Chromatin Regulators and DNA Repair: A Dissertation

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CHROMATIN REGULATORS
AND DNA REPAIR

A Dissertation Presented
By
Gwendolyn M. Bennett

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

December 19th, 2014

Biomedical Science
CHROMATIN REGULATORS AND DNA REPAIR

A Dissertation Presented By
Gwendolyn M. Bennett

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I dedicate this work to my Mom and Dad for fostering my interest in science and encouraging me to pursue it.
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Abstract

DNA double-strand break (DSB) repair is essential for maintenance of genome stability. However, the compaction of the eukaryotic genome into chromatin creates an inherent barrier to any DNA-mediated event, such as during DNA repair. This demands that there be mechanisms to modify the chromatin structure and thus access DNA. Recent work has implicated a host of chromatin regulators in the DNA damage response and several functional roles have been defined. Yet the mechanisms that control their recruitment to DNA lesions, and their relationship with concurrent histone modifications, remain unclear. We find that efficient DSB recruitment of many yeast chromatin regulators is cell-cycle dependent. Furthering this, we find recruitment of the INO80, SWR-C, NuA4, SWI/SNF, and RSC enzymes is inhibited by the non-homologous end joining machinery, and that their recruitment is controlled by early steps of homologous recombination. Strikingly, we find no significant role for H2A.X phosphorylation (γH2AX) in the recruitment of chromatin regulators, but rather that their recruitment coincides with reduced levels of γH2AX. We go on to determine the chromatin remodeling enzyme Fun30 functions in histone dynamics surround a DSB, but does not significantly affect γH2AX dynamics. Additionally, we describe a conserved functional interaction among the chromatin remodeling enzyme, SWI/SNF, the NuA4 and Gcn5 histone acetyltransferases, and phosphorylation of histone H2A.X. Specifically, we find that the NuA4 and Gcn5 enzymes are both required for the robust recruitment of SWI/SNF to a DSB, which in turn promotes the phosphorylation of H2A.X.
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CHAPTER I

Introduction
Introduction

Living cells constantly deal with a wide variety of potentially damaging agents from both external sources and from within themselves. It is vital that the cell protect its genetic material (DNA) from these attacks and accurately repair the DNA whenever it is damaged. DNA damage comes in many forms including bulky adducts, pyrimidine dimers, inter-strand crosslinks, and strand breakages. The most detrimental is the double-strand break (DSB) where both phosphate-sugar backbones are severed. Failure to repair these breaks accurately can have many results such as relatively simple mutations, small and large insertions and deletions, and the egregious chromosomal translocations and fusions. These can then lead to genome instability, cell death, and for multi-cellular organisms, tumorigenesis\textsuperscript{1,2}. Therefore, the cell has established a large and complex system to detect and respond to these DNA lesions. Although much work has been done to resolve the repair mechanisms involving naked DNA, biological systems are not this simple because DNA does not exist by itself within the cell.

In eukaryotic cells, a very high level of compaction is necessary to fit the many megabases of DNA into a nucleus only a few microns across. The main mode of this compaction is to package DNA with proteins into a complex called chromatin. While this complex allows for the necessary compaction, it also forms a barrier to accessing the DNA for various cellular processes like transcription, replication, and repair. Nevertheless, the chromatin structure is astonishingly dynamic, and recent work has been
driven at understanding how the chromatin fiber is altered and functions during DNA repair. This chapter aims to discuss our current understanding of the process of DNA double-strand break repair within the chromatin structure.

**Chromatin structure**

The basic unit of chromatin in the nucleosome, made up of four histones – H2A, H2B, H3 and H4 – each in duplicate. Together they organize 146 base-pairs (bp) of DNA that wraps 1.65 times in a left-handed wrap around the histone octamer. These proteins are highly conserved across eukaryotes, are low in molecular weight, and contain a central histone fold domain which mediates histone-histone and histone-DNA interactions. Each histone also possesses unstructured short N-terminal – and sometimes C-terminal as well – domains, often referred to as histone “tails,” that extend out from the nucleosome core. Even though these tails are not required for nucleosome core particle assembly, they do function in higher order folding of the chromatin fiber. In addition, the tails contain a multitude of sites for post-translational modifications (PTMs) that are key in regulating multiple biological functions.

The primary structure of chromatin is the “beads on a string” structure which represents single nucleosomes in a linear formation as seen by cryo-electron microscopy. This 10-12nm thick structure is then folded into the more common three-dimensional structure termed the 30nm fiber through inter-nucleosomal interactions. This structure is further stabilized by the addition of a fifth class of histone, the linker
histone. Linker histones bind nucleosome at the entry-exit point at a one-to-one ratio and stabilize an additional 20bp of linker DNA\textsuperscript{11}. 30nm fibers can then self-associate into even larger 100-400nm thick structures called chromonema filaments. These structures predominate in the nucleus even during interphase when the need to access DNA is high\textsuperscript{7,12}.

Apart from the four canonical histones (H2A, H2B, H3, and H4) there are also several histone variants that function in additional roles in the cell. Unlike canonical histones which are expressed during S-phase of the cell cycle and are incorporated during replication, these variants are expressed throughout the cell cycle and incorporated independently of replication. Although several variants exist, there are four that exist in all eukaryotes: CenH3, H3.3, H2A.X, and H2A.Z. CenH3 refers to a diverse group of centromere-specific H3 variants that are necessary for kinetochore formation. These variants are much less conserved and are thought to form nucleosomes that are very different in structure from the canonical nucleosome\textsuperscript{13}. H3.3 and H2A.X are very similar to their canonical counterparts, but they are also the major forms of these histones in lower eukaryotes meaning that there is no ‘canonical’ H3 or H2A in these organisms, and so, unlike other variants, are cell cycle regulated in expression\textsuperscript{14,15}. H2A.X is distinct from H2A in its extended C-terminal domain which harbors a Ser-Gln-(Glu/Asp)-Φ motif, where Φ represents a hydrophobic residue. The rapid phosphorylation of this serine in response to a DSB is a hallmark of this lesion and plays important roles in repair. H2A.Z primarily varies from H2A in its extended acidic patch. This variant is
enriched at transcriptional start sites where turnover of histones is very high, thus having roles in transcription, and also implicated in DSB repair\(^{13}\).

**Chromatin Remodeling**

In order to access chromatinized-DNA for basic processes like transcription, replication, or damage repair, the eukaryotic cell has established methods to alter the chromatin structure, such as through the actions of highly conserved enzymes. The first of two classes of these enzymes is the chromatin modifying enzymes, which covalently add or remove post-translational modification to the histones themselves. Lysine residues are subject to acetylation, methylation, sumoylation, and ubiquitination; arginines can also be methylated; and serines and threonines are phosphorylated. Although most of the PTMs occur on the histone tails, there are a few that occur within the histone core. These modifications are associated with a variety of chromatin-mediated events such as transcriptional activation or repression, histone deposition, and repair\(^{8,9,16–18}\). Indeed, there is extensive and on-going research into the regulatory roles of PTMs in these and other chromatin-mediated events.

The second class of enzymes is made up of ATP-dependent chromatin remodeling enzymes. These enzymes use the energy of ATP to disrupt the DNA-histone contacts and thereby alter the chromatin structure. The disruption can take the form of mobilizing nucleosomes *in cis* along the DNA strand, removing nucleosomes completely, or exchanging histone variants in or out of the nucleosome. A defining aspect of these
enzymes is the presence of an ATPase subunit related to the Snf2-family of the SF2-superfamily of helicase-like proteins. The Snf2-family has been further divided into subfamilies based on sequence alignment and characteristics of their remodeling activities\textsuperscript{19,20}. There is a wide variety in the structure of these enzymes, as they range in size from a single catalytic protein to huge multi-subunit complexes. They can also contain an assortment of other domains besides their Snf2-like ATPase, e.g. bromodomains that bind acetylated residues; chromodomain, tudor domains, and plant-homeodomain-finger domains that each bind distinct methylated marks; and AT-hooks that bind the minor groove of A-T rich DNA regions\textsuperscript{16}. The variety in structure and composition of these enzymes also lends them varied functions during cellular events, an area of study which remains under intensive investigation.

Still, it has become clear that the functions of PTMs and chromatin remodeling enzymes do not work in isolation from each other. There is growing evidence that modified histones recruit or stabilize the interaction of various proteins including chromatin remodeling enzymes to areas of DNA. In addition, the recruitment or activity of chromatin remodeling enzymes can affect the modification of histones. The remainder of this chapter will focus on the chromatin modifications and remodeling events that occur during DSB repair.
Double-strand break repair pathways

Cellular nuclei are estimated to withstand many thousands of DNA lesions per day, and several dozen of these are the dangerous double-strand breaks. DSBs can be produced through endogenous events like programmed rearrangements induced by nucleases such as mating-type switching in the budding yeast Saccharomyces cerevisiae, collapsed replication forks, deliberate recombination event during meiosis, V(D)J recombination, and B-cell class-switching. Additionally, DSBs can be caused by exogenous sources, such as UV radiation, ionizing radiation (IR), chemotherapeutic drugs, and environmental toxins. The failure to properly repair these lesions can lead to a multitude of mutations and can lead to the acquisition of characteristics that are universal among tumors. Tumorigenesis is a multi-step process where genome instability – the loss of mechanisms to ensure genome integrity – contributes to this acquisition process, and often leads to cancer. This indicates that the cellular systems to detect and efficiently repair DSBs accurately are critical in preventing the onset of cancer, and so it becomes critical for us to study and understand how DSBs are repaired.

In response to a DSB, the cell has developed complicated signaling systems to sense the lesion, initiate repair, and arrest the cell cycle to allow time for repair to be completed. In the case where the cell has undergone too much or unrepairable damage to be effectively repaired, there are overlapping signaling networks to commence apoptosis to avoid the possibility of tumorigenic instabilities. While there are several pathways to repair DSBs, there are two major ones: non-homologous end joining (NHEJ), and
homologous recombination (HR; Figure 1.1). The latter uses homology found in a sister chromatid or homologous chromosome to copy the missing information and repair the damaged DNA. In diploid cells (like those of mammals) this type of repair could occur during any cell cycle phase, and yet, mammalian cells favor NHEJ throughout the cell cycle. Unfortunately, through its relatively simplistic mode of reattaching the ends, NHEJ tends to be error-prone. In contrast, the haploid yeast cell is prevented from using HR during the G1 cell cycle phase due to the lack of a homologous chromosome. Only after replication do yeast cells use the HR pathway for DSB repair. The choice of repair pathway is controlled not only by DNA ploidy level but also the cell cycle dependent kinase CDK1\(^{23-25}\). This important cell cycle regulator is active during S and G2 phases.

Figure 1.1. Schematic of DSB repair during different cell-cycle phases. See text for details.
and is important for controlling the DNA-end processing (known as resection) that is required for HR-mediated repair.

DSBs are bound by both the MRX and the Ku complexes. These complexes are detectable at a DSB within minutes of break formation and compete in a manner that contributes to the repair pathway choice. The Ku complex is a heterodimeric ring of Ku70 and Ku80, and with other components of the NHEJ machinery, inhibits steps that lead to HR thus directing the pathway towards NHEJ\textsuperscript{25,26}. During the S and G2 phases of the cell cycle, MRX (Mre11-Rad50-Xrs2; MRN in mammals where Nbs1 replaces Xrs2) bind DNA ends with its partner Sae2, which is phosphorylated by CDK1. Although the mechanism isn’t understood, the phosphorylation of Sae2 increases the ability of MRX-Sae2 to overcome the inhibitory effect of Ku to direct repair towards HR\textsuperscript{27,28}. Furthermore, MRX plays an important role in recruiting and activating the apical checkpoint protein Tel1 (ATM in mammals)\textsuperscript{29–31} thereby transducing the DNA damage response to downstream effector kinases\textsuperscript{32}.

MRX-Sae2 together facilitate nucleolytic clipping of the 5’ ends of the broken DNA to create short 3’ single-stranded DNA (ssDNA) overhangs of only a couple hundred base pairs\textsuperscript{33}. This step is considered to be the rate limiting step in end-processing, in part because resection rates are unaffected at distances farther away from the break site in strains lacking MRX\textsuperscript{27,33}. Additionally, Mre11-deficient yeast strains still resect DSBs, but initiation is delayed\textsuperscript{33,34}.

