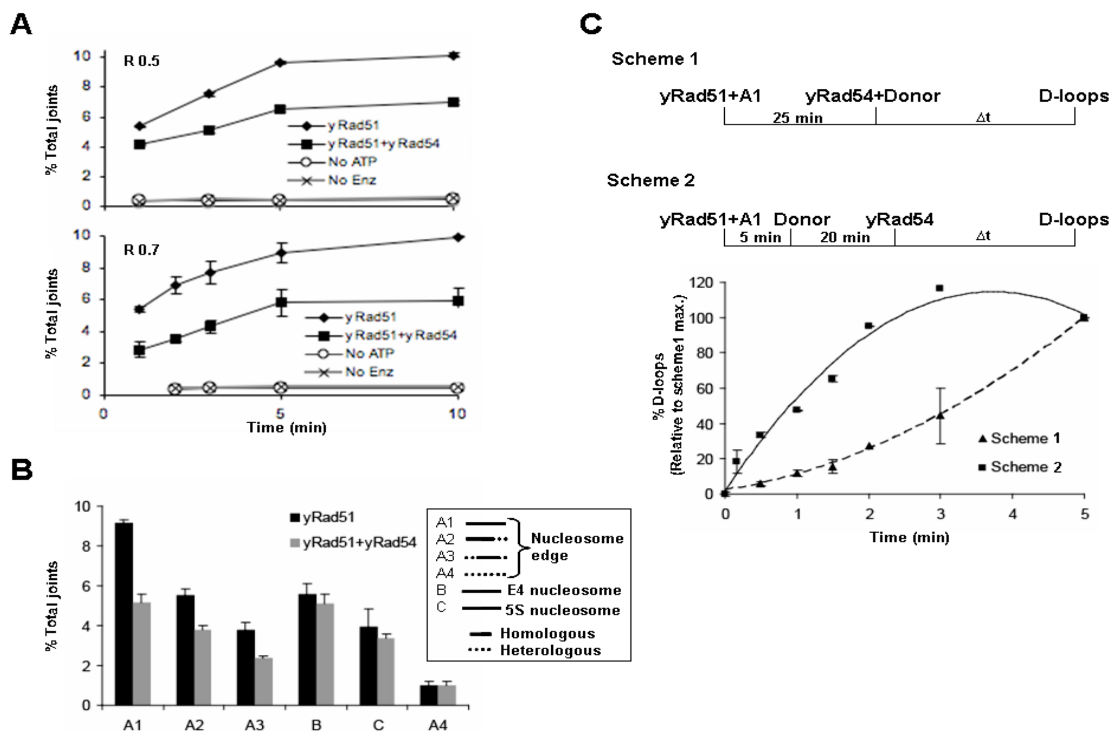


Figure 14. Rad54 does not enhance formation of initial joint molecules. (A.) Kinetics of yRad51-mediated formation of initial joints on subsaturated (Top, R=0.5) or saturated (Bottom, R=0.7) nucleosomal donors in presence or absence of yRad54, as detected by the biotin-streptavidin capture assays using A1 oligonucleotide. Reactions contained ATP, except where indicated and hence lowered the efficiency of formation of joints. (B.) Bar graph represents biotin-streptavidin capture assays with different yRad51 presynaptic filaments on the R=0.7 nucleosomal donor. Grey bars denote reactions that also contained yRad54. Inset panel shows the different positions and varying amounts of homology of the presynaptic filaments. (C.) Top, schematic of reaction timecourses. Bottom, quantification of plectonemic joints (D-loops) assayed by agarose gel electrophoresis of the deproteinized reaction products and autoradiography. Amount of plectonemic joints detected after 5 min (scheme 1) was set to 100%. Reactions in A-C are carried out in presence of ATP as the nucleotide cofactor. Results are from at least three independent experiments; error bars indicate standard deviations.

Moreover, addition of the SWI/SNF chromatin remodeling enzyme did not stimulate formation of protein-dependent or independent joints on nucleosomal donors (data not showed). We considered the possibility that yRad54 might stimulate joint formation at lower levels of nucleosomal density, however yRad54 was also unable to stimulate joint formation on donors that are subsaturated with nucleosomes (R=0.35 and R=0.5; Figures 14A and 15).

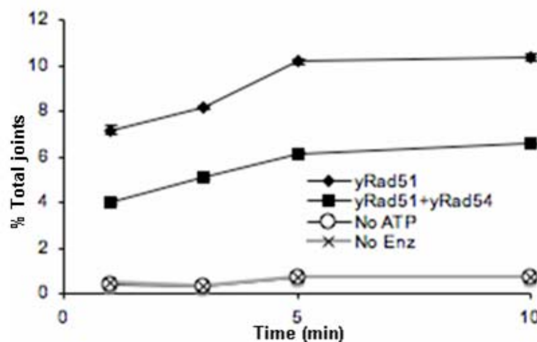


Figure 15. Kinetics of yRad51 mediated formation of initial joints on subsaturated (R=0.35) nucleosomal donors in presence or absence of yRad54, as detected by the biotin-streptavidin capture assays. Reactions contained ATP, except where indicated.

Because the presynaptic filaments that have homology to position A on the nucleosomal donor are predicted to have only the 3'end buried within a positioned nucleosome, we investigated whether the remodeling activity of yRad54 or SWI/SNF might stimulate the homology search when presynaptic filaments are targeted to donor sequences that are predicted to be fully encompassed by the nucleosome. However, like the case for presynaptic filaments directed at position A, neither SWI/SNF nor yRad54 facilitated formation of joints by yRad51-presynaptic filaments targeted at positions B or C which are on the surface of nucleosomes of the donor (Figure 14B and 20B).

These data suggest a linear sequence of events that lead to formation of a stable plectonemic joint (D-loop) on a nucleosomal donor: (1) Rad51 assembles onto ssDNA to form the presynaptic filament; (2) the presynaptic filament searches a chromatin donor

for a homologous DNA duplex and forms an initial, protein-dependent joint; and (3) the yRad54 ATPase converts this initial joint into a stable, protein-independent joint molecule. We envision that this latter step may require a chromatin remodeling role for yRad54. One prediction of this model is that the rate of formation of stable, plectonemic joints should be enhanced by prior formation of the initial, metastable joint. To test this hypothesis, we carried out two types of order of addition experiments (Figure 14C). In scheme 1, yRad54 was added to the yRad51 presynaptic filament prior to addition of nucleosomal donor. This reaction scheme is identical to our typical plectonemic joint assay (e.g. Figure 13B). In scheme 2, the yRad51 presynaptic filament was preincubated with the nucleosomal donor in the absence of yRad54 to allow formation of the initial, metastable joint molecule. yRad54 was subsequently added, and the rate of plectonemic joint formation was assayed. Strikingly, pre-formation of the metastable joint significantly enhanced the rate of stable joint formation following addition of yRad54 (Figure 14C). For instance, within 10 seconds of Rad54 addition, 20% of the maximum numbers of stable joints were formed. In contrast, nearly 2 minutes was required to form a similar amount of joints in the absence of the preincubation step. These data support a linear pathway in which the yRad51 presynaptic filament forms a metastable joint on the nucleosome surface that is subsequently converted to a stable plectonemic joint via the ATPase activity of yRad54.

Discussion

Our study reveals that presynaptic filaments assembled with either prokaryotic or eukaryotic recombinases are sufficient to locate homologous sequences and form joints on the surface of a nucleosome. In this purified system, an ATP-dependent chromatin remodeling enzyme is not required for the homology search process nor does a remodeling enzyme increase the efficiency of this initial step. In contrast, the Rad54p ATPase promotes a subsequent step (formation of a plectonemic joint), which facilitates later steps in homologous recombinational repair.

The mechanism by which presynaptic filaments carry out the homology search in the dense, nucleoprotein environment of the nucleus (or bacterial nucleoid) is largely unknown. Several fundamentally different models for the homology search process have emerged over the years. First, linear diffusion or sliding of RecA presynaptic filaments was proposed in which the presynaptic filament binds to a dsDNA nonspecifically and then linearly diffuses or slides along the dsDNA to locate homology (Gonda and Radding 1983). Later, this model was tested using simple functional assays and the results did not support the sliding model (Adzuma 1998). But recently, the sliding model has re-gained support by the observation that hRad51 can slide bi-directionally on the helical axis of duplex DNA (Graneli et al. 2006). A second model is based on studies with both RecA and hRad51 and proposes that the homology search involves random three-dimensional collisions (Adzuma 1998; Gupta et al. 1999; Folta-Stogniew et al. 2004).

Irrespective of a sliding or collision model, how is homology initially detected? Fluorescence resonance energy transfer analyses with hRad51 indicate that rapid exchange of A:T base pairs between the invading nucleoprotein filament and the duplex donor is key to allow the search for homology to proceed (Gupta et al. 1999). Likewise, kinetic studies using stopped-flow fluorescence and RecA have showed simultaneous switching of A:T bases and the formation of joints in which strand exchange has not been completed (Folta-Stogniew et al. 2004). These data suggest a model for the initial capture of homology in which DNA in the presynaptic filament aligns with the homologous duplex, and bases from the donor rotate out of their stacked conformation to allow the formation of transient pairing interactions. These initial interactions are promoted by the extended structure of ssDNA within the presynaptic filament (Klapstein et al. 2004) and stabilized by RecA or Rad51. In vitro kinetic studies with RecA or hRad51 have supported the view that these initial joints are bona fide intermediates in the pathway that leads to stable plectonemic joints (Riddles and Lehman 1985).

Our data suggest that wrapping DNA onto a histone octamer may not have an adverse effect on such transient base flipping, and that the presynaptic filament can sample homology on the nucleosomal surface. Indeed, previous studies have showed that recognition of homology by a RecA presynaptic filament requires less than one helical turn of DNA (Hsieh et al. 1992). Once extensive homology is detected and the initial joint is formed, it is not clear if the Rad51 filament disrupts nucleosome structure. It seems likely that extensive alignment of the presynaptic filament with nucleosomal DNA

will lead to DNA structures that would be incompatible with the wrapping of DNA around the octamer. Thus, the Rad51 filament may represent a novel type of chromatin remodeling enzyme. However, more extensive disruption of histone-DNA contacts (e.g. by Rad54) may be required to convert these transient interactions into stable joints in which DNA within the presynaptic filament has extensive Watson-Crick base pairs with the complementary strand of the nucleosomal donor. This sequence of events are consistent with recent studies that demonstrate homology-dependent chromatin remodeling by human Rad54 (Zhang et al. 2007).

For both RecA and Rad51, ATP hydrolysis leads to the turnover of presynaptic filaments (Cox 2003; Chi et al. 2006) . Indeed, we found that inclusion of ATP in the reactions led to less stable plectonemic joints (Figure 11) and greatly diminished levels of joints detected by the biotin-streptavidin capture assay (Figures 14A and B). In contrast, inclusion of a nonhydrolyzable analog of ATP, either ATPγS or AMP-PNP, stabilized both protein-dependent and protein-independent joints. In addition to the cycling of Rad51 due to ATP hydrolysis, we also found that the Rad54 ATPase led to a further decrease in the levels of joints detected by the biotin-streptavidin capture assay (Figures 14A, B). These results are consistent with the ability of Rad54 to displace Rad51 from DNA in an ATP-dependent reaction (Solinger et al. 2002). The initial, metastable joints that are detected in the capture assay may be particularly prone to the displacement activity of Rad54.