After this initial short-range clipping of the DNA ends, extensive resection is carried out by two redundant and parallel pathways: Exo1 and Sgs1-Dna2 (BLM-Dna2
in mammals). The role of Exo1, a 5′-3′ processive double-strand exonuclease, in resection was discovered well before its complementary pathway involving Dna2 and Sgs1. However, even though *exo1* mutant strains show reduced resection, they are not compromised in HR repair, implicating another pathway is at work. Eventually, three separate studies identified Sgs1, a RecQ-family helicase, as part of the other pathway. The interacting partners for Sgs1 (Rmi1 and Top3) and the single-strand endonuclease Dna2 work together in this pathway to facilitate long-range resection. These two pathways can create many kilobases of resected DNA to either side of the DSB if repair is inhibited. The resected ends are then used in the genome-wide homology search to complete repair. Although only a few hundred bases of resection is needed for successful gene conversion events, regions several kilobases away from DSB are preferentially used for homology search and repair when sister-chromatid-based repair is inhibited.

The ssDNA tails created during resection are initially coated by the heterotrimeric complex RPA to protect and stabilize it. This ssDNA-RPA complex also has an important role in activating the checkpoint kinase Mec1 (ATR in mammals), furthering the cellular response to the damaged DNA. Subsequently, RPA is replaced with the key recombination protein Rad51 to form nucleoprotein filaments known as the presynaptic filament. The exchange is facilitated by Rad52 with help from Rad55 and Rad57. All of these proteins are part of the Rad52 epistasis group – RAD50, RAD51, RAD52, RAD54, RAD55, RAD57, RAD59, MRE11 and XRS2 – which mediates HR repair. The presynaptic filament searches the genome for a homologous region and,
when found, forms a transient intermediate joint termed the paranemic joint. This structure is then converted to the plectonemic joint, or D-loop, where the 3’ DNA-end invades the double-stranded template, displacing one strand, and serves as a primer for new DNA synthesis. The extension of this template leads to the formation of cruciform structure known as a Holliday Junction, which are resolved by cleavage and ligation, or dissociation of the invading strand and re-annealing to the other side of the DSB completing repair.\(^4\)

In contrast, DSBs that are formed during the G1 cell cycle phase are repaired by a much simpler method. As mentioned above, these breaks are bound by MRX and Ku, but without activated Sae2, MRX is unable to overcome Ku’s activity and thus NHEJ is used to complete repair. Still, MRX plays a role in NHEJ by tethering the broken ends together, and mediating interaction between other components of the NHEJ machinery.\(^4\) Ku functions to protect the DNA ends while also recruiting other proteins – Dnl4, Lif4, Nej1 (Lig4, XRCC4, and DNA-PK in mammals) – that will ligate the ends together and complete repair. Some processing of these breaks does occur in order to prepare the ends for ligation, and so this pathway is typically error-prone.

**Chromatin modifications and remodeling enzymes in DSB repair**

One of the very first chromatin modifications that occurs after DSB formation is the rapid and extensive phosphorylation of histone H2A.X at the serine in its SQE motif of the C-terminal domain (S129 in yeast, S139 in mammals). Conventionally referred to
as γH2AX, this mark is the result of activity by the checkpoint kinases Tel1/ATM and Mec1/ATR, joined by DNA-PK in mammals. γH2AX is dispensable for initial signaling of DSB, but has been shown to be important for the stabilization of repair proteins at the break site\textsuperscript{44}. Additionally, both yeast and mouse cells that are incapable of producing γH2AX suffer from sensitivities to DNA damaging agents and defects in genome stability\textsuperscript{45,46}. In mammals this mark can spread over megabase domains surrounding a DSB\textsuperscript{47}, whereas in yeast it’s been shown to cover smaller regions of about 50 kb\textsuperscript{48}. The spreading of γH2AX in mammals is required for a feed-forward loop with the DNA damage mediator MDC1. γH2AX bound MDC1 recruits MRN which thereby recruits more ATM to phosphorylated additional H2A.X\textsuperscript{49}. However, yeast lack a homolog to MDC1, and so the method of spreading in this system remains unclear. Interestingly, there is some evidence that γH2AX spreading in both yeast and mammals may be through other means that are non-linear such as diffusion of ATM or interactions with genomic regions nearby in 3-dimensional space\textsuperscript{50–53}, although this idea needs further investigation. Another important role of γH2AX is to recruit cohesins to the DSB site in S and G2 cell phases and thereby promote repair by HR\textsuperscript{54,55}.

In yeast, the Rad9 mediator protein is necessary for activation of the main checkpoint effector kinase Rad53 whose phosphorylation is a key step in the signal cascade in checkpoint activation. Rad9 binds to γH2AX via its tandem BRCT domains and to methylated lysine 79 of histone H3 via its tandem Tudor domains\textsuperscript{56,57}. It can also interact with the scaffold protein Dpb11\textsuperscript{58,57}. Rad9 also functions to inhibit single-stranded DNA formation after DNA damage\textsuperscript{59}. Interestingly, cells lacking γH2AX are
defective for G1/S checkpoint activation, but not the G2/M checkpoint, even though Rad9 is required for checkpoint activation through the cell cycle\textsuperscript{58}. In this later cell phase, phosphorylation of Rad9 controlled by CDK1 instead promotes its interaction with Dpb11, and Rad9’s chromatin binding activities become dispensible\textsuperscript{57}.

In higher eukaryotes, $\gamma$H2AX initiates a ubiquitination cascade that leads to the binding of several damage response mediators including the Rad9 homolog 53BP\textsubscript{1}\textsuperscript{60,61}. 53BP\textsubscript{1} shares several characteristics with Rad9 such as tandem BRCT and Tudor domains, phosphorylation sites, inhibition of resection, and importance in checkpoint activation\textsuperscript{62}. However, its recruitment to damaged DNA involves a different mechanism. $\gamma$H2AX leads to the recruitment of MDC1 and activation of RNF8-RNF168-dependent chromatin ubiquitination. 53BP\textsubscript{1} then binds to ubiquitinated H2A at lysine 15 which is catalyzed by RNF168\textsuperscript{62}. Additionally, binding appears to require the dimethylation of histone H4 at lysine 20 which is also induced in response to a DSB\textsuperscript{63}.

Finally, the removal of $\gamma$H2AX is important for checkpoint recovery following repair of a DSB. Dephosphorylation is carried out by a Pph3 (PP2A in mammals), and apparently happens after removal from the chromatin structure\textsuperscript{64,65}. In \textit{Drosophila melanogaster} acetylation of phosphorylated H2Av (the H2A.X ortholog) by the chromatin remodeling enzyme complex TIP60 (which has both histone acetyltransferase and remodeling activities) appears to be a prerequisite for its subsequent removal from chromatin\textsuperscript{66}. Prior to $\gamma$H2AX removal in human cells a similar acetylation is followed by ubiquitination carried out by a DSB-specific complex of TIP60 and the ubiquitin-conjugating enzyme UBC13\textsuperscript{67}. Furthermore, in both cases $\gamma$H2AX removal is TIP60-
dependent, strongly suggesting that TIP60 is the mechanism of $\gamma$H2AX removal from chromatin. However, in yeast, removal of the SWR-C complex, which is able to exchange H2A.X from chromatin like TIP60, instead leads to a decrease in $\gamma$H2AX levels surrounding a DSB rather than an increase (see Chapter II), leaving the mechanism of $\gamma$H2AX removal elusive.

Several other residues have also been reported to be phosphorylated in response to DNA damage. Both S122 and T126 in yeast H2A are phosphorylated in response to various DNA damaging agents$^{68-70}$, although the role of these in the damage response has not been elucidated. In addition to a role in DNA damage, S122 (T120 in mammals) phosphorylation by the tumor suppressor Bub1 is necessary for the localization of Shugoshin to pericentromeric regions, thus playing a key role in the regulatory process for chromosomal segregation during mitosis and meiosis$^{70-72}$. Likewise, yeast H2B has recently been shown to be phosphorylated at T129 in response to an induced DSB$^{53}$. This mark is mediated by the same checkpoint kinases as $\gamma$H2AX (Mec1 and Tel1), and spreads over a similar range, but accumulates at a much slower rate. However, a mutation of this mark had minimal impact on cell viability after treatment with damaging agents, so the impact of this mark on repair remains unknown$^{53}$. Additionally, phosphorylation of H2B in its N-terminal domain also appears to be involved in the damage response. Phosphorylation at S14 in mammalian cells (S10 in yeast) colocalizes with, and is dependent on $\gamma$H2AX after DNA damage$^{73}$. The model presented proposes that this mark promotes chromosomal compaction, however the mechanism remains unknown. Additionally, this mark has been identified in both yeast and mammalian
apoptotic cells, preceded by the deactylation of K15 (K11 in yeast), also playing a role in chromosomal compaction\textsuperscript{74–76}. Finally, the first residue of yeast H4 (serine 1) is also phosphorylated after DSB induction. Catalyzed by CK2, it is thought that this mark occurs during the restoration of the chromatin structure by inhibiting further acetylation (see below)\textsuperscript{77,78}.

In addition to $\gamma$H2AX, another prominent modification of chromatin after DNA damage is acetylation. N-terminal domains of histones H2A.Z, H3, and H4 are acetylated after a DSB and implicated in DNA damage repair\textsuperscript{79}. These modifications are mediated by several histone acetyltransferases including Gcn5 and NuA4 (Tip60 in mammals), which are recruited to the site of a DSB\textsuperscript{46,80}. Removal of these enzymes – or mutation of the acetylatable lysine residues in H3 (K9, 14, 18, 23 & 27) and H4 (K5, 8, 12 and 16) tails – results in DNA damage sensitivities in yeast and mammalian cells\textsuperscript{80–84}. Acetylation is seen to increase after a DSB is induced and to reduce as repair finishes\textsuperscript{80,85}. Many studies have also demonstrated a change in chromatin structure during repair to a more open, less compact conformation\textsuperscript{52} and, since high levels of acetylation can disrupt chromatin compaction\textsuperscript{86,87}, it is thought to promote the changes in chromatin accessibility seen in response to DSBs.

Interestingly, a recent study has identified an activation loop among $\gamma$H2AX, H3 acetylation, and the human SWI/SNF. They show that BRG1 (the catalytic subunit of one of the two human SWI/SNF and RSC complexes) interacts with nucleosomes that contain $\gamma$H2AX through a concomitant acetylation on H3. Additionally, this interaction (mediated by SWI/SNF’s bromodomain) promotes $\gamma$H2AX formation\textsuperscript{88}, demonstrating a
novel crosstalk between histone modifications and chromatin remodeling enzyme recruitment to DSBs. Furthermore, another study showed that acetylation by CBP and p300 contributes to the recruitment of human SWI/SNF, and that SWI/SNF promotes recruitment of the NHEJ factor Ku70/80\textsuperscript{89}, again connecting SWI/SNF with acetylation events at a DSB. In yeast, SWI/SNF is readily recruited to a DSB\textsuperscript{90} but a link with acetylation has yet to be established. However, it is also recruited to the homologous donor region during HR-mediated repair\textsuperscript{90}, and \textit{in vitro} studies have shown that SWI/SNF has a unique ability to aid in homology search and synopsis in heterochromatic structures\textsuperscript{91}.

Rad54, a monomeric ATP-dependent chromatin remodeling enzyme related to SWI/SNF, also has roles in DSB repair. During HR repair, it helps stabilize the formation of the Rad51 nucleoprotein filament\textsuperscript{92,93}, and then later utilizes ATP to help convert the paranemic joint to the stable D-loop, disrupting a single nucleosome\textsuperscript{94}.

The catalytic subunit of the multi-subunit RSC complex, Sth1, shares a great amount of homology with Swi2 (SWI/SNF) but plays very different roles in DSB repair, although there are conflicting reports on exactly what those roles are. RSC is recruited early to a break, but was found only necessary for a very late stage of HR repair after synopsis\textsuperscript{90}. On the other hand, another study found that RSC facilitates the sliding or eviction of a few nucleosomes directly adjacent to a DSB very early after DSB formation\textsuperscript{95}. Additionally, RSC’s role in NHEJ is debatable due to conflicting reports of its affect therein\textsuperscript{90,96}.
Swr1, the eponymous member of its subfamily of Snf2-like ATPases, is the catalytic subunit of the SWR-C complex, and also recruited to a DSB\(^97\). SWR-C is a large multi-subunit chromatin remodeling enzyme and responsible for incorporating H2A.Z into chromatin genome-wide, although this histone variant is only transiently incorporated following a DSB\(^98,99\), even though SWR-C remains associated with the break region for much longer\(^97\). Inactivation of Htz1, the gene encoding for H2A.Z, alone causes only a slight growth defect in rich media, but a high sensitivity to DNA damaging agents and increased genomic instability\(^100,101\), as well as a delay in resection during DSB repair\(^99\). A recent study, using both \textit{in vitro} and \textit{in vivo} data, has nicely shown that SWR-C functions in promoting resection through the Exo1 pathway via its chromatin remodeling activity and also H2A.Z incorporation\(^102\). This is consistent with the actions of the mammalian homolog of Swr1, p400/TIP60, which incorporates H2A.Z into chromatin adjacent to a DSB, resulting in relaxation of the chromosomal conformation as well as additional histone modifications and damage response factor recruitment\(^103\).