Our results are consistent with two previous studies in yeast that employed chromatin immunoprecipitation (ChIP) assays to monitor completion of the homology search process (Sugawara et al. 2003; Wolner and Peterson 2005). Both of these studies used a galactose inducible HO endonuclease to create a unique DSB at the *MAT* locus which could be repaired by HRR using a heterochromatic donor locus (HML). In a wild type yeast strain, the Rad51 recombinase could be detected by ChIP at the HML donor ~40 minutes after DSB formation, reflecting completion of a successful homology search (Wolner et al. 2003). Likewise, and consistent with our in vitro studies, recruitment of Rad51 to the donor locus was also observed in strains that lacked a functional Rad54 ATPase. In contrast, Laurent and colleagues have reported that Rad51 cannot be detected at the HML donor locus in the absence of the SWI/SNF remodeling enzyme, suggesting that this remodeling enzyme is essential for a successful homology search (Chai et al. 2005). Paradoxically, *swi/snf* mutant strains are not extremely sensitive to DSB-inducing agents, suggesting that SWI/SNF is not generally required for HRR. Likewise, our in vitro studies indicate that the chromatin remodeling activity of SWI/SNF does not generally enhance homology search on chromatin donors (Figure 20B, Chapter III). One interesting possibility is that the role of SWI/SNF in HRR is restricted to situations where the donor locus is embedded in highly condensed, heterochromatic chromatin structures, such as the SIR-dependent chromatin at HM loci. These condensed structures may restrict the intrinsic ability of the Rad51 presynaptic filament to capture homology, creating requirements for additional remodeling enzymes.

Experimental procedures

Reagent Preparation:

DNA: Oligonucleotides were obtained from Integrated DNA Technologies, Inc. (Coralville, IA) and were 5' end labeled with ^{32}P using γ-^{32}P -ATP and T4 polynucleotide kinase (New England Biolabs, Inc.; Beverly, MA). For oligonucleotide sequences, see APPENDIX. Plasmid CP943 (p2085S-G5E4) was prepared by alkaline lysis method but extreme caution was taken to minimize the incubation time in the alkaline buffer to maximize the yield of the supercoiled form and minimize the yield of nicked-circular form.

Proteins: Recombinant yRad51 was overexpressed in *E.coli* and purified as described earlier (Zaitseva et al. 1999). GST-yRad54 was expressed in yeast and purified as described earlier (Solinger et al. 2001). RecA and hRad51 were kind gifts from Dr. K.L. Knight (UMMS). Recombinant H2B, H4 and H2AS113C, H3C110A *Xenopus* histones were purified and biotinylated octamer was reconstituted as described earlier (Luger et al. 1999a).

Biotinylation of recombinant mutant octamers: Histone octamers were reconstituted with recombinant histones H3 C110A, H4, H2B, and H2A S113C as previously described (Luger et al., 1999). The cysteine substitution on H2A provides a unique site for biotinylation. To biotinylate octamers, 100 mg of octamer was incubated with 10 nmoles of PEO-iodoacetyl-biotin (Pierce, Rockford, IL) at room temperature for 30min

in the dark in a buffer containing 50mM Tris-HCl, pH 8.0; 5mM EDTA and 2M NaCl. The biotinylated octamers were separated from unincorporated biotin by repeated dilution and concentration in the reaction buffer using a 10 mwco Vivaspin 500 concentrator (Vivascience, Hannover, Germany).

Nucleosome assembly: To prepare the homologous donor, plasmid CP943 (p2085S-G5E4) was used (Ikeda et al. 1999). The supercoiled form of this plasmid is referred to as the naked DNA donor. The circular nucleosomal donor was assembled by gradient salt dialysis of supercoiled plasmid CP943 and the biotinylated recombinant octamer as previously described (Logie and Peterson 1999). Nucleosomes were reconstituted at different ratios of histone octamer per 200 bp of donor DNA (R value).

Analysis of nucleosomal occupancy of donor:

The nucleosomal donor was assembled using supercoiled plasmid CP943 and the biotinylated recombinant octamer. Quantification of the EcoRI analysis involved determination of the amount of mononucleosomal and free 5S repeats. Note that the mononucleosome signal was corrected for the fact that histones quench ethidium bromide staining by 2.5-fold (Carruthers et al., 1999). Based on our previous analytical ultracentrifuge analysis of linear 5S nucleosomal arrays, ~4% free 5S repeats correspond to a saturated nucleosomal array (Logie and Peterson, 1999; see also Carruthers et al., 1999).

In addition, nucleosomal donors were subjected to Micrococcal Nuclease (MNase) digestion followed by Southern hybridization to assess the nucleosomal occupancy specifically at the central E4 promoter sequence of the donor plasmid. To this end, 1μg DNA equivalent of each of the reconstituted donors (R=0.35, R=0.5 or R=0.7) was digested with various amounts of MNase (Worthington), serially diluted from 0.04 units to 0.00125 units. Reactions were incubated at 37°C in a reaction buffer containing 10mM Tris-HCl pH 8.0, 0.5mM $MgCl_2$, 0.3mM $CaCl_2$. After 3 minutes, reactions were stopped by addition of 2.5mM EDTA, 2.5mM EGTA, 1% SDS and 1mg/ml Proteinase K. Reactions were incubated further for 15 minutes and then extracted twice with phenol-chloroform. Purified products were then resolved by 1.2% agarose gel electrophoresis, followed by Southern Hybridization using 32P-labelled oligonucleotide probes. Each preparation of biotinylated nucleosomal donor was also tested for efficiency of streptavidin capture by assembling a mock joint capture reaction (without presynaptic filament) and testing retention of chromatin on streptavidin magnetic beads. In all cases, we observed >80% capture.

Joint capture assays:

To form the presynaptic filaments, 1mM of recombinase, RecA or Rad51 was incubated with radio-labeled oligonucleotides (3mM nucleotides) in a buffer containing 35mM Tris-HCl, pH 7.5, 2.5mM MgCl2, 30mM KCl, 1mM DTT at 30°C for 5 min in a 10ml reaction volume. As a nucleotide cofactor, 1mM AMP-PNP, 0.3mM ATP-ϒ-S, or 2mM

ATP with an ATP regeneration system consisting of 20mM creatine phosphate and 30 µg/ml creatine phospho-kinase was used as indicated. Then, 2µl of naked DNA or nucleosomal donor at a final concentration of 33nM DNA (plasmid molecules) was added to the reaction and allowed to form joint molecules. Thus, the presynaptic filament and duplex donor DNA are present at equimolar ratios. Reactions were supplemented with an additional 12.5mM $MgCl_2$ at the time of addition of the donor and then incubated at 30°C (for yRad51) or at 37°C (for RecA and hRad51) for the indicated time. In the reactions containing yRad54, 200nM yRad54 (~1 yRad54 per presynaptic filament) was added during formation of presynaptic filaments after incubating the oligonucleotides with yRad51 for 2min and then were incubated for an additional 3 min. One half of the reaction mixture was deproteinized by incubating with 2%SDS and 2mg/ml ProteinaseK for 5' at 37°C, electrophoresed on a 0.9% agarose gel, and plectonemic joints detected by autoradiography (Jaskelioff et al. 2003). % Plectonemic jojnts were calculated as the proportion of the homologous donor converted into radioactive joints.

To capture all joints (metastable and stable), half of the reaction mixture was added to 20 µl of streptavidin coated magnetic beads (Dynabeads M-280 Streptavidin, Dynal Biotech ASA, Oslo, Norway) that had been preblocked in reaction buffer containing 1 µg/ml BSA and 1mM sonicated salmon sperm DNA. After 5 minutes, joints were captured with a magnetic concentrator, washed three times with the reaction buffer and the unbound and bound fractions were counted in a scintillation counter. % Total joints were calculated as proportion of bead-bound radioactivity to total (unbound + bound) radioactivity added into each reaction.

Preface to Chapter III

The data presented in this chapter has been submitted in the form of a manuscript for peer review as:

Recombinational repair within Sir3- heterochromatin requires chromatin remodeling. Sinha, M., Johnson, A., Moazed, D. and Peterson, C.L.

Aaron Johnson, Ph.D. from Dr. Danesh Moazed's laboratory at Harvard Medical School provided purified ySir3 for this study.

CHAPTER III

RECOMBINATIONAL REPAIR WITHIN SIR3-HETEROCHROMATIN REQUIRES CHROMATIN REMODELING

CHAPTER III

Recombinational repair within Sir3- heterochromatin requires chromatin remodeling

SUMMARY

Heterochromatin plays a key role in protection of chromosome integrity by suppressing homologous recombination. In *Saccharomyces cerevisiae*, Sir3 is a structural component of heterochromatic structures found at telomeres and the silent mating type loci. Here we have investigated whether incorporation of Sir3 into minichromosomes regulates early steps of recombinational repair in vitro. We find that Sir3 eliminates yRad51-catalyzed formation of joint molecules, and that this repression requires histone residues that are known to be critical for silencing in vivo. Moreover, we demonstrate that the SWI/SNF chromatin remodeling enzyme facilitates joint molecule formation by displacing Sir3, thereby promoting subsequent Rad54-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.

Introduction

The eukaryotic genome is organized into two structurally distinct chromatin states, heterochromatin and euchromatin. Early cytological studies defined heterochromatin as the regions of the genome that remain visibly condensed and deeply stained throughout the cell cycle, whereas euchromatin undergoes decondensation as the cells progressed from metaphase to interphase (Passarge 1979). The regions that remain condensed throughout the cell cycle 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, suppressed mitotic and meiotic recombination, 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 (Henikoff 2000; Richards and Elgin 2002; Grewal and Jia 2007). Heterochromatin plays several key roles in chromosome maintenance and segregation. Heterochromatin is required for the organization and function of centromeres (White and Allshire 2008), protects telomeres, and limits telomere length in Drosophila (Savitsky et al. 2002). Consequently, heterochromatin assembly is essential for proper chromosome segregation. Heterochromatin also 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; Barton et al. 2008; Bisht et al. 2008; Peng and Karpen 2008). Heterochromatic regions also show suppressed crossing-over frequencies during meiosis (Klar and Miglio 1986; Nakaseko et al. 1986; Thon and Klar 1993; Allshire et al. 1994; Westphal and Reuter 2002; Barton et al. 2008), and repressed mitotic recombination (Jaco et al. 2008). It has been proposed that the general suppression of recombination within heterochromatic regions may represent a mechanism to focus the recombination machinery on gene-rich regions (Holmquist and Ashley 2006). However, recombination does occur at measurable frequencies within heterochromatin (Paques and Haber 1999; Luo et al. 2002; Jaco et al. 2008), and in the case of the yeast transposon Ty5, transposition events into heterochromatin are actually preferred (Ebina and Levin 2007).

In the budding yeast *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, Sir2, Sir3, and Sir4, which play a key role in establishing a domain of transcriptional silencing. The assembly of Sir-dependent heterochromatin is a stepwise process in which DNA binding proteins like Rap1 or the ORC complex recruits Sir4, which is required for subsequent recruitment of Sir2 and Sir3 (Hoppe et al. 2002; Luo et al. 2002; Rusche et al. 2002). All three Sir proteins are then required for spreading of the heterochromatic domain. Sir2 is a NAD^+-dependent histone deacetylase whose HDAC activity is required for heterochromatin assembly (Imai et al.

2000; Landry et al. 2000; Smith et al. 2000). Sir2 removes the acetyl group from histone H4 K16, thereby promoting the binding of Sir3 and Sir4 to the hypoacetylated histone H4 N-terminal domain. Multiple cycles of histone deacetylation and Sir3/Sir4 binding are thought to control the spreading of the heterochromatin domain from the initial point of recruitment (Rudner et al. 2005).

Several studies indicate that Sir3 is the dominant structural component of Sir-dependent heterochromatin and that it can function, at least in part, independently of Sir2 and Sir4. *In vitro*, Sir3 can bind to DNA (Georgel et al. 2001; McBryant et al. 2008) and to nucleosomes, and nucleosome binding requires H4K16 (Fry et al. 2006; Onishi et al. 2007). Binding of Sir3 to nucleosomes also requires histone H3 K79, as well as several other surrounding histone residues (Fry et al. 2006; Onishi et al. 2007). Methylation of H3 K79 (Ng et al. 2002) or acetylation of H4 K16 (Liou et al. 2005) also disrupts the binding of Sir3 to chromatin. These and other studies have defined a putative nucleosome binding surface for Sir3 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 Sir3 extends the domain of transcriptional silencing (Renauld et al. 1993; Hecht et al. 1996; Grunstein 1997). Within the extended domain, Sir3 appears to spread in the absence of Sir2 or Sir4. Furthermore, inactivation of the Sas2 histone acetylase leads to spreading of Sir3 at telomeres in the absence of Sir2 (Kimura et al. 2002; 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 (*HMLα* and *HMRa*) on each end of the same chromosome, with *MATa* and *MATα* strains preferentially using information from *HML* and *HMR*, respectively (Haber 1998b). Previous studies have showed that capture of the homologous donor and formation of the initial joint between the DSB at *MAT* and the heterochromatic *HMLα* donor requires two different ATP-dependent chromatin remodeling enzymes, Rad54 and SWI/SNF (Chai et al. 2005). In contrast, several other ATP-dependent chromatin remodeling enzymes, RSC, INO80.com and SWR1.com, are not required for completion of this early step (Chai et al., 2005; Papamichos-Chronakis et al., 2006). Whereas Rad54 is known to be a general feature of HR, SWI/SNF typically plays roles in transcription and had not previously been linked to DSB repair (Osley et al. 2007). Recently, we hypothesized that SWI/SNF might only be required for HR when the homologous donor was assembled into Sir-dependent heterochromatin (Sinha and Peterson 2008).

In this study, we wished to study the mechanism for how heterochromatin-like structures suppress homologous recombination and to test the hypothesis that the ATP-dependent chromatin remodeling enzyme, SWI/SNF, can counteract such repression. To this goal, Sir3p-nucleosome complexes were reconstituted with purified components, and they were used as substrates in an assay that monitors the early steps of HR. We find that Sir3-nucleosome complexes are sufficient to block formation of the initial, Rad51-mediated

joint molecule, and that this repression requires the histone H4 N-terminal domain, as well as the key histone residues, H4 K16 and H3 K79. Furthermore, we demonstrate that the chromatin remodeling activity of SWI/SNF can displace Sir3, facilitating formation of a DNA joint and a strand invasion product. Hence, our findings recapitulate the repression of recombination mediated by heterochromatin in vivo, and they provide insight into how chromatin remodeling enzymes can play essential roles during recombinational repair when the homologous target is buried in heterochromatin.

Results

Repair of a DSB by homologous recombination (HR) requires that the Rad51 recombinase assemble onto ssDNA that flanks a DSB to form a presynaptic filament that 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 template that contains two head-to-tail arrays of five 5S rDNA nucleosome positioning sequences that flank a di-nucleosome length E4 promoter (Top panel, Figure 16A; see also Sinha and Peterson, 2008). The Rad51-presynaptic filament is then assembled with recombinant yRad51p and a radioactively end-labeled, 90-nucleotide long oligonucleotide that is homologous to DNA sequences encompassed within one of the E4 promoter nucleosomes (arrow, top panel, Figure 16A). A typical joint formation assay involves incubation of the presynaptic filament with the nucleosomal donor, and joint molecules are captured with streptavidin magnetic beads and quantified by scintillation counting. Using this assay, joint molecules are formed on 20-30% of the nucleosomal donors in the absence of chromatin remodeling enzymes (Sinha and Peterson, 2008; see also Figure 16C).

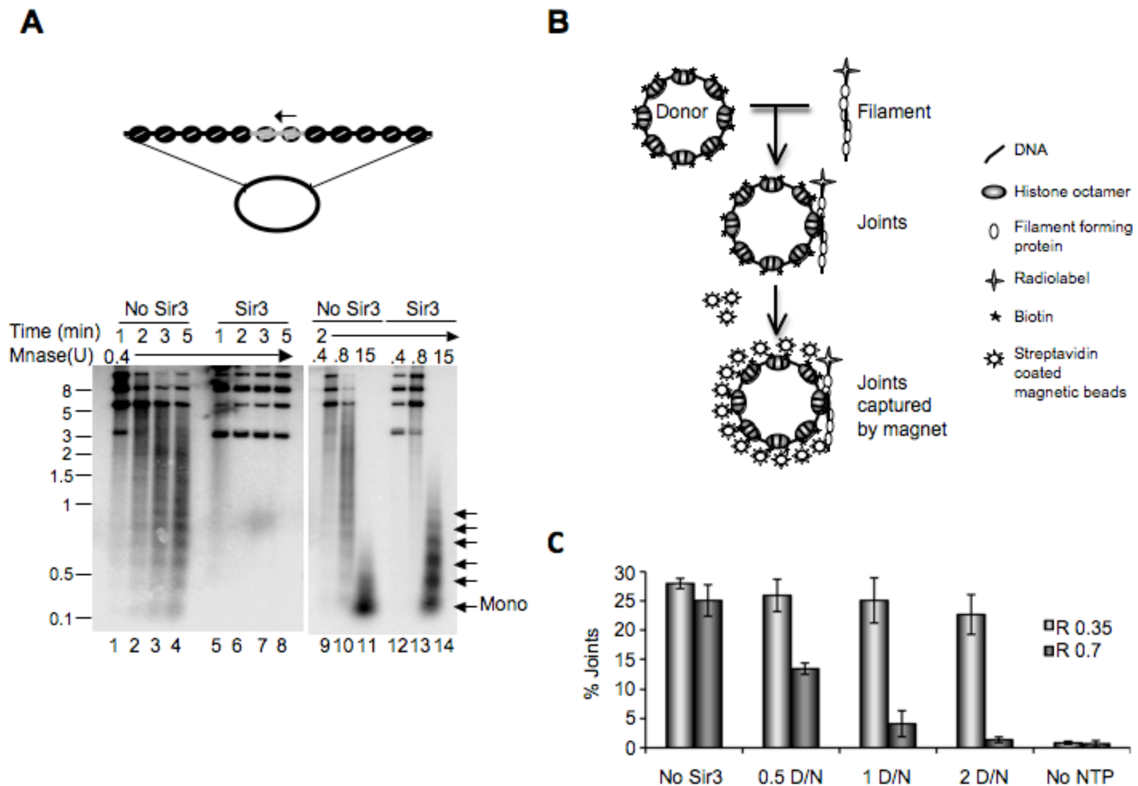
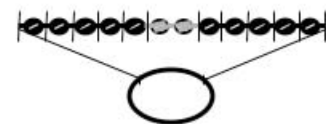


Figure 16. Sir3-nucleosomes pose a barrier to nucleosomes accessibility and Rad51-catalyzed formation of joints: (A) Top panel: Schematic of the nucleosomal minichromosomes donor. Black ovals: nucleosomes on 5S rDNA positioning elements; grey ovals, nucleosomes on E4 sequence; black arrow, 87-nucleotide long probe. Bottom panel: Minichromosomes, reconstituted at a ratio of 0.7 octamers per 200bp of DNA were analyzed in absence (lanes 1-4, 9-11) or presence (lanes 5-8, 12-14) of Sir3 by MNase digestion. The digested products were deproteinized, run on 1.2% agarose gel and Southern blotted with E4 oligonucleotide probe. Units of MNase enzyme and time of incubation at 23°C are indicated on top of the gels. DNA size markers in kilobases are drawn on left. Lanes are indicated at bottom. Black arrows on the right point to banding pattern of MNase sensitive sites. (B) Schematic of the strategy of biotin-streptavidin joint capture assay. (C) Results of biotin-streptavidin joint capture assays, targeting yRad51 filament to the E4 nucleosome for joint formation on Sir3-minichromosomes that are either subsaturated (R 0.35) or saturated (R 0.7). Sir3 bound both minichromosomes, unless indicated otherwise at a molar ratio of two dimers of Sir3 per nucleosome (for R0.7) or 200b of DNA (for R0.35). AMP-PNP was used as the NTP cofactor. % Total joints were calculated as the proportion of bead-bound to total radioactivity in each reaction. Results are from at least three independent experiments; error bars indicate standard deviations. D/N denotes dimers per nucleosome.

Sir3 inhibits early steps of HR

To study the impact of heterochromatin on the early steps of HR, FLAG-tagged Sir3 was purified from yeast and bound to nucleosomal donors. Previous studies have indicated that Sir3 is a dimer in solution (Liou et al. 2005; McBryant et al. 2008), thus we added Sir3 at ratios of 0.5, 1.0, or 2.0 dimers per nucleosome (D/N), yielding nucleosomal donors with different levels of bound Sir3. These nucleosomal donors were first analyzed by *EcoR*I analysis to ensure that all donors had comparable nucleosome densities. *EcoR*I restriction sites flank each 5S rDNA repeat, and therefore extensive *EcoR*I 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, *EcoR*I digestion released mononucleosomes and very little free, 5S DNA repeats (Figure 17). These results are indicative of a 5S nucleosomal array in which >90% of the 5S rDNA repeats are occupied by nucleosomes.



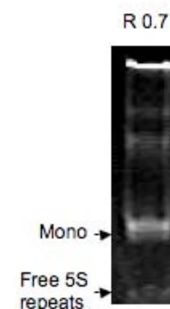


Figure 17. Schematic and analysis of minichromosome donor. Top: Vertical lines denote *EcoR*I restriction sites that flank each 5S rDNA repeats. Bottom: *EcoR*I analysis of minichromosome. Ethidium bromide stained 4% native polyacrylamide gel shows the nucleosomal occupancy of the minichromosomes, reconstituted at a ratio of 0.7 octamers per 200 bases of DNA (R 0.7).

One hallmark of heterochromatin is a diminished accessibility to nucleases and restriction enzymes. Indeed, a previous study demonstrated that Sir3-containing heterochromatin is less accessible to

nuclease digestion within isolated nuclei (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 16A). When this analysis was performed on minichromosomes that lacked Sir3, the E4 oligonucleotide hybridized to mononucleosomal DNA and to an extensive nucleosomal ladder (Figure 16B, lane1-3), confirming that this E4 promoter sequence is fully encompassed by nucleosomes, as showed previously (Sinha and Peterson, 2008). Strikingly, minichromosomes assembled with two Sir3 dimers per nucleosome were more resistant to MNase digestion, requiring >10-fold higher concentrations of MNase before significant digestion products were released (Figure 16B, compare lanes 2 and 5). However, at these higher MNase concentrations, the E4 oligonucleotide also detected an extensive nucleosomal ladder (Figure 16B, lane 6). Interestingly, the nucleosomal ladder was more distinct in the presence of Sir3, suggesting that the binding of Sir3 may lead to more homogeneous positioning of the E4 promoter and/or 5S nucleosomes. Thus, the binding of Sir3 to nucleosomal donors is sufficient to create a chromatin structure that is less accessible to MNase digestion.