A SWR-C related complex, INO80, is thought to reverse the incorporation of H2A.Z by SWR-C by exchanging H2A.Z dimers with free H2A dimers\(^98,101\). INO80 arrives to a DSB relatively slowly and covers up to 10kb away\(^97,104,105\). Both its unique Nhp10 subunit and its shared Arp4 subunit have been implicated in this recruitment mechanism\(^46,105\). The Arp4 subunit, shared with SWR-C and NuA4, is suggested to interact directly with \(\gamma\)H2AX at breaks\(^46\), but there are spatial and temporal discrepancies that cast doubt on the role of this interaction in repair. Indeed, we have recently shown
that the majority of recruitment of this enzymes, as well as five others – SWI/SNF, RSC, 
NuA4, SWR-C, and Fun30 – relies on Rad51 rather than γH2AX106 (see Chapter II). 
Interestingly, this puts the recruitment of INO80 and others after initial resection events 
during HR, indicating that the long-held “access, repair, restore” model for chromatin 
modifying enzyme involvement in DNA repair is not so simple. Additionally, another 
recent study has indicated that tethering INO80 to a genomic locus is sufficient to 
enhance movements of undamaged chromosomes within the nucleus107. Further, INO80, 
SWR-C and H2A.Z are necessary for chromosomal movements after DSB 
formation107,108. Moreover, two other studies have shown that such chromosomal 
mobility following a DSB is dependent on Rad51109,110 (which is necessary for INO80 
recruitment to a DSB) furthering the evidence for INO80 in this role.

The effect of INO80 on DSB repair is still controversial, specifically its role in the 
fate of nucleosomes and resection rates. A prevailing view of DSB processing is that 
nucleosomes are evicted because of, or to facilitate, resection. In support of this theory, 
two studies report an approximate two-fold loss of histones at a DSB in a manner 
dependent on MRX and INO8097,111. In contrast, there are two additional studies that 
found no significant loss of histones after DSB induction48,112. These discrepancies are 
evidence of the difficulties in interpreting data from chromatin immunoprecipitation 
assays at processed DSBs, as it is unclear how the cross-linking efficiency critical to 
these assays is affected by the formation of ssDNA during processing. Moreover, early 
biochemical studies demonstrated that nucleosome-like molecules can form on 
ssDNA113,114, suggesting that nucleosomes may interact with the ssDNA formed after
resection machinery pass through an area. However, whether this occurs in vivo at DSBs is still unknown. Finally, the effect of INO80 on the resection process is also controversial. One group reports that resection in the absence of a functional INO80 complex is reduced\textsuperscript{97}, while another reports that resection is normal but Rad51 recruitment is slowed\textsuperscript{111}.

Finally, a burst of recent research has been directed at the chromatin remodeling enzyme Fun30 and its involvement in DSB repair. Fun30, a member of the Swr1-like subfamily of ATPases\textsuperscript{20}, is unlike other well known remodeling enzymes in that it has no known binding partners that form a large complex, but instead is suggested to form a homodimer\textsuperscript{115}. In vitro assays have shown Fun30 to possess both nucleosome sliding and histone dimer eviction activities\textsuperscript{116,117}. Additionally, three recent studies have shown that Fun30 is important for promoting DNA-end processing after DSB formation\textsuperscript{118–120}. The activity became dispensable in the absence of the checkpoint mediator protein Rad9, indicating that Fun30 may function to remove Rad9 from chromatin in order to allow repair machinery access\textsuperscript{118}.

**Concluding Remarks**

The studies outlined above indicate that a wide variety of chromatin modifications occur in response to a DSB, as well as that many chromatin regulators are involved in the DNA damage response. Together they work to overcome the inherent barrier that the chromatin structure places on DNA-mediated events such as DNA repair. However, the
specific roles of those modifications and the chromatin regulators involved require additional study to fully understand. Many questions remain regarding how the enzymes are recruited to DSBs, their functional part in mediating repair, and how these many events interact with each other. This work attempts to address some of those questions.
CHAPTER II

DNA repair choice defines a common pathway for recruitment of chromatin regulators

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Abstract

DNA double-strand break (DSB) repair is essential for maintenance of genome stability. Recent work has implicated a host of chromatin regulators in the DNA damage response and, although several functional roles have been defined, the mechanisms that control their recruitment to DNA lesions remain unclear. Here, we find that efficient DSB recruitment of the INO80, SWR-C, NuA4, SWI/SNF, and RSC enzymes is inhibited by the non-homologous end joining machinery, and that their recruitment is controlled by early steps of homologous recombination. Strikingly, we find no significant role for H2A.X phosphorylation (γH2AX) in the recruitment of chromatin regulators, but rather their recruitment coincides with reduced levels of γH2AX.

Introduction

Cell viability and genomic stability are frequently threatened by chromosomal DNA double-strand breaks (DSBs). DSBs can be induced by endogenous free oxygen radicals, collapsed replication forks, or by exposure to DNA damaging agents, such as ionizing radiation (IR), UV light, and chemicals. 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. Consequently, cells have developed complex signaling networks that sense DSBs, arrest the cell cycle, and activate repair pathways.
Eukaryotic cells have evolved two major mechanisms that repair chromosomal DSBs, non-homologous end joining (NHEJ) and homologous recombination (HR). NHEJ is the predominant DSB repair mechanism in the G1 phase of the cell cycle, whereas HR predominates in the S and G2 phases\textsuperscript{23,24,26,122,123}. In the case of NHEJ, the broken DNA ends are recognized and bound by the Ku70/Ku80 heterodimer which subsequently recruits other factors to facilitate ligation of the ends\textsuperscript{124–126}. In contrast, DSB repair by HR relies on sequence homology from an undamaged sister chromatid or a homologous DNA sequence to use as a template for copying the missing information. The first step of HR involves extensive processing of the DSB such that the 5' ends of the DNA duplex that flank the DSB are resected to generate long, 3' single-stranded tails\textsuperscript{127}. Notably, extensive processing of the DSB ends is inhibited in G1 phase cells by the Ku70/80 complex\textsuperscript{26}, and increased CDK activity at the G1/S boundary activates DSB processing during later cell cycle phases\textsuperscript{23–25}.

DSB processing regulates the differential recruitment of two functionally related, checkpoint kinases ATM and ATR (Tel1 and Mec1, respectively in budding yeast). ATM recruitment does not require extensive DSB processing, while recruitment of the ATR/ATRIP (scMec1/Ddc2) checkpoint kinase complex requires the binding of the single stranded binding protein RPA to the processed DNA\textsuperscript{17,18}. One of the most intensively studied targets for checkpoint kinases is the histone variant H2A.X, which is phosphorylated at a C-terminal serine residue (H2A S129 in yeast or H2A.X S139 in higher eukaryotes; termed γH2AX). The formation of γH2AX is one of the earliest events at a DSB, and this mark spreads over at least a megabase of chromatin adjacent to
each DSB in mammalian cells, and up to 50 kb on each side of a DSB in budding yeast. Although γH2AX is not essential for the initial recruitment of DSB response factors, it plays a role in stabilizing the binding of checkpoint factors to DSB chromatin. Besides its role in the DNA damage checkpoint, γH2AX has also been proposed to recruit chromatin regulatory factors, namely the ATP-dependent chromatin remodeling complexes INO80 and SWR-C. These results have established γH2AX as both a ubiquitous hallmark and regulator of the chromatin response to DSBs.

In budding yeast, the DSB recruitment of chromatin regulators has been monitored primarily in asynchronous cell populations, and thus it is unclear if these events are linked to NHEJ or HR. In order to investigate whether the chromatin response to DNA damage is defined by a specific DSB repair pathway, we induced a single DSB within yeast cells synchronized in either G1 or G2/M cell cycle phases, and chromatin immunoprecipitation (ChIP) assays were performed to follow recruitment of many chromatin regulators. We surprisingly find that subunits of the INO80, SWR-C, NuA4, SWI/SNF, and RSC enzymes are primarily recruited outside of G1 phase, with the key NHEJ factor Ku70 inhibiting the recruitment of each of these enzymes in G1 cells. Furthermore, we find that recruitment of all chromatin regulators requires DSB processing and the Rad51 recombinase. In contrast to previous reports, we find that γH2AX plays no significant role in the recruitment of chromatin regulators to DSBs in either G2/M or asynchronous cells, though our data do suggest that chromatin regulators may enhance γH2AX dynamics during the HR process.
Results

Recruitment of chromatin regulators is cell cycle regulated

We use an established yeast system that has proven invaluable for monitoring the DSB recruitment of repair factors and chromatin regulators by chromatin immunoprecipitation (ChIP) analyses. This system allows for a single, persistent DSB to be induced on chromosome III by galactose-dependent expression of the HO endonuclease in a yeast strain that lacks homologous donor sequences\(^\text{128}\) (hml\(\Delta\) hmr\(\Delta\); Figure 2.1a). To investigate whether recruitment of chromatin regulators might be linked to the NHEJ or HR repair pathways, cells were first synchronized in G1 phase with alpha-factor mating pheromone (\(\alpha\)F), and then released into three different media conditions: (1) galactose and alpha factor to induce a DSB in G1 cells; (2) galactose and hydroxyurea to induce a DSB as cells exit G1 phase and arrest in S phase; and (3) galactose and fresh media to induce a DSB as cells exit G1 and subsequently arrest at the G2/M DNA damage checkpoint. Cell cycle arrest was confirmed by flow cytometry analysis (Supplementary Figure 2.S1a). Confirmation of robust formation of a DSB was done via qPCR using primers that span the cut site, whereby disappearance of product indicates cleavage. In this initial study we followed recruitment of the Arp5 subunit of the INO80 chromatin remodeling enzyme. Surprisingly, recruitment of Arp5 was very low in G1 cells and in cells arrested in S phase. In contrast, Arp5 recruitment was robust in cells that had received a DSB outside of G1 phase and accumulated at the G2/M cell cycle checkpoint (Figure 2.1b). To further investigate these results, cells were arrested in
Figure 2.1. Cell cycle regulated recruitment of chromatin modifying enzymes to an induced DSB. (a) Schematic of chromosome III of a donorless yeast strain harboring a galactose inducible HO endonuclease. Primers used during ChIP analyses are indicated according to their distance from the DSB, and designated with “-” for centrosomal-proximal and “+” for centrosomal-distal. (b) A wild-type, donorless strain was arrested in G1 using F, and then split into three cultures: maintained in αF-arrest (“G1”), released
into fresh media containing 0.2M hydroxyurea ("S(HU)"), or released into fresh media alone ("G2"). Galactose was also added at this time to induce a single DSB. Arp5 recruitment to areas surrounding the HO cut site was monitored by ChIP. (c,d) A wild-type, donorless strain was arrested with either αF ("G1") or nocodazole ("G2/M"), after which a DSB was induced by addition of galactose for the indicated times. Recruitment of various chromatin remodeling complexes to the DSB region was monitored by ChIP using antibodies to the indicated enzyme subunit. Fold enrichment reflects the %IP values normalized to the ACT1 locus, relative to time zero values. (e) H2A phosphorylation (γH2AX) is cell cycle regulated. Cells were treated as in panel (c) and levels of γH2AX were determined and normalized to levels of histone H3 also determined by ChIP. Data shown represent at least two biological replicates; error bars represent s.e.m.

either G1 phase with alpha factor or in G2/M with nocodazole, followed by galactose addition to induce a DSB. Initial cell cycle arrest was confirmed by flow cytometry (Supplementary Figure 2.S2a). Once again, recruitment of INO80, monitored by both Arp5 and the catalytic Ino80 subunit, was robust only when a DSB was induced in G2/M cells, with low levels of recruitment observed at a DSB induced in G1 cells (Figure 2.1c and Supplementary Figure 2.S2c). Consistent with previous findings in asynchronous cultures, recruitment of INO80 in G2-arrested cultures as well as asynchronous cultures was observed within a 10 kb chromatin domain adjacent to the DSB, and recruitment continued for at least 4 hours after DSB formation (Figure 2.1c and Supplementary Figure 2.S2g). Importantly, recruitment of the NHEJ factor yKu70 was also monitored, and in this case DSB recruitment was equal in both G1 and G2/M cells, similar to previous studies (Supplementary Figure 2.S2f). Given the unanticipated result of differential recruitment of INO80 during the cell cycle, we conducted further
ChIP assays to monitor recruitment of several other chromatin regulators, including subunits of the SWR-C, SWI/SNF, RSC, and Fun30 remodeling enzymes, as well as the NuA4 histone acetyltransferase complex. Interestingly, the recruitment of each of these chromatin regulators was much more robust outside of G1 phase, compared to G1 arrested cells (Figure 2.1d and Supplementary Figure 2.S2d). These data suggest that there may be a common, cell-cycle regulated mechanism for recruitment of multiple chromatin regulators to a DSB.