Biotin-streptavidin capture assays were performed with yRad51 presynaptic filaments and nucleosomal donors that contain different amounts of Sir3. Markedly, Sir3 inhibited yRad51p-mediated joint formation in a concentration dependent manner (Figure 16D). At

a ratio of 0.5 Sir3 dimers per nucleosome, joint formation was reduced 2-fold, but addition of Sir3 at a ratio of 2 dimers per nucleosome decreased joint formation by at least 20-fold (Figure 16D). Importantly, incorporation of Sir3 into the minichromosomes had no influence on the capture efficiency of the minichromosomes by the streptavidin beads. Since Sir3 has been showed to bind cooperatively and nonspecifically to DNA (McBryant et al. 2008; Adkins et al. 2009), we investigated whether Sir3 would also inhibit joint formation in assays where the joint molecule 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 subsaturated nucleosomal donor in which the E4 promoter is nucleosome-free (Sinha and Peterson 2008). In this case, the presence or absence of Sir3 yielded a similar level of joint molecule formation, even at a ratio of 2 dimers of Sir3 per 200 bp of DNA (Figure. 16D). Importantly, the binding of Sir3 to saturated and subsaturated nucleosomal donors was equivalent (Figure 18). Thus, Sir3-dependent inhibition of joint formation requires that the homologous donor sequences be encompassed by nucleosomes.

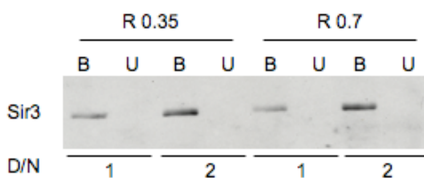


Figure 18. Sir3 binds subsaturated and saturated minichromosomes with similar efficiency. Representative Immunoblot of Sir3 probed with anti-FLAG antibody is showed. Reactions contained biotinylated subsaturated (R 0.35) or saturated (R0.7) minichromosomes assembled at ratios 0.35 octamers or 0.7 octamers per 200 bases of DNA respectively. Reactions contained biotinylated minichromosomes (17nM) and Sir3 at molar ratios of 0.5 or 2 dimers per nucleosome, indicated as D/N. Streptavidin conjugated magnetic bead-bound fractions are denoted as B and supernatant fractions are denoted as U.

Sir3-dependent inhibition requires key histone residues, the H4 N-terminal domain, and the Sir3 BAH domain

In vivo studies have demonstrated that assembly and function of Sir3-containing heterochromatin is disrupted when the N-terminal domain of histone H4 is truncated or if H4 K16 is substituted or acetylated (Johnson et al. 1990; Hecht et al. 1995; Hecht et al. 1996; Carmen et al. 2002; Liou et al. 2005). Similarly, substitution or methylation of lysine 79 within the globular domain of histone H3 disrupts Sir3-dependent heterochromatin in vivo (Ng et al. 2002). To investigate whether Sir3-dependent inhibition of joint formation might reflect assembly of heterochromatic-like structures, nucleosomes were reconstituted with histone octamers that either lacked the H4 N-terminal domain (H4ΔN), or contained H4K16Q or H3K79E substitution derivatives. First, the ability of Sir3 to bind to mononucleosomes that harbor each of these derivatives was tested by gel shift assay. As showed in Figure 19A, each of these histone alterations significantly decreased the binding affinity of Sir3 for these mononucleosome substrates (Figure 19A). Whereas 1 dimer of Sir3 per nucleosome showed robust binding to the wild type nucleosomal substrate, at least 2- to 4-fold more Sir3 was required to observe comparable binding to each of the other substrates.

H4ΔN, H416Q, and H3K79E histone octamers were next assembled into nucleosomal donors that were used in biotin-streptavidin capture assays. In each case, Sir3-dependent inhibition of joint formation was strongly alleviated (Figure 19B). Specifically, donors

assembled with nucleosomes that lacked the H4 N-terminal domain or contained H4K16Q were remarkably resistant to Sir3-dependent inhibition compared to wild type nucleosomal donors, whereas donors assembled with H3 K79E were able to respond weakly to Sir3-dependent repression (Figure 19B). Interestingly, the binding of Sir3 to minichromosomes reconstituted with octamers carrying either wild type (WT) or modified histone H4 (H4 K16Q) was equivalent (Figure 19D). Thus, Sir3-dependent inhibition of joint formation not only requires nucleosomes, but inhibition also requires key histone residues that are known to regulate heterochromatin assembly and function *in vivo.*

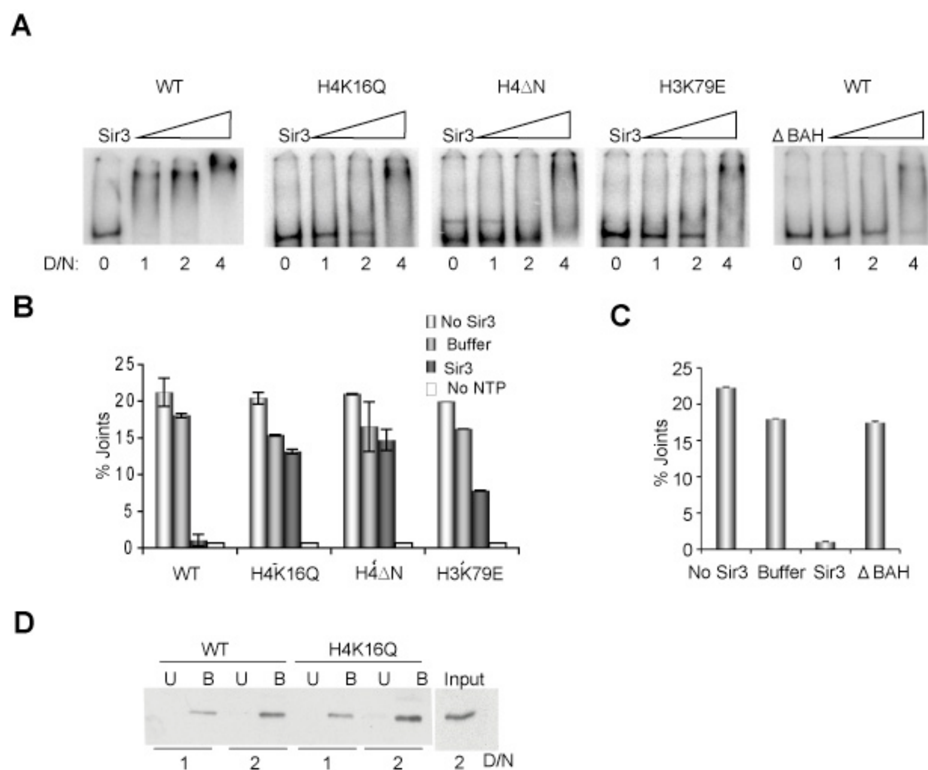


Figure 19. Histone residues and BAH-domain of Sir3 regulate Sir3-dependent inhibition of yRad51-mediated formation of joints: (A) Gel-shift assays show the difference in electrophoretic mobility of mononucleosomes reconstituted on “601” template, due to binding of increasing amounts of Sir3 or Sir3 lacking the BAH domain (ΔBAH) as indicated on top of the gels. D/N: dimers of Sir3 or Sir3pΔBAH per nucleosome. Mononucleosomes were assembled using octamers that carried either wild type (WT) or

truncated histone H4 (H4ΔN) or site-specifically modified histones H4 (H4K16Q) or H3 (H3K79E). (B-C) Results of biotin-streptavidin joint capture assays, targeting yRad51p filament to the E4 nucleosome for joint formation on saturated (R 0.7) minichromosomes. In all reactions, AMP-PNP was used as the NTP cofactor. % Total joints were calculated as the proportion of bead-bound to total radioactivity in each reaction. Results are from at least three independent experiments; error bars indicate standard deviations. (B) Minichromosome donors contained either wild type (WT) or truncated histone H4 (H4ΔN) or site-specifically modified histones H4 (H4K16Q) or H3 (H3K79E) as indicated at the bottom. In reactions having Sir3, Sir3 was present at a molar ratio of two dimers of Sir3 per nucleosome of minichromosomes. (C) Wild type minichromosomes were targeted for formation of joints in absence or presence of Sir3 or truncated Sir3 (ΔBAH), which was present at a molar ratio of two dimers per nucleosome of minichromosomes. (D) Representative immunoblot analysis of Sir3, probed with anti-FLAG antibody. Reactions contained biotinylated minichromosomes, reconstituted with octamers carrying either wild type (WT) or site-specifically modified histones H4 (H4K16Q). Streptavidin conjugated magnetic bead-bound fractions are denoted as B and supernatant fractions are denoted as U.

Previous studies have shown that the conserved bromo-adjacent homology (BAH) domain within Sir3 is essential for heterochromatin function *in vivo* and that this domain binds to nucleosomes *in vitro* (Onishi et al. 2007). Furthermore, the Sir3 BAH domain interacts with the N-terminus of histone H4 and binding is regulated by acetylation or methylation of H4 K16 and H3 K79, respectively (Onishi et al. 2007). As expected, a derivative of Sir3 that lacks the BAH domain (Sir3ΔBAH) does not bind well to nucleosomes (Figure 19A, right panel; see also (Onishi et al. 2007). To determine if the Sir3- BAH domain plays an essential role in the inhibition of joint formation, we added Sir3ΔBAH to nucleosomal donors and performed biotin-streptavidin capture assays. As shown in Figure 19C, the BAH domain is essential for Sir3-dependent inhibition of Rad51-mediated joints; addition of 2 dimers of Sir3ΔBAH per nucleosome had no affect on the ability of Rad51 presynaptic filaments to capture the homologous duplex (Figure 19C). Taken together, these results indicate that the Sir3-dependent inhibition of Rad51-catalyzed joints has many of the hallmarks of functional Sir3-containing heterochromatin.

ATP-dependent chromatin remodeling disrupts Sir3-containing heterochromatin

Mating type switching in *Saccharomyces cerevisiae* requires the recombinational repair of a DSB with a Sir-containing, heterochromatic HM donor loci (*HMLα* and *HMRa*). Previous studies have shown that formation of the initial joint between the DSB at *MAT* and the *HMLα* donor requires two different ATP-dependent chromatin remodeling enzymes, Rad54 and SWI/SNF (Chai et al. 2005). As an initial test for whether SWI/SNF might only be required for HR 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 $MAT\mathbf{a}_{inc}$ 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 (Moore and Haber 1996). This strain also lacks both *HMLα* and ***HMRa***, and thus the DSB is repaired using the euchromatic $MAT\mathbf{a}_{inc}$ donor. Both DSB induction and the formation and extension of the initial strand invasion product at the $MAT\mathbf{a}_{inc}$ locus are monitored by PCR (See Figure 20A schematic). In a *SWI2*+ strain, DSB induction occurred within 1.5 hours, and appearance of the initial strand invasion/extension product at the $MAT\mathbf{a}_{\mathrm{inc}}$ donor occurred by 5 hours (Figure 20A, left panel). Inactivation of SWI/SNF led to slower kinetics of DSB induction (7-8 hours; Figure 20A, 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% wild type levels by 12

hours (Figure 20A, right panel). These data confirm that SWI/SNF is not generally required for recombinational repair, and furthermore that SWI/SNF may only be required for early steps of HR when the homologous donor is assembled into Sir3-containing heterochromatin.