\textit{γH2AX is dispensable for recruitment of chromatin regulators}

Previous ChIP studies have indicated that formation of γH2AX is required for efficient DSB recruitment of INO80 and SWR-C within asynchronous cell populations\textsuperscript{97,104}. To understand how this mechanism interfaces with cell cycle regulation, we monitored the levels of γH2AX in chromatin surrounding DSBs formed in our experiments. Surprisingly, the levels of γH2AX surrounding the DSB were much lower in cells outside of G1 compared to those arrested in G1 (Figure 2.1e, and Supplementary Figures 2.S1b and 2.S3a). These contrasting levels of γH2AX are not due to changes in nucleosome density, as levels of H3 and H2B were reduced only ~2-fold in G2/M samples compared to G1, presumably due to DSB processing (Supplementary Figure 2.S3d, see below). Levels of γH2AX were also reduced in G2/M samples at early time points after DSB induction, when end processing has not progressed significantly (e.g. 30’), and when ChIP samples were processed in buffers containing 0.5M NaCl
(Supplementary Figure 2.S3b,c). Furthermore, we monitored formation of γH2AX following exposure of synchronized cells to the DSB-inducing agent phleomycin and again observed more robust γH2AX formation in G1 cells compared to G2/M cells, indicating that these cell cycle differences are not unique to an HO-induced DSB (Supplementary Figure 2.S3e,f). The data suggest that γH2AX levels or dynamics may be dramatically altered in chromatin surrounding DSBs formed within G2/M cells. Furthermore, these results imply that the levels of γH2AX and chromatin regulators are anti-correlated, indicating that γH2AX may not be involved in their recruitment.

In order to re-examine the role of γH2AX in recruitment of chromatin regulators, we monitored recruitment events in two different strains that lack γH2AX: a strain expressing a derivative of H2A (bulk yeast H2A is the equivalent to mammalian H2A.X) where serine 129 has been changed to an alanine residue (hta1,2-S129A)98, and a strain expressing a truncated H2A derivative that removes the final four C-terminal amino acids, including the Mec1/Tel1 phosphorylation site (hta1,2-S129AΔ4)46. Importantly, both of these strains exhibited similar sensitivity to the DNA damaging agent methyl methanesulfonate (MMS), as expected from previous studies129 (Supplementary Figure 2.S4a). Surprisingly, neither H2A-S129A nor H2A-S129AΔ4 reduced INO80 recruitment, irrespective of whether a DSB was induced in asynchronous or G2/M arrested cells (Figure 2.2a and Supplementary Fig. S4c). Indeed, recruitment of the Arp5 subunit of INO80 was slightly elevated in the strain expressing the C-terminal H2A truncation (Figure 2.2a). Similar results were found for Sth1 (RSC), Eaf1 (NuA4), Eaf3...
Figure 2.2. γH2AX is not essential for recruitment of chromatin regulators to a DSB. (a,b,c) Isogenic, donorless wild-type (wt), hta1,2-S129A (S129A), and hta1,2-S129Δ4 (S129Δ4) strains were arrested in G2/M using nocodazole, and analyzed by ChIP for recruitment of the indicated chromatin modifying enzyme subunits to the DSB region at the indicated time points after DSB induction. Data shown represent at least two biological replicates; error bars represent s.e.m.
(NuA4/Rpd3S), Swi2 (SWI/SNF), and Yaf9 (NuA4/SWR-C) (Figure 2.2b,c and Supplementary Figure 2.S4d,e). Interestingly, however, recruitment of the Snf6 subunit of SWI/SNF complex was markedly decreased in the absence the H2A C-terminus, even though its recruitment is not affected by the H2A-S129A substitution (compare Figure 2.2b and Supplementary Figure 2.S4d), implicating other residues within the H2A C-terminus. Why recruitment of the Swi2 and Snf6 subunits of SWI/SNF differentially respond to the H2A C-terminus remains unclear. However, when taken together, the data indicate that γH2AX does not regulate recruitment of chromatin regulators.

Although our hta1,2-S129A4 and hta1,2-S129A alleles were created within the same JKM strain background as two previously published studies, our ChIP data are contradictory97,104. We obtained the previously published hta1,2-S129* strain (also a four residue truncation; GA282418) and found that this strain shows similar sensitivity to DNA damaging agents as our hta1,2-129Δ strain (Supplementary Figure 2.S4a). However, strain GA2824 also exhibits an unexpected, severe growth defect in raffinose or lactate media, and liquid cultures arrested growth at low cell densities (e.g. OD600=0.4). Flow cytometry analysis also demonstrates that asynchronous cultures of GA2824 grown in raffinose media accumulate in the G1 phase of the cell cycle, and furthermore, this cell cycle distribution does not change following galactose addition to induce the HO endonuclease (Supplementary Figure 2.S4b). These growth defects precluded our ability to obtain high quality, reproducible ChIP data with this strain. Previous studies with the GA2824 strain have also indicated that γH2AX is required for efficient DSB processing18. However, a recent study shows that γH2AX inhibits DSB processing118,
and we also observe increased levels of RPA adjacent to a DSB in our \textit{hta1,2-S129Δ4} and \textit{hta1,2-S129A} strains (Supplementary Figure 2.S4f), consistent with a negative role for γH2AX in DSB processing. Since DSB processing is restricted in G1 cells, and INO80 and SWR-C are also poorly recruited in G1 cells, it seems likely that the aberrant slow growth and G1 accumulation phenotypes of the GA2824 strain were the cause of the previously observed defects in both DSB end processing and chromatin regulator recruitment, rather than a lack of γH2AX.18

\textit{Chromatin regulator recruitment requires DNA-end processing}

Our results indicate that the cell cycle regulation of the DSB response plays a key role in the recruitment of chromatin regulators to the DSB. Recruitment of chromatin factors outside of G1 coincides with the binding of RPA to ssDNA that is formed by the extensive processing of the DSB by the redundant Sgs1/Dna2 and Exo1 resection pathways24,33,34,37 (Supplementary Figure 2.S2d). Notably, this relationship is also consistent with the poor recruitment of Arp5 (INO80) to a DSB induced within HU treated cells (Figure 2.1b), as HU activates cell cycle checkpoints that inhibit DSB processing130. To examine the possibility that DSB recruitment of chromatin regulators requires resection, we monitored recruitment in isogenic \textit{sgs1Δ}, \textit{exo1Δ}, and \textit{sgs1Δ exo1Δ} strains. Strikingly, recruitment of Arp5 (INO80), Snf6 (SWI/SNF), Sth1 (RSC), Eaf3 (NuA4/Rpd3S), or Yaf9 (NuA4/SWR-C) was greatly reduced in the \textit{sgs1Δ exo1Δ} double mutant, with significant reductions occurring 500 bp distal to the DSB and reducing to
basal levels by 2.5 kb distal (Figure 2.3a). Importantly, the sgs1Δ exo1Δ strain showed a cell cycle profile identical to the wild-type strain (Supplementary Figure 2.S5a).

Consistent with the functional redundancy of these processing enzymes, only a minor defect in recruitment of INO80 to a DSB was observed in exo1Δ or sgs1Δ single mutants (Supplementary Figure 2.S5b). Fun30 has also been shown recently to depend on Exo1 and Sgs1 for its recruitment to a DSB118. Interestingly, γH2AX levels were also

**Figure 2.3.** DNA-end processing is required for recruitment to a DSB. Isogenic, donorless wild-type (wt) and exo1Δ sgs1Δ strains were grown asynchronously, and analyzed by ChIP in the region surrounding the DSB for (a) recruitment of the indicated chromatin modifying enzyme subunits 4 hours after induction (dotted line indicates the HO cut site), and (b) levels of γH2AX at the indicated time points after induction. Data shown represent at least two biological replicates; error bars represent s.e.m.
increased in the double mutant, most notably distal from the break (Figure 2.3b). These results suggest that DSB processing is required for optimal recruitment of multiple chromatin regulators and that their recruitment correlates with decreased γH2AX levels.

Previous studies have shown that the Ku70/80 heterodimer inhibits DSB processing in G1 cells, limiting DNA-end resection and promoting NHEJ\textsuperscript{26}. In addition, loss of Ku70 allows Rad52- and Rad51-dependent recombination events to occur efficiently in G1 arrested cells\textsuperscript{131}. To test whether the decreased recruitment of chromatin factors in G1 is due to limited DSB processing, an \textit{yku70Δ} strain was arrested in G1, and ChIP assays were performed at an HO-induced DSB. Strikingly, recruitment of all chromatin regulators was restored to high levels in the G1-arrested \textit{yku70Δ} cells (Figure 2.4a). In contrast, γH2AX levels were reduced in the G1-arrested \textit{yku70Δ} strain to levels previously seen in G2/M cultures, once again displaying an inverse relationship to the recruitment of chromatin regulators (Figure 2.4b). These results suggest that yKu70/80 inhibits recruitment of chromatin regulators in G1 cells, and furthermore, that recruitment is independent of cell cycle position. Notably, inactivation of Ku70 does not restore the recruitment of INO80 and SWI/SNF in the absence of Sgs1 and Exo1 (Supplementary Figure 2.S5d), strongly supporting the idea that DSB processing facilitates the recruitment of chromatin regulatory factors at the DSB. Previous work has shown that the yeast Mre11/Rad50/Xrs2 (MRX) complex directs processing of the initial ~100 bp of DNA proximal to the DSB\textsuperscript{33}. Indeed, we find high levels of the ssDNA binding protein RPA proximal to the DSB in \textit{exo1Δ sgs1Δ} cells (Supplementary Figure 2.S5c), a result consistent with MRX-dependent resection. To test whether MRX-
dependent processing might be responsible for the residual recruitment of chromatin regulators observed in the sgs1Δ exo1Δ double mutant, Arp5 (INO80) recruitment was
monitored in wild-type and \textit{mre11}\textDelta cells arrested in G2/M. Note that an \textit{exo1}\textDelta \textit{sgs1}\textDelta \textit{mre11}\textDelta strain was not constructed due to the expected growth defects of this strain. As shown in Supplementary Figure 2.S5e, Arp5 (INO80) recruitment was lost from DSB proximal chromatin in the absence of Mre11, and overall levels are similar to those found in G1 cells. Taken together, these data suggest a model in which DSB processing controls the recruitment of chromatin regulators, either through direct interactions with ssDNA or by subsequent events of the HR or the DNA damage checkpoint signaling pathway.

\textit{Rad51 is required for recruitment of chromatin regulators}

Following DSB processing, the ssDNA ends are initially bound by the single strand DNA binding protein RPA, which is subsequently replaced by the key recombinase, Rad51\textsuperscript{132}. Therefore, we tested whether Rad51 is key for recruitment of chromatin regulators. Strikingly, recruitment of Arp5 (INO80) was nearly eliminated in the G2/M arrested \textit{rad51}\textDelta strain, with a reduction to the two-fold recruitment level seen in G1 cells (Figure 2.5a). Furthermore, recruitment of Swi2 (SWI/SNF), Snf6 (SWI/SNF), Sth1 (RSC), Eaf3 (NuA4/Rpd3S), and Yaf9 (NuA4/SWR-C) were also nearly abolished (Figure 2.5a and Supplementary Figure 2.S6a). In addition, levels of \(\gamma\text{H2AX}\) were also increased in the absence of Rad51, consistent with one or more chromatin regulators controlling \(\gamma\text{H2AX}\) dynamics (Figure 2.5c). Importantly, Rad51 is
Figure 2.5. Rad51 is necessary for recruitment of chromatin regulators. Isogenic, donorless wild-type (wt), and (a) rad51Δ or (b) rad54Δ strains were arrested in G2 with nocodazole, and analyzed by ChIP for recruitment of the indicated chromatin modifying enzyme subunits to the region the DSB region at the indicated time points after DSB induction. A dotted line indicates the HO cut site. (c,d) Levels of γH2AX determined by ChIP of experiments described in panels (a) and (b), respectively. Data shown represent at least two biological replicates; error bars represent s.e.m.
not required for DSB processing or for establishing the checkpoint response\textsuperscript{33,132,133} (Supplementary Figure 2.S6b), indicating that formation of ssDNA is not sufficient for recruitment of chromatin regulators or for decreased levels of γH2AX.