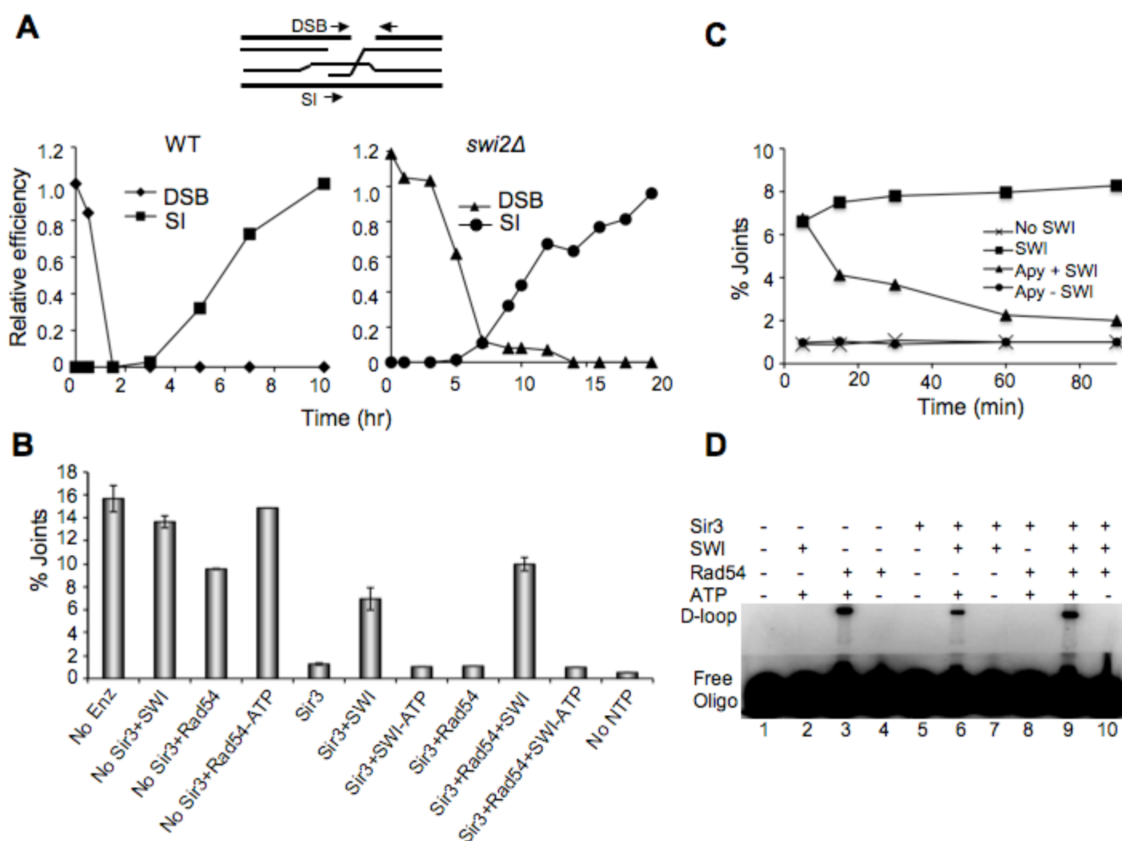


Figure 20. SWI/SNF antagonizes Sir3-dependent inhibition of HR. (A) Top Panel: Schematic of a DNA intermediate of HR pathway. Black arrows indicate primers used for monitoring double strand break (DSB) and strand invasion/extension (SI) product formation. Bottom panels: Graphs show kinetics of efficiency of DSB formation at *MAT* and SI product formation in wild type (WT) or *SWI2*-deleted (*swi2*Δ) strains, which contain a $MATa_{inc}$ donor locus at an ectopic site on chromosome V and lack both *HMLα* and *HMRa* donor loci on chromosome III. Efficiency of DSB formation and strand invasion was arbitrarily set to 100% for the highest WT DSB product i.e. at 0 timepoint. All values were normalized to a *Pho5* internal control. (B) Graph shows results of biotin-streptavidin joint capture assays, targeting yRad51 filament to the E4 nucleosome for joint formation on saturated (R 0.7) minichromosomes in absence or presence of Sir3 at a molar ratio of two dimers per nucleosome. In all reactions, yRad51-presynaptic filaments were formed in presence of 0.3mM AMP-PNP and subsequently supplemented with 2mM ATP during joint formation unless otherwise indicated. Indicated reactions were also supplemented with 30nM SWI/SNF or 200nM Rad54 as described in Methods. (C) Graph shows results of biotin-streptavidin joint capture assays, on saturated (R 0.7) minichromosomes in presence of Sir3 at a molar ratio of 2 dimers per nucleosome.

yRad51-presynaptic filament was assembled in presence of 0.3mM AMP-PNP and allowed to form joints on Sir3-minichromosomes pre-incubated with 30nM SWI/SNF and 2mM ATP, unless indicated otherwise. After 5 min of incubation, half of the reaction received 1U of Apyrase (Apy) and kinetics of joint formation was assayed for 90 min. (D) Representative autoradiograph shows yRad51-catalyzed D-loop formation on minichromosomes in presence or absence of Sir3 at a molar ratio of 2 dimers per nucleosome. Reactions contained 2mM ATP as nucleotide cofactor unless indicated and either 30nM SWI/SNF and/or 200nM Rad54 as indicated.

To directly test whether SWI/SNF might antagonize Sir3-dependent inhibition of HR, we employed biotin-streptavidin capture assays (Figure 20B). As we shown previously, SWI/SNF remodeling activity does not enhance joint formation with nucleosomal donors (Figure 20B). However, addition of a low concentration of SWI/SNF (1 SWI/SNF per 15 nucleosomes) restored joint formation on the Sir3-nucleosomal donors to 50% of the level observed for the nucleosomal donor (Figure 20B). 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 20B). Interestingly, Rad54 was not able to substitute for SWI/SNF, but addition of both SWI/SNF and Rad54 further stimulated joint formation (Figure 20B). Thus, these data indicate that SWI/SNF-dependent chromatin remodeling is essential for early steps of HR with a heterochromatic donor, and that the ATPase activity of Rad54 can augment SWI/SNF function.

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 Sir3-nucleosomal donor was reversible in vitro (Figure 20C). Joint formation assays were assembled that contained yRad51 presynaptic filament, Sir3-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 remodeling activity (Figure 21).

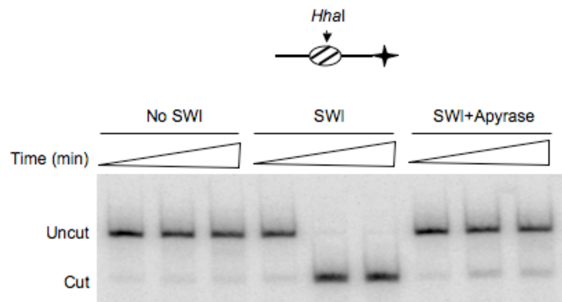


Figure 21. Apyrase abolishes catalytic activity of SWI/SNF remodeling complex. Top: Schematic representation of "601"-mononucleosome radio-labeled at the 5'-end. Oval denotes nucleosomes, black line denotes DNA, star denotes radio-label, and black arrow denotes *Hha*I restriction site. Bottom: Restriction enzyme accessibility assay monitors remodeling activity of SWI/SNF complex for a time period of 15 min. Reactions contain 2nM 601-mononucleosomes and 2nM SWI/SNF or buffer and 2mM ATP. A subset of reactions contains 1U of Apyrase as indicated.

Reactions were further incubated and aliquots removed and joints captured on magnetic beads at time intervals. As shown in Figure 20C, inactivation of SWI/SNF led to the time-dependent decrease in joint formation, reaching near background levels by 60 minutes of apyrase treatment. These data indicate that once SWI/SNF is inactivated, Sir3 can re-establish nucleosomal structures that block joint formation.

The biotin-streptavidin capture assay measures formation of the initial, unstable DNA joint as well as the stable plectonemic joint (Riddles and Lehman 1985) in which the joint molecule displaces the non-complementary strand and forms what is known as a D-loop. Whereas the initial joint is stabilized by protein-DNA interactions, the D-loop is stable even in the absence of protein (e.g. yRad51). To monitor formation of stable D-loop products, reactions were de-proteinized and DNA products separated on polyacrylamide gels. As we showed previously, Rad54 is essential for formation of stable D-loops on nucleosomal donors, whereas SWI/SNF could not substitute for Rad54 nor does it stimulate Rad54 activity (Figure 20D, lanes 2 and 3; see also (Jaskelioff et al. 2003). In

contrast to nucleosomal donors, Rad54 did not promote formation of a D-loop product on the Sir3-nucleosomal donor (Figure 20D, lane 8), as expected from the biotin-streptavidin capture assays (Figure 20B). Strikingly, addition of SWI/SNF and ATP led to detectable levels of D-loop product (Figure 20D, lane 6), and further addition of Rad54 led to levels of D-loops that paralleled the amount of joint molecules formed in the absence of Sir3 (Figure 20D, lane 9). Since SWI/SNF does not promote D-loops on a nucleosomal donor that lacks Sir3, these data indicate that assembly of a Sir3-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 Sir3

To investigate how SWI/SNF antagonizes the repressive effects of Sir3 on HR, we tested whether SWI/SNF can remodel Sir3-nucleosomal substrates. One hallmark of ATP-dependent chromatin remodeling enzymes is their ability to mobilize ("slide") nucleosomes in cis (Smith and Peterson 2005). This ATP-dependent mobilization reaction can be monitored by native gel electrophoresis of mononucleosome substrates, as the position of a nucleosome on a DNA fragment determines the extent of migration. Centrally-positioned mononucleosomes were assembled on a 343 bp DNA fragment that contains a '601' nucleosome positioning element. Mononucleosomes or Sir3-mononucleosome complexes (see Figure 19A) 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