Formation of the Rad51-ssDNA nucleoprotein filament plays a key role in the subsequent search and capture of a homologous DNA duplex. Rad51 also recruits Rad54 which is a member of the Snf2/Swi2 family of ATPases and exhibits weak chromatin remodeling activity \textit{in vitro}\textsuperscript{134}. Rad54 plays at least two roles during HR. First, Rad54 has an ATP-independent activity that facilitates Rad51 loading onto DNA proximal to the DSB\textsuperscript{92,94}, and second, Rad54 plays an ATP-dependent role to convert the initial joint molecule into a stable, strand invasion product that can be extended by DNA polymerases\textsuperscript{94,135}. To investigate possible roles for Rad54 in the recruitment of chromatin regulators, a DSB was induced in G2/M arrested \textit{rad54}Δ or \textit{rad54 K341R} strains, the latter of which contains an allele of \textit{RAD54} that inactivates its ATPase activity\textsuperscript{136}. ChIP assays for Arp5 (INO80), Swi2 (SWI/SNF), Sth1 (RSC), Eaf3 (NuA4/Rpd3S), or Yaf9 (NuA4/SWR-C), indicate a small but reproducible role for Rad54. In all of these cases, there is a defect in recruitment at locations proximal to the DSB, but less of an effect at distal locations (Figure 2.5b and Supplementary Figure 2.S7a). However, very few recruitment defects were observed in the strain harboring the ATPase defective version of Rad54 (Supplementary Figure 2.S7b). In contrast, recruitment of Snf6 (SWI/SNF) was nearly abolished in the absence of Rad54, or when the ATPase activity of Rad54 was inactivated (Supplementary Figure 2.S7). Thus, recruitment of the Snf6 subunit of SWI/SNF is distinct from both the Swi2 catalytic
subunit and from other chromatin regulators, requiring both Rad51 and the ATPase activity of Rad54.

**Discussion**

We have shown here that the recruitment of at least six chromatin regulatory enzymes – INO80, SWR-C, SWI/SNF, RSC, Fun30 and NuA4 – are recruited to a DNA double-strand break in a cell cycle-dependent manner, with at least five fold higher levels observed in G2/M cells compared to G1 cells. Our results indicate that recruitment is inhibited in G1 cells by the Ku70/80 complex, and that robust recruitment outside G1 is promoted by early steps of the HR process that lead to formation of the Rad51 nucleoprotein filament (Figure 2.6). Our data are not inconsistent with roles for chromatin regulators during NHEJ, as recruitment of chromatin regulators is low but not entirely abolished in G1. Indeed, recruitment of the INO80 complex in G1 cells is not affected by loss of Rad51, suggesting an independent mode for recruitment of chromatin regulators at this cell cycle phase. However, Rad51 is at least partially required for recruitment of INO80 in G1 cells that lack Ku70 (Supplementary Figure 2.S6c). Our data strongly support the view that chromatin regulators primarily impact repair events such as HR that occur following S phase. This idea is consistent with the known roles for the RSC, SWI/SNF, INO80, SWR-C, and Fun30 remodeling enzymes in distinct steps of HR and in cell cycle checkpoint control\(^{90,95,98,107,109,118-120}\).
Whereas recruitment of human INO80 to DSBs does not require γH2AX, three studies previously implicated γH2AX in the recruitment of the yeast INO80 and SWR-C remodeling enzymes. This conclusion was based primarily on three results: (1) ChIP assays using a strain harboring an H2A C-terminal truncation allele (hta1,2-S129*); (2) ChIP assays in a mec1 tel1 double mutant; and (3) co-purification of INO80 with γH2AX from cells treated with DNA damaging agents. Our current data indicate that the interpretation of previous ChIP data were confounded by the cell cycle distribution of the strain used: the previously employed H2A-S129* strain exhibits an aberrant accumulation of cells in G1, conditions where recruitment of INO80 and
SWR-C is poor. Likewise, we envision that the lack of G2 checkpoint arrest in the *mec1 tel1* double mutant led to a similar issue. Furthermore, we note that purification of INO80 in low salt buffers leads to co-purification of all four core histones\(^ {105}\), so it is expected that some level of $\gamma$H2AX will be associated with INO80 under DNA damage conditions. It is perhaps not surprising that $\gamma$H2AX does not control recruitment of INO80 or SWR-C, since their recruitment requires hours, whereas formation of the $\gamma$H2AX domain occurs within minutes. In addition, loss of $\gamma$H2AX leads to relatively little sensitivity to DNA damaging agents, whereas inactivation of INO80 causes a strong impact on the DNA damage response\(^ {104,129}\). Furthermore, as shown here and previously, the chromatin distribution of $\gamma$H2AX and INO80 do not coincide at DSBs\(^ {48,97}\), and furthermore, our ChIP data show an anti-correlation in the recruitment of chromatin regulators and $\gamma$H2AX signal. Although it remains a possibility that $\gamma$H2AX may play a role within G1 cells, our data do not support a dominant role of $\gamma$H2AX in recruitment of chromatin regulators to DSBs.

Previous studies in budding yeast have demonstrated high levels of $\gamma$H2AX in both asynchronous cell populations and in cells arrested in G1. However, no previous studies have reported $\gamma$H2AX levels for DSBs induced in cells synchronized outside of G1 phase. We were quite surprised to find a dramatic decrease in $\gamma$H2AX levels for DSBs induced with G2/M cells. This decrease does not appear to be due solely to DSB processing, as $\gamma$H2AX levels remain high in *rad51Δ* cells where DSB resection occurs normally. We envision that $\gamma$H2AX may be established at normal levels in G2/M cells, but that it is subject to enhanced dynamics, likely catalyzed by one or more chromatin
regulators. One possibility is that the low levels of γH2AX reflect dynamic exchange of H2A for H2A.Z by the SWR-C complex\textsuperscript{98,138,139}, although we find that γH2AX levels are not increased in a G2/M-arrested \emph{swr1Δ} strain (Supplementary Figure 2.S8). Removal of γH2AX, particularly in G2/M cells, is consistent with recent studies, and our own data, indicating a negative impact of γH2AX on DSB processing\textsuperscript{118} (Supplementary Figure 2.S4f).

We note that, although all the chromatin modifying complexes examined share a common set of requirements for recruitment to a DSB, only the Snf6 subunit of SWI/SNF shows a strong requirement for both the H2A C-terminus and Rad54, though neither was needed to recruit the Swi2 catalytic subunit. Our previous study indicated that Snf6 is uniquely associated with SWI/SNF\textsuperscript{140}, so it seems unlikely that it is recruited to DSBs by an independent mechanism. We favor a model in which the Snf6 subunit does not directly associate with nucleosomal DNA, and thus its cross-linking during the ChIP procedure is highly sensitive to small changes in SWI/SNF chromatin interactions.

How might Rad51 control the recruitment of chromatin regulators? One simple possibility is that the Rad51-ssDNA filament functions as an assembly platform for chromatin regulators and that each of these enzymes may directly interact with Rad51. Although there are no subunits held in common among all of the chromatin regulators that we have monitored, we note that each enzyme does harbor a member of the Actin-Related Protein (ARP) family that may provide a common interaction surface\textsuperscript{141}. Alternatively, it is possible that only a limited number of regulators interact directly with Rad51, and the activity of these few enzymes control recruitment of other complexes.
Testing this latter possibility may require the development of strains where multiple essential regulators can be removed simultaneously.

Recently, studies in *Drosophila* and yeast have demonstrated that DSB processing and the Rad51 recombinase are required for long-range, intra-nuclear movements of DSB chromatin during the homology search step of HR\textsuperscript{109,110}, and to regulate repair of heterochromatic DSBs by HR\textsuperscript{142}. Interestingly, yeast studies indicate that the ATPase activity of Rad54 is also essential for enhanced DSB mobility\textsuperscript{110}. Although the INO80 enzyme has been suggested as a candidate factor that catalyzes DSB mobility\textsuperscript{107}, our recruitment data suggests that other remodeling enzymes may also contribute, as all of the enzymes tested require Rad51 for their recruitment to DSBs. How ATP-dependent remodeling might promote chromosome dynamics is currently unclear, though the orchestration of such a complex event may provide one explanation for why so many chromatin regulators are recruited to a DSB.

**Methods**

*Yeast strains*

All strains are derivatives of JKM139 or JKM179\textsuperscript{128}, and were generated by the one-step PCR disruption method. Disruptions were confirmed by PCR analysis. Full genotypes are available in the appendix. All strains were grown at 30 °C in lactate media (1% yeast extract, 2% bactopeptone, 2% lactic acid, 3% glycerol, 0.05% glucose, pH6.6) or YPR (1% yeast extract, 2% bactopeptone, 2% raffinose) pre- induction. HO induction
was achieved by adding 2% galactose to each culture. Cultures were arrested in G1 using 1 µM alpha-factor (αF) treatment for 4 hours (bar1Δ strains). G2/M arrest was achieved using 30 µg/ml nocodazole for 4-5 hours. Arrests were confirmed by visual microscopy, followed by budding indices.

**Chromatin Immunoprecipitation**

Chromatin immunoprecipitations were done with some modifications as previously described:  50 mL of mid-log phase cells were cross-linked by adding 1% (final) formaldehyde for 15 minutes at room temperature, followed by neutralization with 150 mM (final) glycine for 5 minutes. Cell pellets were washed twice in cold TBS (50 mM Tris-Cl pH 7.5, 150 mM NaCl), and then resuspended in cold 400 µl FA-lysis buffer (50 mM HEPES-KOH pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate) plus 1X fresh “Complete” protease inhibitor cocktail (PIC; Roche). Cells were lysed with an equal volume of glass beads at 4 °C. After glass bead removal, samples were sonicated to shear DNA to an average size of 500 bp. An additional 1 mL FA-lysis buffer plus PIC was added and the chromatin lysate was purified by centrifugation at 14000 rpm for a total of 1.5 hours at 4 °C.

For most immunoprecipitations (IPs), 100-200 µl of the purified chromatin lysate was diluted up to 400ul with FA-lysis buffer plus PIC, and 1-2 µl antibody added. For SWI/SNF IPs, 1% (final) sarkosyl was also added. For high salt γH2AX IPs, FA-lysis buffer was replaced by FA-500 buffer (50 mM HEPES-KOH pH7.5, 500 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate) All IPs were incubated overnight at 4 °C, followed by incubation with 15 µl equilibrated sepharose protein A
beads (50% slurry; Rockland) for 2 hours at 4 °C. Pelleted beads were washed at room
temperature, for 5-10 minutes each, sequentially with FA-lysis buffer (except high salt
γH2AX IPs), FA-500 buffer, LiCl wash buffer (10 mM Tris-Cl pH 8.0, 250 mM LiCl, 1
mM EDTA, 0.5% NP-40, 0.5% sodium deoxycholate), and TE (10 mM Tris-Cl pH 7.5, 1
mM EDTA), followed by elution in Elution buffer (50 mM Tris-Cl pH 7.5, 10mM
EDTA, 1% sodium dodecylsulfate) shaking for 10 minutes at 65 °C. For input samples,
10 µl purified chromatin lysate was diluted in 450 µl TE. All samples (IPs and inputs)
were treated with proteinase K (0.2 mg/mL final) at 42 °C for 2 hours, cross-links
reversed by incubation at 65 °C for ≥ 5 hours, and purified by phenol-chloroform
extraction and ethanol precipitation. Input samples were diluted 20x over IP samples
during DNA purification.

The IP and input DNA was analyzed by quantitative real-time PCR with iTaq
SYBR Green Supermix with ROX (Biorad). Primer sequences are available in the
appendix. Fold enrichment represents the ratio of recovered DNA to input DNA of the
break region, normalized to the same ratio obtained for the ACT1 ORF. These ratios
were additionally normalized to pre-induction (0 hr) values and corrected for DSB
induction. Percent IP (for anti-RPA only) represents the ratio of IP DNA to input DNA
corrected for dilution, and is not normalized to a control region, because those values
approached zero. Error bars indicate standard error of the mean from at least 2
independent biological replicas and four PCR reactions.
**Western blotting**

Whole cell extracts were prepared by trichloroacetic acid precipitation and proteins were separated by sodium dodecyl sulfate-polyacrylamide electrophoresis in 18% acrylamide gels. Samples were blotted onto polyvinylidene difluoride membranes and probed with antibodies using standard methods.