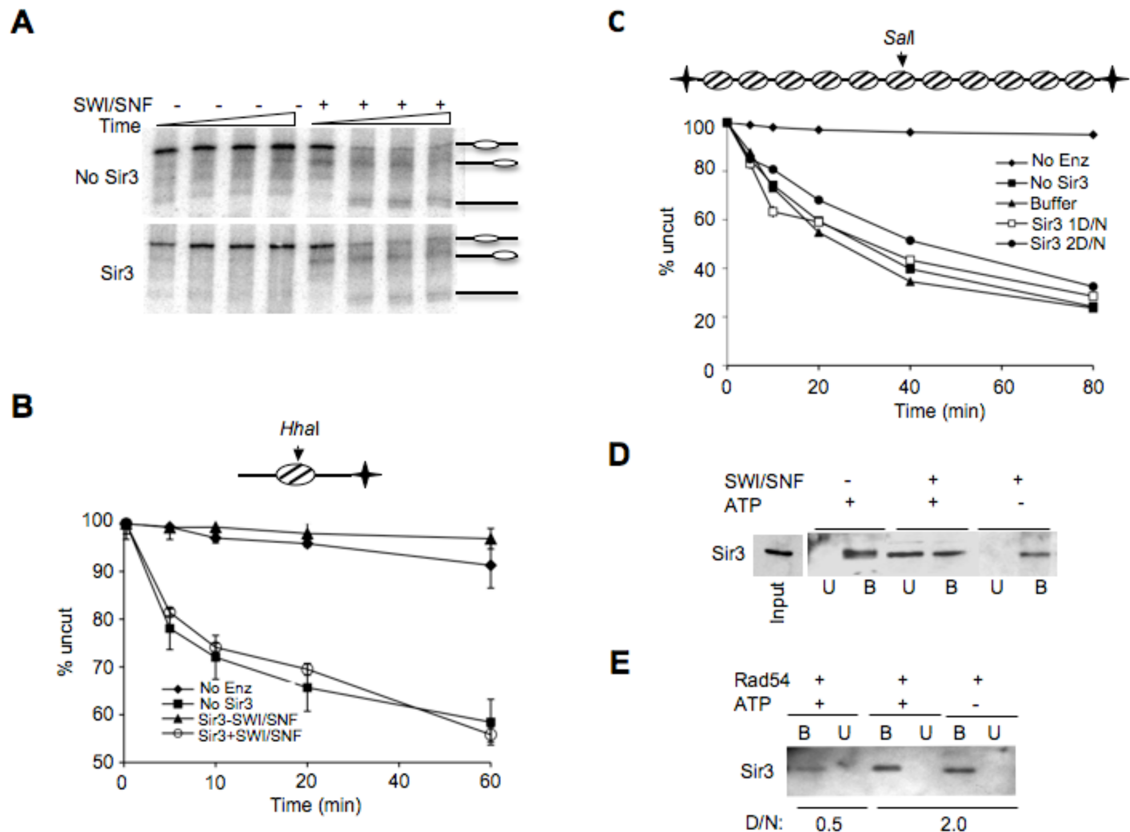


Figure 22. SWI/SNF remodels Sir3p-heterochromatin by evicting Sir3. (A) Representative native PAGE analysis of a 343-bp mononucleosome (2nM) with (Bottom) or without (Top) two dimers of Sir3, incubated with 1nM SWI/SNF or buffer as indicated. Schematics of mononucleosome species migrating at different rate as a result of SWI/SNF catalyzed nucleosome "sliding" are drawn on the right of the gels. (B) Graphical representation of a unique restriction enzyme (*Hha*I) accessibility assay on the 601-mononucleosome (2nM) in absence or presence of two dimers of Sir3 and 1nM SWI/SNF as indicated. Schematic of the assay is shown on top; oval denotes nucleosomes, black line denotes DNA, black arrow denotes *Hha*I restriction site and star denotes 5'-end radio-label (C) Graphical representation of a unique restriction enzyme (*Sal*I) accessibility assay on 2nM 208-11 nucleosomal array. Schematic of the assay is shown on top; ovals represent nucleosomes, black line represents DNA, black arrow denotes *Sal*I restriction site and stars represent 5'-end radio-label. Reactions contained 2nM SWI/SNF and Sir3 at a molar ratio of 1 or 2 dimers per nucleosome of the array, unless indicated otherwise. Results in B, C are summarized from at least three independent experiments, error bars represent standard deviations. (D, E) Representative Immunoblot analysis of Sir3, probed with anti-FLAG antibody. Reactions contained biotinylated minichromosomes (17nM), 40nM SWI/SNF complex (D) or 200nM yRad54 (E) and 2 mM ATP where indicated. Streptavidin conjugated magnetic bead-bound fractions are denoted as B and supernatant fractions are denoted as U. Molar ratio of Sir3 dimers per nucleosome is indicated as D/N in (E).

loading to remove SWI/SNF from the substrate; DNA addition also removes Sir3. As shown in Figure 22A, SWI/SNF action mobilizes the nucleosomal substrate, leading to formation of faster migrating species and the appearance of a free DNA product. Notably, addition of 2 dimers of Sir3p 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 the kinetics of *Hha*I digestion of the 601 mononucleosome substrate (Figure 22B) and the kinetics of *Sal*I digestion of a nucleosomal array substrate (Figure 22C). 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 22B, C). However, SWI/SNF-dependent remodeling leads to greatly enhanced digestion rates for both mononucleosomal and array substrates (Figure 22B, C). Furthermore, incorporation of 1-2 dimers of Sir3 per nucleosome had very little impact on the apparent rates of SWI/SNF remodeling activity (Figure 22B, C). Taken together, these data indicate that SWI/SNF is quite effective at remodeling Sir3-nucleosome substrates.

Previous studies have suggested that Sir3, in concert with Sir2 and Sir4, may form extended filaments on nucleosomal substrates (Onishi et al. 2007), and thus it seemed unlikely that SWI/SNF could remodel Sir3-nucleosomes by simply mobilizing individual Sir3-nucleosome complexes. Thus, we tested whether SWI/SNF action might lead to the displacement of Sir3 from the minichromosome donors. The Sir3-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 Sir3. As shown in Figure 22D, Sir3 is exclusively found in the bound (B) fraction in the absence of SWI/SNF, but addition of SWI/SNF and ATP catalyzed the eviction of at least 50% of the Sir3 into the supernatant (U) fraction (Figure 21D). The Sir3 displacement activity of SWI/SNF is robust, as SWI/SNF is added to reactions at only ~1 SWI/SNF per 15 nucleosomes. Interestingly, the ATPase activity of Rad54 cannot evict Sir3, even when Sir3 is present at a low ratio of Sir3 dimers per nucleosome (0.5 D/N; Figure 22E). Thus, SWI/SNF seems uniquely able to evict Sir3 from nucleosomal templates, an activity that enhances chromatin accessibility and facilitates joint formation.

Discussion

Here we have assembled Sir3-containing minichromosomes that share several of the functional properties of yeast heterochromatin. Sir3-nucleosomal arrays are refractory to digestion by micrococcal nuclease, and this chromatin structure inhibits early steps of homologous recombination. Furthermore, Sir3-dependent inhibition of HR requires the presence of nucleosomes, the histone H4 N-terminal domain, histone residues H4K16 and H3K79, and the Sir3 BAH domain, all factors that are known to be crucial for heterochromatin assembly and function *in vivo*. Moreover, assembly of Sir3-nucleosomal donors creates an absolute requirement for the chromatin remodeling activity of SWI/SNF in joint molecule formation. This novel *in vitro* requirement recapitulates the requisite activity of SWI/SNF during a mating type switching event *in vivo* when strand invasion specifically occurs within a heterochromatic donor locus.

In vivo, assembly of Sir-dependent heterochromatin requires Sir2, Sir3, and Sir4, and these three proteins form a complex *in vitro* (Moazed 2001; Rusche et al. 2003; Rudner et al. 2005). 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* (Johnson et al., 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. However, in our *in vitro* study, we employed histones that lack

posttranslational modifications, obviating the need for the HDAC activity of Sir2. Likewise, Sir3 is bound to all nucleosomes within the minichromosome substrate, eliminating the need for the known targeting activity of Sir4 (Rudner et al. 2005). Consequently, our results reveal a dominant role for Sir3 in heterochromatin assembly, a role that may be augmented by additional functions provided by Sir2 and Sir4.

Our data suggest a model that explains the sequence of events for recombinational repair in the context of euchromatic and heterochromatic donor loci (Figure 23). After formation and exonucleolytic processing of a DSB, Rad51 polymerizes onto the resulting ssDNA tail with the help of mediators such as Rad52. The Rad51 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 suggests that the Rad51 presynaptic filament is no

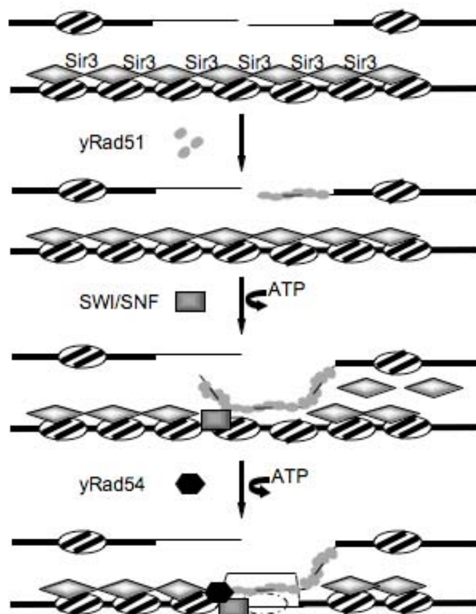


Figure 23. Model depicting the sequence of early events for recombinational repair in the context of a Sir3-heterochromatic donor locus. Formation and exonucleolytic processing of a DSB, result in generation of ssDNA tails (thin black lines) flanking the break. yRad51 polymerizes onto the ssDNA tail to assemble an active presynaptic filament. In order for the yRad51 presynaptic filament to capture homology within a Sir3-heterochromatic region and form initial joints, ATP-dependent remodeling enzyme, SWI/SNF has to disrupt the heterochromatin structure, by evicting Sir3. Once the initial joint is formed, the Rad54 ATPase converts this initial joint into a stable D-loop in which the 3' end of the presynaptic filament is engaged in base-pairing interactions with the complementary strand of the donor and thus entails subsequent events of recombinational repair. Note that cartoons are drawn not to scale and not to implicate any specific stoichiometry or structures.

longer sufficient to locate homology and form an initial joint. In this case, an ATP-dependent remodeling enzyme, such as SWI/SNF, plays an essential role in the initial capture event, disrupting heterochromatin structure. Once the initial joint is formed, the Rad54p ATPase helps to convert 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.

We were surprised to find that SWI/SNF was able to displace a large percentage of the Sir3 from minichromosomes, even when SWI/SNF was present at a low ratio of enzyme to nucleosome. How does SWI/SNF action evict Sir3? 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 believed to be key for mobilizing nucleosomes (Shundrovsky et al. 2006; Cairns 2007). Thus, SWI/SNF might displace Sir3 simply by its DNA translocation activity. However, the Rad54 ATPase also appears to be a potent DNA translocase (Van Komen et al. 2000; Jaskelioff et al. 2003), but Rad54 is unable to evict Sir3 from nucleosomal substrates. Recent studies indicate that SWI/SNF binds to nucleosomes in a defined orientation such that DNA translocation is initiated ~2 DNA turns from the dyad axis (Dechassa et al. 2008). Thus, we favor a model in which a DNA translocation event is initiated within a Sir3-nucleosome complex and this reaction leads to displacement of Sir3. Displacement could be the result of the altered histone-DNA interactions or to the destabilization of histone-histone interactions, which also occur during SWI/SNF remodeling (Yang et al. 2007). If Sir3 binds

cooperatively to the nucleosomal array, displacement of one Sir3 dimer may destabilize additional molecules of Sir3, which may explain why SWI/SNF shows such robust displacement activity.

Numerous studies have demonstrated that formation of a stable D-loop joint molecule requires the combined actions of Rad51 and the Rad54 ATPase, even when naked DNA is used as the donor molecule. Sung and colleagues have shown that Rad54 can alter DNA topology, presumably through its DNA translocation activity, and changes in topology are likely to drive DNA duplex opening and strand invasion (Van Komen et al. 2000). SWI/SNF is unable to substitute for Rad54 with either a DNA or nucleosomal donor, even though SWI/SNF can also function as a translocase (Jaskelioff et al. 2003; Zhang et al. 2006). Based on these previous results, it was unexpected that SWI/SNF activity was able to drive formation of D-loops on the Sir3-nucleosomal donor in the absence of Rad54. This suggests that the Sir3 that is not evicted from the donor plays an active role in facilitating SWI/SNF action. We envision that Sir3-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.