**Antibodies**

Polyclonal antibodies to HA tag (ab9110), Arp5 (ab12099), Yaf9 (ab4468), Eaf3 (ab4467), H2A-S129phos (γH2AX; ab15083), and Myc tag (ab9132) are commercially available from Abcam. Anti-H2B (39237) is available from Active Motif. Anti-Snf6 and anti-Swi2, Anti-RPA, Anti-Sth1, Anti-Eaf1, and Anti-Ku antibodies were kind gifts from J. Reese (Pennsylvania State University), V. Borde (Institut Curie), B. Cairns (University of Utah), J. Cote (Laval University Cancer Research Center), and S.E. Lee (University of Texas Health Science Center at San Antonio), respectively.

**Flow cytometry**

Approximately 1 mL of mid-log phase (~1-2 x10^7) cells were collected per sample, washed in water, fixed in 100% ethanol, and incubated at 4 °C rocking overnight. After fixation, cells were again washed in water, resuspended in 50 mM Tris pH 8.0, containing 200 μg/mL RNase A and incubated at 37 °C for 2-4 hours. Samples were then pelleted and resuspended in 50 mM Tris pH 7.5 containing 2 mg/mL Proteinase K and incubated at 50 °C for 30-60 minutes, followed by resuspension in 500 μl FACS buffer (200 mM Tris pH 7.5, 200 mM NaCl, 78 mM MgCl_2). Approximately 100 μl of each
sample was then incubated for 10 minutes at room temperature with 1 mL Sytox solution (50 mM Tris pH7.5, 1 µM Sytox Green (Molecular Probes S-7020)) and sonicated gently for approximately 30 seconds directly before analysis on a BD FACSCalibur flow cytometer. Data analysis and preparation completed with FlowJo.
Supplementary Information

Figure 2.S1. Cell cycle dependence of γH2AX. Cells were treated as described in Figure 1b and (a) analyzed by flow cytometry for cell cycle distribution, and (b) analyzed by ChIP for levels of γH2AX surrounding the break site. Data represent at least two biological replicates; error bars represent s.e.m.
Figure 2.S2. Cell cycle dependent recruitment to a DSB. (a) Flow cytometry analysis showing effective cell cycle arrest using alpha-factor mating pheromone (α-factor) to arrest cells in G1, and nocodazole to arrest cells in G2/M. (b) Representative double-strand break induction for cell cycle arrested ChIP experiments. Percent uncut was calculated using qPCR signal achieved from primers spanning the HO cut site and normalized to a control region. (c-f) Cells were treated as described in Figure 1c and analyzed by ChIP for recruitment of the indicated proteins in the regions surrounding an induced DSB. (g) Wild-type, donorless cells were grown asynchronously and assayed by ChIP for recruitment of Arp5 to the region surrounding the DSB. Data shown represent at least two biological replicates; error bars represent s.e.m.
Figure 2.S3. Cell cycle regulation of H2A.X phosphorylation. (a,b) Cells were treated as described in Figure 1c and analyzed by ChIP for levels of γH2AX at the indicated time points after DSB induction. (c) As in (a) except that the initial antibody binding buffer conditions contained 500mM NaCl, rather than 140mM NaCl. (d) Cells were treated as described in Figure 1c and analyzed by ChIP for occupancy of histones H3 and H2B in the regions surrounding a DSB. ChIP data shown represent at least two biological replicates, with exception of panels (b) and (c) which represent one biological experiment each; error bars represent s.e.m. (e,f) Wild-type W303 (CY1343) or JKM139 (CY1508) cells, respectively, were arrested in G1 with αF or G2/M with nocodazole, then exposed to 10 µg/ml phleomycin and samples taken after two and four hours of exposure. Western-blot analysis was then performed using antibodies to phosphorylated histone H2A-S129 and unmodified histone H2B.
Figure 2.54. Analysis of H2A.X phosphorylation-deficient mutants. (a) Phosphorylation mutants show expected sensitivity to methyl methanesulfonate (MMS). Serial dilutions of the indicated strains were spotted onto rich media or media containing 0.02% MMS. (b) Strain GA2824 accumulates in the G1 cell phase. FACS analysis in rich media containing 2% raffinose of wild-type (wt), our H2A C-terminal truncation strain (hta1,2S129A; CY1722), the previously described hta1,2-S129* strain (GA2824), and GA2824 after four hours of exposure to galactose. (c) H2A phosphorylation does not affect recruitment of INO80 to an induced DSB, regardless of cell cycle. A DSB was induced in freely cycling isogenic, donorless wild-type (wt) and hta1,2-S129A (S129A) cells and analyzed by ChIP for recruitment of an HA-tagged Ino80 to the region surrounding the DSB. (d) H2A phosphorylation does not affect recruitment of Eaf3 (NuA4/Rpd3S) to an induced DSB. Cells were treated as in Figure 2b and analyzed by ChIP. (e) H2A phosphorylation does not affect recruitment of Snf6 (SWI/SNF) to an induced DSB. Cells were treated as in Figure 2c and analyzed by ChIP. (f) H2A phosphorylation inhibits resection. Cells were treated as in Figure 2a and recruitment of RPA surrounding the DSB was determined by ChIP. Data shown represent at least two biological replicates; error bars represent s.e.m.
**Figure 2.S5.** DNA-end processing is needed for recruitment to a DSB.  (a) Flow cytometry analysis confirms that wild-type (wt) and exo1Δsgs1Δ strains have identical cell cycle distributions.  (b) Isogenic, donorless wild-type (wt), exo1Δ, and sgs1Δ cultures were grown asynchronously and assayed by ChIP for recruitment of HA-tagged Ino80 to regions surrounding an induced DSB.  (c) Levels of RPA and histone H3 confirm a defect in long range resection.  Cells treated as in Figure 3 and analyzed by ChIP.  (d) Ku inhibition of recruitment is epistatic to end processing.  Isogenic, donorless wild-type (wt) and exo1Δsgs1Δ yku70Δ cultures were grown asynchronously and analyzed by ChIP for recruitment of Arp5 and Snf6 to a DSB.  (e) MRX promotes recruitment of chromatin modifying enzymes.  Isogenic, donorless wild-type (wt) and mre11Δ cells were arrested in G2/M using nocodazole and analyzed by ChIP for levels of Arp5 and γH2AX to a DSB.  For all line graphs: data represent samples taken four hours post DSB induction; a dotted line indicates the HO cut site.  Data shown represent at least two biological replicates; error bars represent s.e.m.
Figure 2.S6. Rad51 is required for recruitment to a DSB. (a) Cells were treated as in Figure 5a and analyzed by ChIP for Eaf3 and Snf6 to the DSB region. (b) Quantification by qPCR of input DNA from experiments described in Figure 5a relative to a control region (ACT1). (c) An independent recruitment pathway for INO80 exists in the G1 cell phase. Donorless, isogenic wild-type (wt), rad51Δ, yku70Δ, and rad51Δyku70Δ strains were arrested in G1 with αF and assayed by ChIP for recruitment of Arp5 surrounding an induced DSB. Data shown represent at least two biological replicates; error bars represent s.e.m.
Figure 2.S7. Effects of Rad54 on recruitment to a DSB. Isogenic, donorless wild-type (wt) and either (a) rad54Δ or (b) rad54K341R (a catalytically-dead version of Rad54) cultures were arrested in G2/M using nocodazole and analyzed by ChIP for recruitment of the indicated proteins to the DSB region. (c) γH2AX levels determined by ChIP in cultures described in (b). Data shown represent at least two biological replicates; error bars represent s.e.m.
Figure 2.8. SWR-C deletion does not increase $\gamma$H2AX at a DSB. Isogenic, donorless wild-type (wt) and swr1Δ cultures were arrested in G2/M with nocodazole and assayed by ChIP for levels of $\gamma$H2AX surrounding an induced DSB. Data shown represent two biological replicates; error bars represent s.e.m.
CHAPTER III

The effects of Fun30 on histone occupancy and γH2AX at DNA double-strand breaks
Abstract

DNA damage repair is crucial for genome stability and cell survival. Particularly damaging is the DNA double-strand break (DSB). Many recent studies have shown the involvement of chromatin remodeling enzymes in DSB repair. Among them, the Snf2-related Fun30 has very recently been shown to participate in the repair by promoting DNA-end processing after break formation. However, it was unclear if Fun30’s ability to exchange histone dimers played a role in this activity. Here we investigated the effects of Fun30 on nucleosome occupancy and the phosphorylation of histone H2A.X. We find that, while nucleosome occupancy adjacent to a break is altered in the absence of Fun30, strikingly, there was a very limited effect on the histone phosphorylation.

Introduction

The repair of DNA damage is very important for the survival of any cell. Without proper repair, damaged DNA can lead to not just local mutation, but also cell death and, for multicellular organisms, cancerous tumorigenesis. While there are many forms of DNA damage, the most detrimental to the cell is the DNA double-strand break (DSB). These lesions are primarily repaired via two distinct pathways: non-homologous end joining during the G1 cell phase and homologous recombination during later cell phases after DNA synthesis. The hallmark of homologous recombination is the processing of the DNA ends to reveal 3’ single stranded DNA overhangs (known as
resection) that subsequently searches for a homologous region of the genome to use as a template for repair\textsuperscript{127}. A major hurdle for repair in this process is the chromatin structure, which must be modified to allow for repair machinery to access the DNA. However, details of what happens to the chromatin structure during this repair, and the fate of nucleosomes that are altered is still unclear.

In response to a DSB, the cell launches a complex and immediate response. One of the very first steps in this is the recruitment and activation of checkpoint kinases Mec1 and Tel1\textsuperscript{17,18}. While these kinases have many targets, one very important target is the histone variant H2A.X which is phosphorylated at its C-terminal end (termed $\gamma$H2AX). $\gamma$H2AX forms in large domains to either side of a DSB is considered\textsuperscript{47,48} and also participates in binding stabilization of several DNA damage response factors\textsuperscript{44}. Among these factors is the checkpoint mediator protein Rad9, which has a conserved role in inhibiting resection at DSBs\textsuperscript{58,59,144–147}. $\gamma$H2AX persists throughout the repair process, and is only removed after the removal of the histone from chromatin\textsuperscript{64}. This process possibly occurs through the action of a chromatin remodeling enzyme, but which one, if any, remains to be identified. Notably, we have also shown that $\gamma$H2AX is detected at much lower levels in G2/M cell phase when compared to G1\textsuperscript{106}, but the function and mechanism of this difference remains unexplained.

The relatively unstudied chromatin remodeling enzyme Fun30 has received a burst of recent study including evidence that it is involved in DNA repair processes. Fun30, unlike other well known remodeling enzymes, has no known binding partners that form a large complex, but instead is suggested to form a homodimer\textsuperscript{115}. \textit{In vitro} assays
have shown Fun30 to possess both nucleosome sliding and histone dimer eviction activities\textsuperscript{116,117}. Additionally, three recent studies have shown that Fun30 is important for promoting DNA-end processing after DSB formation\textsuperscript{118–120}. However, the activity is dispensable in the absence of the checkpoint mediator protein Rad9, indicating that Fun30 may function to remove Rad9 from chromatin in order to allow repair machinery access\textsuperscript{118}. Indeed, Fun30’s function in resection is also partially dispensable in a mutant where one of Rad9’s binding platforms, γH2AX, does not form\textsuperscript{118}. Moreover, we observe that Fun30 is recruited to DSBs at greater levels during G2/M when histone disruption is at its greatest (Figure 2.S2). This led us to hypothesize that Fun30 functions to promote resection in DSB repair by removing histone dimers containing γH2AX in regions near to the damage site. Here we investigated the role of Fun30 in histone dynamics at a DSB and find that while it does have a significant function in removing histones from DNA surrounding a break, this action is constrained to just several hundred base pairs to either side of the DSB. In addition, we find that γH2AX dynamics are only moderately affected by Fun30 in a narrow region just a few kilobases away from the DSB.

\textbf{Results}

\textit{Fun30 has a significant effect on histone occupancy close to a DSB}

We have already reported that Fun30 is detected at higher levels at a break during the G2/M cell cycle phase, while at the same time γH2AX is significantly reduced when
compared to amounts observed in G1-arrested cultures (Figure 2.1), so we conducted our experiments in cultures arrested in G2/M with nocodazole. As in our previous studies, we used a yeast strain that allows us to create an inducible DSB which cannot be repaired because the homologous regions are deleted\textsuperscript{128}. Using chromatin immunoprecipitation (ChIP), we first investigated the effects Fun30 has on histone occupancy next to an induced DSB in on chromosome III at the \textit{MAT} locus. According to our hypothesis we would not expect loss in histone occupancy adjacent to the break. Indeed, when we monitored histone H2B in \textit{fun30Δ} cells we found a significant increase in occupancy (Figure 3.1a). This effect was limited to less than one kilobase from the break, which agrees with our previous results indicating H2B occupancy isn’t affected at distances beyond this\textsuperscript{106}.

Given that Fun30 genetically interacts with the histone variant H2A.Z and components of SWR-C\textsuperscript{148}, the complex that incorporates it into chromatin\textsuperscript{138,148}, we were also interested in the effects on H2A.Z occupancy. Similarly to H2B, the absence of Fun30 caused a defect in the removal of histone H2A.Z (Figure 3.1b). Interestingly, H2A.Z was only detected at low levels beyond one kilobase from the DSB, similar to levels found at the control region, the open reading frame of \textit{ACT1}. In contrast, H2A.Z enrichment was two to four fold higher over the control locus at regions just 500 bp from the break (Figure 3.1c). Since H2A.Z is enriched at gene promoters rather than within open reading frames\textsuperscript{149,150}, this suggests that at the \textit{MAT} locus H2A.Z is only enriched in regions directly adjacent to the HO cut site. In light of this, it was not surprising to find that the effect of Fun30 on H2A.Z removal was limited to this same region.
Figure 3.1. Fun30 promotes histone eviction close to a DSB. Wild-type and fun30Δ cultures were arrested in G2/M with nocodazole, and analyzed by ChIP for levels of (a) histone H2B, (b) histone variant H2A.Z, and (d) histone H3 surrounding the DSB at the indicated time points after DSB induction. (c) H2A.Z levels surrounding the DSB site relative to the control locus (ACT1) before galactose addition. Asterisks indicate where the change from 0 h has a p-value < 0.05.
Finally, we examined the occupancy of histone H3 in a \textit{fun30Δ} mutant. Again we found the same failure to remove histones from the break proximity (Figure 3.1d), suggesting that whole nucleosomes are failing to be removed from the break region. Taken together, these data indicate that Fun30 plays a significant role in nucleosome eviction during DSB repair during the G2/M cell phase.

\textit{Fun30 has a limited effect on γH2AX near a DSB}

We next investigated the effect of Fun30 on the presence of γH2AX surrounding a DSB. We found that in \textit{fun30Δ} cells, there was a significant increase in γH2AX levels within a few kilobases of the break site (Figure 3.2a). Interestingly, the effect on γH2AX levels in the mutant was not observed at distances farther from the break (Figure 3.2a) where γH2AX usually accumulates at much higher amounts\textsuperscript{46,48,106}. Because we have found that overall histone occupancy levels are affected in a \textit{fun30Δ} mutant, we needed to evaluate the effect on γH2AX in this context. Therefore, we normalized the γH2AX amounts in each experiment to the concurrent H2B levels (Figure 3.2b). After this analysis, we can see that any apparent effect on γH2AX close to the break in \textit{fun30Δ} cells is due to the simultaneous accumulation of histones in that region. However, in regions one to three kilobases away from the DSB, where there were no effects on histone occupancy in the mutant, the impact on γH2AX levels by Fun30 remains real. Taken together, although Fun30 does apparently inhibit γH2AX levels, this effect is limited to a narrow region just a few kilobases long.
Discussion

In this study we investigated the impact of Fun30 on histone dynamics at DNA double-strand break. We have found that Fun30 promotes the removal of nucleosomes from areas surrounding an induced DSB in the G2/M cell phase, but this is limited to less than one kilobase in distance. Furthermore, in the absence of Fun30 each histone monitored – H2B, H2A.Z, and H3 – was not only not removed from the break region, but...
occupancy increased significantly when compared to unbroken DNA (Figure 3.1).

Considering that our previous observations suggest that even in wild type cells not all histones are removed from regions proximal to the break\textsuperscript{106}, the increase in histone occupancy offers a model where nucleosomes are slid in cis away from the break site in the absence of Fun30. Thus, in wild type cells Fun30 would function to disrupt nucleosomes to either side of a DSB.

While examining histone occupancy in our experiments, we also found H2A.Z enrichment at distances farther from the break to be similar to quantities found at our control region and to unbroken MAT DNA. However, these quantities are significantly above those found in a strain lacking Swr1, where no incorporation of H2A.Z occurs, indicating a low level of integration. Importantly, we did not observe any increase in H2A.Z enrichment after break induction at any of the loci tested. Although a previous study did find that H2A.Z is incorporated at the MAT locus after break induction, this was a transient event\textsuperscript{99}. Further, our data is not inconsistent with this finding because our cultures are synchronized in the G2/M cell phase rather than freely cycling. This may indicate that H2A.Z incorporation in response to a DSB is specific to the repair response outside of G2.

Our results indicate that Fun30 does not have a large effect on \(\gamma\)H2AX dynamics, in contradiction with our original hypothesis. It is important to note that the little effect Fun30 does have on \(\gamma\)H2AX is limited to a few kilobases from the break site. This was unexpected because Fun30 has the greatest defect in long range DNA resection. Yet, because Fun30 is detected at higher levels closest to the break site\textsuperscript{118,120}, it remains
possible that its effect on long range resection is due to activities within these regions adjacent to the DSB.

**Methods**

**Yeast strains**

All strains are derivatives of JKM139\textsuperscript{128}, and were generated by one-step PCR disruption and confirmed by PCR analysis. Full genotypes are available in the appendix. All strains were grown at 30 °C in lactate media (1% yeast extract, 2% bactopeptone, 2% lactic acid, 3% glycerol and 0.05% glucose, pH 6.6) pre-induction. HO induction was achieved by adding 2% galactose to each culture. G2/M arrest was achieved using 30 mg/ml nocodazole for 4-5 hours.

**Chromatin Immunoprecipitation**

Lysates were prepared as previously described\textsuperscript{106}. Briefly, mid-log phase cells were cross-linked by adding 1% (final) formaldehyde for 15 minutes at room temperature, followed by neutralization with 150 mM (final) glycine for 5 minutes. Lysed cells were sonicated to obtain an average DNA fragment size of 500 bp. The lysate was subsequently purified by centrifugation at 14000 rpm for a total of 1.5 hours at 4°C. Immunoprecipitations (IPs) were performed by adding 1-2 µl of antibody to diluted chromatin lysate and incubated overnight at 4°C diluted. Sarkosyl (1% final) was also added for IPs with SWI/SNF antibodies. All IPs were incubated with sepharose protein A beads (50% slurry; Rockland) for 2 hours at 4°C, washed several times, and eluted for
10 minutes at 65°C. DNAs were purified by treatment with proteinase K, cross-links reversal at 65°C, and phenol-chloroform extraction and ethanol precipitation. 10% input samples were diluted 20x over IP samples during DNA purification. The recovered IP and input DNA was analyzed by quantitative real-time PCR with iTaq Universal SYBR Green Supermix with ROX (Biorad). Primer sequences are available in the appendix. Fold enrichment represents the ratio of IP to input DNA at the break region, normalized to the same ratio obtained for the ACT1 ORF. These values were corrected for DSB induction and normalized to pre-induction (0 h) values. Error bars indicate standard error of the mean from at least 2 independent biological replicates and at least four qPCRs.

**Antibodies**

Rabbit polyclonal antibody H2A-S129phos (γH2AX; ab15083) is commercially available from Abcam. Rabbit polyclonal antibodies H2B (39237), Htz1 (H2A.Z; 39647), and H3 (39163) are commercially available from Active Motif.
CHAPTER IV

SWI/SNF recruitment to double-strand breaks by NuA4-dependent acetylation

This chapter was published as the following, presented here with minor additions:

Gwendolyn Bennett and Craig L. Peterson.

Author Contributions:
G.B. designed and performed all experiments, and assisted in manuscript preparation;
C.L.P. acted in project overview, data interpretation, and manuscript preparation.
Abstract

The DNA damage response to double-strand breaks (DSBs) is critical for cellular viability. Recent work has shown that a host of chromatin regulators are recruited to a DSB, and that they are important for the DNA damage response. However, the functional relationships between different chromatin regulators at DSBs remain unclear. Here we describe a conserved functional interaction among the chromatin remodeling enzyme, SWI/SNF, the NuA4 and Gcn5 histone acetyltransferases, and phosphorylation of histone H2A.X (γH2AX). Specifically, we find that the NuA4 and Gcn5 enzymes are both required for the robust recruitment of SWI/SNF to a DSB, which in turn promotes the phosphorylation of H2A.X.

Introduction

DNA damage repair is essential for cell viability and genomic stability. The most severe form of DNA damage, the double-strand break (DSB), can arise from exposure to damaging chemicals, ultraviolet or ionizing radiation, free oxygen radicals, or DNA replication errors, and failure to properly repair DSBs can lead to genome instability and cell death. Upon DSB formation, the cell launches a complex network of signals to elicit cell cycle arrest and repair functions. In addition, the complex compaction of eukaryotic DNA into chromatin necessitates the involvement of a diverse group of regulators to modify this structure and enable access for DNA repair.
A key first step of the DNA damage response is the activation of the checkpoint kinases (Mec1 and Tel1 in yeast, or ATM and ATR in mammals) that catalyze the phosphorylation of histone H2A.X at a C-terminal serine residue (S129 in yeast and S139 in mammals). This is a hallmark of the damage response, and although it does not directly recruit repair proteins or chromatin regulators, it does play an important role in stabilizing the binding of damage response factors to the DSB lesion\textsuperscript{44,106}. In addition, several other chromatin modifications are formed in regions surrounding a break and have been shown to be important for DSB repair. Indeed, acetylation of H3 and H4 N-terminal domains has been shown to be important for DNA repair and facilitate factor accessibility to DNA\textsuperscript{46,80,81,129}. In support of the importance of acetylation to DSB repair, the deletion of factors responsible for the acetylation and deacetylation of these residues, including the yeast histone acetyltransferases Gcn5 and Esa1, severely impacts repair and cell survival after damage\textsuperscript{80,81,124}.

Several ATP-dependent chromatin remodeling enzymes have also been shown to be recruited to a DSB \textit{in vivo}, and facilitate various aspects of repair. INO80 and SWR-C have been shown to be important for DNA end processing and checkpoint regulation\textsuperscript{97,98,102,104}, while RSC and SWI/SNF have been implicated in remodeling nucleosomes during repair\textsuperscript{90,95,96}. However, the interplay between chromatin remodeling and histone modifications is still under investigation. Interestingly, a study has indicated that human SWI/SNF facilitates the phosphorylation of H2A.X by interacting with nucleosomes acetylated by Gcn5\textsuperscript{88}. Here we describe a similar functional interaction in yeast, but that, in addition to Gcn5 acetylation, activity of the Esa1-containing histone
acetyltransferase, NuA4, plays a key role in recruiting SWI/SNF to the region surrounding a DSB. Furthermore, the subsequent action by SWI/SNF to promote γH2AX is dependent on its ability to interact with acetylated histone residues through a bromodomain within the Swi2 subunit.

Results

Gcn5 promotes recruitment of chromatin regulators to a DSB, H2A.X phosphorylation, and DSB resection.

In order to study DSB responses in yeast, we used an established genomic system which places the endogenous HO endonuclease under the galactose promoter which allows for galactose-dependent induction of a single double-strand break (DSB) at the MAT locus (Figure 4.1a). These strains also lack homologous donor loci at either end of the chromosome, so the DSB cannot be repaired by homologous recombination, and continuous HO expression ensures that the DSB remains unrepaired throughout the experiment. Since the Gcn5 acetyltransferase is key for recruitment of SWI/SNF to a DSB in human cells, we tested whether Gcn5 plays a similar role in yeast. We first deleted the GCN5 locus, and performed chromatin immunoprecipitation assays to measure the recruitment of chromatin regulators. Because it has been previously shown that the majority of chromatin regulators are recruited to a DSB during G2/M^{106}, we synchronized cultures in G2/M with nocodazole and then induced a DSB by galactose addition. Importantly, the induction of the break was not affected by the mutation (Table
Figure 4.1. Gcn5 promotes γH2AX and the recruitment of chromatin remodelers to a DSB. (a) Schematic of the donorless yeast strain harboring a galactose inducible HO endonuclease, which cuts at the MAT locus of chromosome III. The approximate locations of regions amplified for ChIP analyses to the right (“+”) of the break are shown in red and labeled according to their distance from the DSB in kilobases (kb). (b, c) Wild-type (wt), and gcn5Δ strains were arrested in G2/M using nocodazole, and analyzed by ChIP for recruitment of the indicated chromatin modifying enzyme subunits to the DSB region at the specified time points after DSB induction. (d) Input DNA at the break site relative to an unbroken locus (ACT1) and normalized to pre-induction levels. (e) As in (b), γH2AX levels determined by ChIP at the indicated time points after break induction.
We monitored the recruitment of two subunits of SWI/SNF, Swi2 and Snf6, and found that their recruitment was dramatically lower in the absence of Gcn5 (Figure 4.1b), mirroring the behavior of human SWI/SNF. We also monitored the levels of two other chromatin regulators, Yaf9, a shared subunit between the NuA4 and SWR-C enzymes, and Arp5, a subunit of INO80. Surprisingly, recruitment of Yaf9 was also decreased by the lack of Gcn5 (Figure 4.1c), even though the removal of Gcn5 has previously been shown to have no negative effect on SWR-C occupancy. On the other hand, Arp5 was not significantly affected (Figure 4.1c). Taken together, these data suggest that Gcn5 has a role in the recruitment of several chromatin regulatory complexes to a DSB.