One hallmark of heterochromatin is the silencing of transcription directed by RNA polymerase II. Indeed, expression of the a1, a2, and α1 transcription factors that are encoded within *HMR* and *HML* is silenced by Sir-dependent heterochromatin (Laurenson

and Rine 1992). However, if transcription-associated chromatin remodeling enzymes like SWI/SNF can disrupt heterochromatin, what blocks their recruitment to genes located in heterochromatin? During transcriptional activation, SWI/SNF is recruited to target genes by direct interactions with the activation domains of gene-specific activators (Yudkovsky et al. 1999; Neely et al. 2002; Govind et al. 2005). Within a heterochromatin domain, silenced chromatin is likely to block the binding of transcriptional activators to the promoters of embedded genes, precluding recruitment of remodeling enzymes like SWI/SNF. In contrast, during recombinational repair of a DSB, SWI/SNF is recruited to the presynaptic filament which then appears to target SWI/SNF to the heterochromatic donor locus (Chai et al. 2005). Thus, the structure of heterochromatin blocks transcription-dependent recruitment of remodeling enzymes and guarantees that its repressive structure remains intact unless remodeling enzymes are targeted during a recombinational repair event.

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;Savitsky et al., 2002; Peng and Karpen, 2007; Bisht et al., 2008; Jaco et al., 2008; Allshire et al., 1994; Nakaseko et al., 1986). 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 (Chai et al. 2005), 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'CAAAATAGCACCCTCCCGCTCCAGAA CAACATACAGCGCTTCCACAGCGGCAGCCATAACAGTCAGCCTTACCAGTAA AAAAGAAAA) 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 overexpressed in *E.coli* and purified as described earlier (Zaitseva et al. 1999). GST-yRad54, TAP-SWI/SNF and Sir3-FLAG were purified from yeast as described earlier (Solinger et al. 2001; Smith et al. 2003; Buchberger et al. 2008). Recombinant H2B, H3, H4, H2AS113C, H4K16Q, H3K79E and globular domain of H4 (H4ΔN) *Xenopus* histones were purified and several octamers containing different modified histones were reconstituted as described earlier (Luger et al. 1999a).

Biotinylation of recombinant octamers: Histone octamers were reconstituted containing recombinant histone H2A S113C as previously described (Luger et al. 1999b). The cysteine substitution on H2A was used for the unique site of biotinylation. To biotinylate octamers, 100 mg of octamer was incubated with 10 nmoles of PEO-iodoacetyl-biotin (Pierce, Rockford, IL) at room temperature for 30min in the dark in a

buffer containing 50mM Tris-HCl, pH 8.0; 5mM EDTA and 2M NaCl. The biotinylated octamers were separated from unincorporated biotin by repeated dilution and concentration in the reaction buffer using a 10 mwco Vivaspin 500 concentrator (Vivascience, Hannover, Germany).

Nucleosome assembly: To assemble all the nucleosomal templates, salt step dialysis of supercoiled or linear DNA and recombinant octamers were used as previously described (Logie and Peterson 1997). Nucleosomes were reconstituted at different ratios of histone octamer per 200 bp of donor DNA (R value). The circular minichromosome was assembled by using plasmid CP943 and the biotinylated recombinant octamer. To prepare nucleosomal array template for the restriction enzyme accessibility assay, plasmid CP589 was digested with *Not*I and *Hind*III 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 *Hind*III. 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. Nucleosomal occupancy of the 5S repeats within the minichromosome donor and linear 208-11array template was evaluated by EcoRI analysis (Logie and Peterson, 1997; Carruthers et al., 1999). Reconstituted mononucleosomes were analyzed by native polyacrylamide gel electrophoresis in 1xTBE buffer. In reactions containing Sir3, minichromosomes, mononucleosomes or linear nucleosomal arrays were incubated with Sir3 at different molar ratios of Sir3 (D/N: dimers per nucleosomes) in a buffer containing 35mM Tris-HCl, pH 7.5, 2.5mM MgCl2,

30mM KCl, 1mM DTT at room temperature for 30 min. Binding of Sir3 to mononucleosomes was analyzed by subjecting the products to 4% native polyacrylamide gel electrophoresis in 0.5x TBE.

MNase assay:

Minichromosomes, bound or unbound to Sir3 were subjected to Micrococcal Nuclease (MNase) digestion followed by Southern hybridization. To this end, 200ng DNA equivalent of each of the minichromosomes (Sir3p bound or unbound) was digested with various amounts of MNase (Worthington), serially diluted from 15 units to 0.375 units. Reactions were incubated at 23°C in a reaction buffer containing 10mM Tris-HCl pH 8.0, 0.5mM $MgCl_2$, 0.3mM $CaCl_2$, and 75mM NaCl. After 2 minutes, reactions were stopped by addition of 2.5mM EDTA, 2.5mM EGTA and extracted with phenol-chloroform. Purified products were then resolved by 1.2% agarose gel electrophoresis, followed by Southern Hybridization using ^{32}P-labelled oligonucleotide E4 probe.

Joint capture assays:

Joints were captured essentially as described before with some minor modifications (Sinha and Peterson 2008). Specifically, to form the presynaptic filaments, 3µM of yRad51p was incubated with radio-labeled oligonucleotide B (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 Sir3 at indicated ratios or unbound) at a final concentration of 17nM DNA (~400nM nucleosomes) was added to the reaction and allowed to form joint molecules. Thus, the presynaptic filament is present at twice the molar ratio to duplex donor DNA. Reactions were supplemented with an additional 12.5mM $MgCl_2$ at the time of addition of the donor and then incubated at 30°C for 15 min. In the reactions containing yRad54, 200nM yRad54 (~1 yRad54 per presynaptic filament) and 2mM ATP were added during formation of presynaptic filaments after incubating the oligonucleotide with yRad51 for 2 min and then were incubated for an additional 3 min. In the reactions containing SWI/SNF, 30nM SWI/SNF (~1 SWI/SNF per 15 nucleosomes) was preincubated with minichromosome donors in presence of 2mM Mg-ATP at 30°C for 15 min.

To capture all joints (initial and stable), the reaction mixture was added to 20 ml of streptavidin coated magnetic beads (Dynabeads M-280 Streptavidin, Invitrogen) that had been preblocked in reaction buffer containing 1 mg/ml BSA and resuspended in 5 μl volume . After 5 minutes, joints were captured with a magnetic concentrator, washed three times with the reaction buffer and the unbound and bound fractions were counted in a scintillation counter. % Total joints were calculated as proportion of bead-bound radioactivity to total (unbound + bound) radioactivity added into each reaction.

To visualize stable joints, reaction mixtures were deproteinized by incubating with 2%SDS and 2mg/ml ProteinaseK for 5' at 37°C, electrophoresed on a 0.9% agarose gel, and detected by autoradiography (Jaskelioff et al. 2003).

Remodeling assays:

Mononucleosome accessibility and mobility assays were done as described (Smith and Peterson 2005). Restriction enzyme–accessibility assay of 208-11 arrays was done as described (Logie and Peterson 1997).

Sir3 displacement assay:

Standard Sir3-containing heterochromatin was assembled in 3μl with 17nM DNA equivalent of biotinylated minichromosomes and Sir3 at a molar ratio of 1 dimer per two nucleosomes or two dimers per nucleosome at room temperature for 30 minutes in the same reaction buffer as in joint-capture assay. Subsequently, 200nM yRad54 or 30nM SWI/SNF 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 as described before (See Joint Capture assays), 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, used in Figure 3) lacks *HMLα* or HMRa on Chr III and the donor sequence is a *MATa* locus containing an incleavable HO site (*MAT*a-inc) on Chr V (Vaze et al. 2002). The *SWI2, RAD54* genes were disrupted in this strain background by replacing the coding regions with KAN-MX6 gene that confers resistance to the drug G418 (Longtine et al. 1998). Kinetics of DSB formation and strand invasion was analyzed on a PCR-based primer-extension assay as described before (White and Haber 1990; Keogh et al. 2006a). 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 that have been previously described (Keogh et al. 2006a). Efficiency of DSB formation and strand invasion was arbitrarily set to 100% for the highest WT DSB product i.e. at 0 timepoint. All values were normalized to a *Pho5* internal control.

CHAPTER IV

PERSPECTIVES

CHAPTER IV

Summary

The role of chromatin in the regulation of cellular response and repair of chromosomal DNA DSBs has only begun to be fully appreciated. Research in the last decade has identified an evergrowing number of chromatin modifications and chromatin remodeling enzymes that have emerged as key regulators of DNA accessibility to factors responsible for eliciting a cellular response to and repairing a chromosomal DNA DSB. A great emphasis has fallen onto the important functions of the ATP-dependent chromatin remodeling enzymes in repairing DNA DSBs by multiple pathways and together maintaining genomic integrity. Interestingly, several aspects and functions of these enzymes are conserved throughout eukaryotes. The research presented in this thesis has focused on understanding how the activities that remodel chromatin structure are integrated with the events associated with repair of chromosomal DNA DSB by the HR pathway.

The backdrop of this thesis

The picture that has emerged from recent studies about the particular roles of chromatin remodeling enzymes in the repair of a DSB in the *MAT* locus of *S. cerevisiae* is as follows. After the DSB formation and rapid marking of the chromatin domain that flanks the break by the phosphorylation event of the histone H2AX, four members of the chromatin remodeling enzyme family, RSC, SWI/SNF, INO80.com and SWR1.com are recruited to the site of the break in an ordered fashion (See also Figure 5 in CHAPTER I). Amongst these chromatin remodeling enzymes, RSC is reported to come first to the DSB. RSC seems to be playing multifunctional roles during DSB repair. First, it associates with both donor and broken chromatin and plays a role in the post-synaptic phase of HR, during new DNA synthesis and ligation of the repaired strands (Chai et al. 2005). Recent work has suggested that RSC remodels nucleosomes at the DSB site to facilitate loading of repair machinery (Shim et al. 2007). SWI/SNF comes next, around the same time as Rad52 and Rad54 are detected at *MAT* and plays an important role in the synapsis event of *MAT* and *HM* loci (Chai et al. 2005). INO80.com comes to the site of the break around the same time or a little later. However, reports suggest that like RSC, the INO80.com complex remodels chromatin around the DSB; particularly causing loss of nucleosomes near the HO induced DSB at *MAT* (Tsukuda et al. 2005). This seems rather paradoxical; firstly because INO80.com is recruited at a time when repair process is already well in progress, obviating the need of additional nucleosome remodeling at the site of the break.

Secondly, complete eviction of nucleosomes around the site of the break seems inefficient use of cellular energy as an elegant set of *in vitro* studies by Bruce Alberts in late seventies had shown that histone octamers can form nucleosome-like structures on ssDNA as efficiently as on dsDNA, suggesting that nucleosome core particles may transiently become bound to stretches of ssDNA as they arise during different DNA mediated processes (Palter and Alberts 1979; Palter et al. 1979). The reason for the popular belief that INO80.com is responsible for nucleosome loss at the site of the break can be attributed to imperfect interpretation of data obtained from chromatin immunoprecipitation experiments (Chen et al. 2008), which could have been misleading due to altered crosslinking efficiency of histones to ssDNA. However, a number of studies agree on the points that INO80. COM.com influences the DNA end-processing at the site of the break via MRX complex and thus, also affects efficient formation of the Rad51 presynaptic filament. Finally, its key role is underscored in damage checkpoint activation and escape or adaptation in case of a persistent DSB (Papamichos-Chronakis et al. 2006). Very recently, INO80.com complex has also been reported to promote nucleosome eviction at the donor HM loci during synapsis to facilitate strand invasion by the *MAT* presynaptic filament (Tsukuda et al. 2009). However, paradoxically the efficiency of mating-type switching remains moderately unchanged in cells lacking INO80.com as compared to the wild type cells. In addition, the nucleosome that is detected to be lost in this study resides at the opposite side of the direction of new DNA synthesis. Hence, it is tempting to speculate that INO80.com may have a role in branch migration of the joint intermediate which would actually affect the length of gene

conversion tracts, as observed by this study. Owing to the presence of two subunits of INO80.com remodeling complex related to the bacterial RuvB helicase (Shen et al. 2000), this is indeed an intriguing speculation that awaits further experimental corroboration.

Like INO80.com remodeling complex, SWR1.com that shares a number of subunits with the former, also gets recruited to the DSB in γ-H2AX dependent manner (van Attikum et al. 2007a) at the same time as INO80.com. SWR1.com has a unique chromatin remodeling activity in that SWR1.com catalyzes the incorporation of histone variant H2A.Z into chromatin at promoters, centromeres and subtelomeric regions affecting gene expression (Krogan et al. 2003; Kobor et al. 2004; Mizuguchi et al. 2004; Raisner et al. 2005). Whether SWR1.com mediated exchange of histone variants near the site of a DSB is an occurrence with any consequences in the repair processes, is yet to be established. On the other hand, recent reports show evidence that SWR1.com is involved in recruitment of yKu80 and thus contribute to NHEJ pathway of a DSB repair (van Attikum et al. 2007a). In this regard, role of SWR1.com overlaps with that of RSC complex, which also contributes to NHEJ by recruiting yKU70 (Shim et al. 2007).

As mentioned earlier, RSC complex additionally contributes to the HR pathway of DSB repair during the post-synaptic phase by regulating the dissociation of the invading presynaptic filament from the HM loci and ligation of the presynaptic filament with the DNA strand on the other side of the break (Chai et al. 2005). Thus, RSC might be

involved in the "restoration" of chromatin state upon completion of repair.

Summary of the experimental findings of this thesis

The studies presented in this thesis address a fundamental question in the field of the repair of a chromosomal DNA DSB. What is the role of chromatin remodeling enzymes during the search and capture of homology in chromatin and heterochromatin-like condensed nucleo-protein structures in the genome? Specifically, the studies have investigated whether and when chromatin remodeling is necessary during this fundamentally essential cellular process. Importantly, the studies have been able to recapitulate *in vitro* the formation of recombination intermediates, central to *in vivo* recombinational repair of a DNA DSB, by using purified yeast proteins and reconstituted nucleosomal templates. The data in Chapter II of this thesis demonstrate that the central recombinase, Rad51, when assembled onto single-stranded DNA in the form of an active nucleoprotein filament is sufficient to capture homology on the nucleosomal surfaces. This study also indicates that chromatin remodeling by chromatin remodeling enzymes like Rad54 or SWI/SNF do not facilitate the initial capture of homology on the nucleosomal surface. Experimental data support the notion that Rad54 is required to convert the initial protein-dependent DNA intermediates into stable protein-independent intermediates (Sinha and Peterson 2008). Moreover, the data in chapter III of this thesis demonstrate that chromatin remodeling is essential to capture homology on a heterochromatin-like more complex nucleo-protein structure. Unlike on euchromatin, the

recombinase assembled into nucleoprotein filament is not sufficient to search homology on heterochromatin-like structure. The experimental data in this chapter suggest that the chromatin remodeling enzyme, SWI/SNF remodels the heterochromatin by displacing key structural component of heterochromatin to form the initial intermediate. Additionally, Rad54 plays a synergistic role with SWI/SNF in converting the initial intermediates into stable protein-independent ones, from which subsequent steps of recombinational repair can begin.

Questions awaiting further investigations

Despite providing seminal information in the field of recombinational repair of a chromosomal DNA DSB, the studies described in this thesis raise many more questions that demand much more work to fully understand the intricacies of the HR process in the context of chromatin.

A key question that yet awaits further investigation is what happens to the nucleosome on which a Rad51-presynaptic filament synapses to form the intermediate joint? Common sense as well as some preliminary experimental data on the minimal requirement of homology length for formation of Rad51 mediated joints on a nucleosome predict at least local disruption of histone-DNA interactions within the nucleosome (Figure 24). This conclusion is based on the structure of the Rad51-nucleoprotein filament in which the ssDNA is extended 1.5 times, which has to align and strand exchange with dsDNA that is

wrapped around an octamer of histone proteins. By definition, alteration or

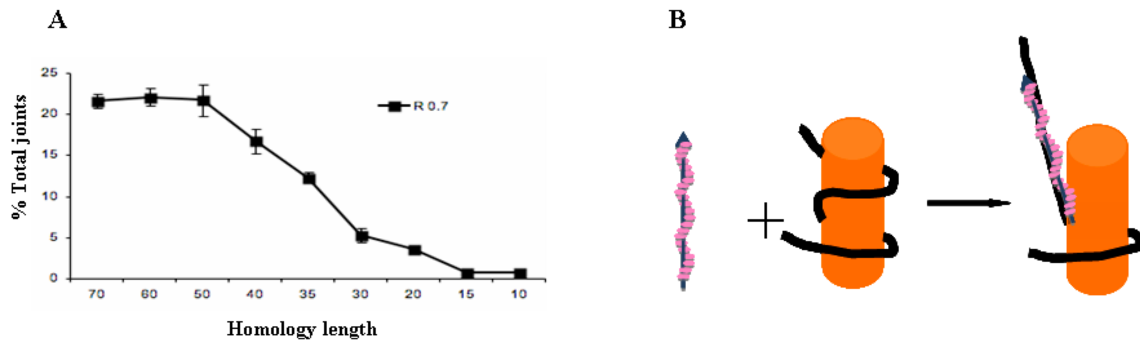


Figure 24. Minimal homology length requirement for Rad51 mediated formation of joints on a nucleosome. (A) Graph shows length of homology requirement for joint formation as captured by biotin-streptavidin assays. (B) Schematic shows proper alignment of 50-nucleotide long (green arrow) Rad51 (Pink ovals)-presynaptic filament causing disruption of histone-DNA (black solid line) interactions on the surface of the histone octamer (orange cylinder).

disruption of histone-DNA contacts within a nucleosomes core particle is nucleosome remodeling. How is this nucleosome remodeling being achieved? Can any drastic change of nucleosome positioning or any subtle change in nucleosomal DNA topology and thus accessibility of nucleosomal DNA be experimentally detected? Related to this, there is another question: does Rad54 affect the nucleosome remodeling mentioned above? In addition, how does Rad54 use its ATPase activity to convert the Rad51 catalyzed initial protein-dependent joint into the protein-independent stable joint on both naked and nucleosomal DNA?

The finding in chapter III that heterochromatin poses further constraints on the repair machinery and demands for additional nucleosomal remodeling activity by SWI/SNF to counteract the repressive state is fascinating by itself. However, a question that immediately arises is whether other members of the family of remodeling enzymes like

RSC or INO80.com or SWR1.com or even ISWI can replace the function of SWI/SNF in this regard? Furthermore, in our purified system, we were able to bypass the requirement of the complete SIR-complex mediated formation of heterochromatin by using recombinant histones that lack posttranslational modifications, obviating the need for the HDAC activity of Sir2 and also eliminating the need for the targeting activity of Sir4 and investigated the repressive nature of Sir3-heterochromatin. How chromatin remodeling activity contends with the more protein-associated complex heterochromatin structure *in vivo* still remains un-addressed.

The more the merrier?

In addition to the specific questions related to the studies in the preceding two chapters of this thesis, a lot more questions remain to be addressed in the field of repair of DSBs in context of chromatin. Why are so many chromatin remodeling complexes required for the repair of a DSB? Are these enzymes performing somewhat redundant or completely different and specific functions? In addition, why have they evolved with numerous subunits? Are there any associated advantages with respect to dealing with chromatin? More importantly, are the different subunits of all these multi-subunit protein complexes making individual and specific contributions to the overall roles of each of these enzymes? It is also tempting to speculate that different subunits may in fact regulate the functions of these chromatin remodeling enzymes at different events, thus making a single complex to be involved in multiple steps of the same biochemical pathway.

It is interesting to note that the same histone modification, namely γ-H2AX is able to recruit different remodeling complexes like INO80.com and SWR1.com. In addition, these complexes are members of the same class of remodeling enzymes. Likewise other histone modifications may also recruit directly or indirectly other classes of chromatin remodeling enzymes. Therefore, it is intriguing to investigate if the specificity of each of these chromatin remodeling complexes is being governed by external cues. External cues might result in different chromatin modifications, which in turn would allow for combinatorial control by integrating multiple cellular signals to achieve a dynamic regulation of the repair process. On the other hand, if it is not the chromatin modifications, then what are the factors that modulate recruitment of chromatin remodeling enzymes to the site of the break? In this regard, it indeed remains unknown how SWI/SNF remodeling complex is recruited to the DSB at the *MAT* locus. Is it just the structure of the DNA ends harboring a junction of ssDNA and dsDNA that is generated from nucleolytic processing of the break that attracts SWI/SNF like dsDNA dependent ATPase machines?

An interesting possibility regarding the specificity of the chromatin remodeling enzymes could also be that the function of a particular chromatin remodeling enzyme is context dependent. Consistent with this idea, the study in this thesis show that SWI/SNF is mainly required for Rad51-dependent synapsis event, only when the nucleosomal homology is encompassed in a heterochromatin-like structure.

Concluding Remarks:

Although there has been a rapid increase in our knowledge of physiological role of chromatin remodeling enzymes in fundamental chromatin mediated cellular processes, continued investigations are in demand to characterize mechanistically how these enzymes influence specific biochemical steps in the damage repair pathways. Undoubtedly, further research will provide a molecular basis for understanding the mechanism of preserving our genomic integrity in the face of continuous genotoxic insults. Ultimately, chromatin remodeling enzymes may hold the promise of becoming targets for future gene or drug therapies designed to predict, diagnose and treat human pathologies associated with genomic instability.

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APPENDIX

Table 1: Sequences of the Oligonucleotides

Name	Sequence (5'-3')	Comments
1	CCTTTTTTACACTGTGACTGATTGAGCTGGTGCCGTGTCGA GTGGTGTTTTTTTAATAGGTTTTCTTTTTTACTGGTAAG	Type1, Position A
2	CCTTTTTTACACTGTGACTGATTGAGCTGGTGCCGTGTCGA GTGGTGTTTAGTTATTCTTATTGTACTTGTGATTAGTT	Type 2, Position A
3	CGTGTCAGTATCATCTGCTATCGTCCTGTGACTGATTGAGC TGGTGCCGTGTCGAGTGGTGTTTTTTTAATAGGAGTTATTC TTATTGTACTTGTGATTAGTT	Type 3, Position A
4	TTGATAAGAGGTCATTTTTGCGGATGGCTTAGAGCTTAATT GCTGAATCTGGTGCTGTAGCTCAACATGTTTTAAATATGCA A	Type 4, Position A
Position B	CAAAATAGCACCCTCCCGCTCCAGAACAACATACAGCGCT TCCACAGCGGCAGCCATAACAGTCAGCCTTACCAGTAAAA AAGAAAA	
Position C	CTTGCTTGATGAAAGTTAAGCTATTTAAAGGGTCAGGGAT GTTATGACGTCATCGGCTTATAAATCCCTGGAAGTTATTC	