Recruitment of chromatin modifying enzymes is tightly linked to DNA-end processing, where the 5' strand is resected and produces a long 3' ssDNA tail, and is a hallmark of the homologous recombination pathway that is favored in G2/M. To monitor DSB resection, quantitative PCR of ChIP input DNA was used to compare the DNA signal at the DSB site to an undamaged locus (ACT1). Since resection will eliminate one strand of DNA, this leads to a 2-fold decrease in the relative PCR amplification signal. While the wild-type strain showed 60% resection of the DSB locus by 4 hours, resection in the gcn5Δ strain was only decreased to about 30% (Figure 4.1d). Therefore, one possibility is that the decrease in resection rates in the gcn5 mutant may be responsible for the decrease in SWI/SNF and Yaf9 recruitment.

DSB resection rates and the phosphorylation of histone H2A.X typically show an inverse relationship. Thus, extensive resection generally leads to a decrease in γH2AX, and defects in resection are associated with increased levels of γH2AX.
Notably, although the \textit{gcn5} mutant showed decreased levels of resection, there was a significant reduction of $\gamma$H2AX after 4 hours of DSB induction (Figure 4.1e). Taken together, these data suggest that Gcn5 has a broad impact on early events at a DSB where it appears to promote DSB resection, chromatin regulator recruitment, and $\gamma$H2AX formation.

\textit{The NuA4-dependent acetylation promotes the recruitment of SWI/SNF to a DSB, as well as H2A.X phosphorylation.}

Next we examined the role of another histone acetyltransferase, NuA4, which – along with its mammalian homolog, Tip60 – has been shown to be important for DSB repair and $\gamma$H2AX levels\textsuperscript{83}. Since the catalytic subunit of NuA4, Esa1, is essential for yeast viability, we utilized an inducible degron system to degrade Esa1 before DSB induction in order to study its role in SWI/SNF recruitment. We fused the auxin-inducible degron (AID) cassette to the C-terminus of \textit{ESA1} in a strain where the \textit{Arabidopsis thaliana (At)} \textit{TIR1} gene is under constitutive expression. The binding of \textit{AtTIR1}, an F-box protein, to the highly conserved Skp1 protein is promoted by auxin, and forms an E3 ubiquitin ligase that targets Esa1-AID for degradation. We grew \textit{ESA1-AID}-containing cells in nocodazole prior to the addition of either synthetic auxin (1-naphthaleneacetic acid; NAA) to induce degradation, or ethanol as a control (Figure 4.2a). Cells were maintained at 22°C during the auxin treatment since \textit{AtTIR1} has
Figure 4.2. NuA4 promotes SWI/SNF recruitment to a DSB and γH2AX.  
(a) Schematic representing the yeast culture growth and treatments.  
(b) Western blot of samples from ESA1-AID containing yeast strains treated with either ethanol or 500µm NAA.  untr. = untreated samples.  
(c,d) The ESA1-AID yeast strain treated with ethanol or NAA and analyzed by ChIP for the indicated chromatin modifying enzyme subunits after DSB induction.  
(e) γH2AX levels determined by ChIP at the indicated time points after break induction.  
(f) Input DNA at the break site relative to an unbroken locus (ACT1) and normalized to pre-induction levels.
optimal activity at this lower temperature. At this lower temperature, the timecourses were extended to 6 hours of DSB induction, and Gal-HO induction remained similar between the cultures (Table 4.S2). This protocol resulted in a robust depletion of Esa1 (Figure 4.2b).

After Esa1 degradation (+NAA), the DSB recruitment of Swi2 and Snf6 were significantly reduced compared to the ethanol control (Figure 4.2c). These results paralleled the effects seen in the absence of Gcn5 (Figure 4.1b). Importantly, there was no decrease in the levels of Arp5 surrounding the break, indicating that INO80 recruitment does not require NuA4 (Figure 4.2d). Likewise, Eaf3, a shared subunit of both NuA4 and the Rpd3S histone deacetylase complex, also showed no defect in recruitment levels (Figure 4.2d). This may indicate that the antibody preferentially detects the Rpd3-bound form of Eaf3. We also monitored recruitment of Yaf9 (SWR-C and NuA4), and in this case, Esa1 depletion led to a reduction in DSB recruitment at 4 hours, but Yaf9 levels returned to wild-type levels by 6 hours (Figure 4.2d). A reduction in SWR-C recruitment is consistent with data demonstrating that NuA4 acetylation of H2A and H4 promotes SWR-C recruitment via its bromodomain-containing subunit Bdf1\textsuperscript{151,153}. However, the lack of greater changes in the Yaf9 and Eaf3 data, even though functional NuA4 is absent, is compatible with data indicating that the smaller NuA4 complex, Piccolo NuA4 (Esa1, Epl1, and Yng2)\textsuperscript{154}, displays distinct recruitment kinetics from that of bulk NuA4\textsuperscript{155}.

Similarly to Yaf9, γH2AX was also reduced at the 4 hour time point but returned to wild-type levels by 6 hours (Figure 4.2e). DSB resection was also monitored by
quantitative PCR, but the extent of resection in both the Esa1-depleted and control cells was quite low, likely due to the lower growth temperature (Figure 4.2f). The lower amount of resection may explain the overall low recruitment levels for each of the

![Figure 4.3.](image) NuA4 acetylation of chromatin specifically promotes SWI/SNF recruitment to a DSB. (a,b) ChIP analyses of the indicated chromatin modifying enzyme subunits after an induced break in isogenic \( wt \) and \( yng2\Delta \) yeast strains. (c) \( \gamma H2AX \) levels determined by ChIP at the indicated time points after break induction. (d) Input DNA in at the break site relative to an unbroken locus (\( ACT1 \)) and normalized to pre-induction levels.
chromatin modifying enzymes. These data indicate that the NuA4 enzyme promotes recruitment of SWI/SNF.

To further investigate the role of NuA4, we performed ChIP analyses in a yng2 strain. Yng2 is a subunit of NuA4 that is required for optimal nucleosomal HAT activity\(^{156,157}\). Since yng2 mutants are hypersensitive to microtubule destabilizing agents\(^{158}\), the yng2 mutant could not be synchronized in G2/M by treatment with nocodazole, so experiments were performed in asynchronous cultures. Gal-HO induction was somewhat less efficient under these conditions, but remained robust and comparable between the two cultures (Table 4.S3). Similar to the Esa1 depletion studies, recruitment of SWI/SNF to areas surrounding the induced DSB was dramatically decreased in the yng2 mutant (Figure 4.3a). However, there was also a moderate effect on Arp5 (INO80) recruitment, although only after 4 hours of break induction (Figure 4.3b). Meanwhile, Yaf9 (SWR-C & NuA4) exhibited only a marginal defect at farther distances from the break (Figure 4.3b), again consistent with bulk NuA4 displaying distinct recruitment kinetics from Piccolo NuA4\(^{155}\). In addition, the yng2 mutant showed a defect in apparent resection rates (Figure 4.3d), but also only after 4 hours of break induction. This coincides with the defects seen in Arp5 and Yaf9 recruitment, suggesting that Arp5 and Yaf9 defects may be due to the resection deficiency. In contrast, the recruitment of SWI/SNF subunits was already affected by 2 hours after break induction (Figure 4.3a). Furthermore, H2A.X phosphorylation was again negatively affected, although the contributions from very high levels of γH2AX in G1 cells may obscure a greater defect. Importantly, while only having minor effects on INO80 and SWR-C, both NuA4 mutants
produced major defects in the recruitment of SWI/SNF to a DSB, indicating that NuA4 dependent acetylation plays a significant role in SWI/SNF recruitment.

*The Swi2 bromodomain is necessary for SWI/SNF recruitment to a DSB.*

The ATPase subunit of SWI/SNF, Swi2, contains a bromodomain which recognizes acetylated histone residues\(^\text{159}\) and has been shown previously to stabilize the interaction of SWI/SNF with chromatin acetylated by both Gcn5 and NuA4\(^\text{160,161}\). We asked if the Swi2 bromodomain could also stabilize SWI/SNF interaction with the chromatin surrounding a DSB. To address this point, we inserted a stop codon just after Swi2 residue 1554, effectively removing the bromodomain of Swi2 (*swi2Δbr*). Notably, previous studies have shown that a similar C-terminal truncation has no impact on SWI/SNF assembly\(^\text{162}\). Consistent with previous work, recruitment of SWI/SNF to the *GAL1* promoter was decreased in the absence of the bromodomain (Figure 4.4a)\(^\text{163–165}\), although this defect did not alter the induction of the galactose-induced DSB (Table 4.S4). Importantly, the recruitment of both Swi2 and Snf6 to the DSB region was defective, particularly close to the break after 4 hours (Figure 4.4b). In contrast, other chromatin regulators were unaffected (Figure 4.4c). Notably, removal of the Swi2 bromodomain did not affect DSB resection (Figure 4.4e). But in contrast, there was a significant defect in γH2AX levels (Figure 4.4d) emulating the effect seen in human cells. This effect indicates that SWI/SNF has an important role in promoting the high levels of γH2AX surrounding a DSB in G2/M.
Figure 4.4. SWI/SNF’s bromodomain is key for DSB recruitment and promotes γH2AX. (a) Isogenic wt and swi2Δbr yeast strains were synchronized in G2/M and analyzed by ChIP for the indicated chromatin modifying enzyme subunits at (a) the GAL1/10 promoter region, and (b,c) the double-strand break site. (d) γH2AX levels determined by ChIP at the indicated time points after break induction. (e) Input DNA at the break site relative to an unbroken locus (ACT1) and normalized to pre-induction levels.
Discussion

In this study, we have investigated the effects of two histone acetyltransferases on the recruitment of several chromatin regulators to a DSB. We found that the removal of histone acetyltransferase Gcn5 negatively effects the recruitment of at least three chromatin remodeling complexes (SWI/SNF, INO80, and SWR-C), while also significantly affecting DNA resection rates and levels of γH2AX. We further found that, even though removal of the histone acetyltransferase NuA4 also affected resection rates, it primarily affected SWI/SNF recruitment to DSBs with only minor effects on the other complexes tested. Finally, we showed that the acetyl-binding bromodomain of SWI/SNF is required for association of SWI/SNF with chromatin flanking a DSB. These results are consistent with early studies demonstrating that Gcn5 and Esa1 are recruited to an HO-induced DSB and that histones H3 and H4 are acetylated following DSB formation.80 Furthermore, the defect in SWI/SNF recruitment led to a defect in the phosphorylation of H2A.X, indicating that SWI/SNF plays an important role in promoting the creation or maintenance of this important histone mark.

The dramatic effect of Gcn5 on DNA resection surrounding the break (Figure 4.1d) was unexpected but consistent with results from two recent studies. In one, inactivation of the fission yeast Gcn5 homolog led to a significant defect in DSB resection as well as a reduction in chromatin accessibility.166 The authors associated this effect with the acetylation of histone H3 at lysine 36, catalyzed by Gcn5, and in direct opposition to the methylation of the same residue, whose inhibition had similarly opposite effects166. In a second study, the authors found that inhibition of histone
deacetylases caused impaired resection and the rapid degradation of resection machinery, which was partially recovered when Gcn5 was also removed\textsuperscript{167}. From this data we can expect that the effects of acetylation on repair are complex and that a balance of Gcn5-dependent acetylation and deacetylation is required for proper DNA resection and repair\textsuperscript{167}. Furthermore, we have previously shown that defects in resection substantially impact the association of chromatin remodeling complexes with DSB\textsuperscript{106}, and so the observed defect in resection due to the loss of Gcn5 likely contributes to the negative effect on chromatin remodeling complex recruitment to the DSB in that mutant (Figure 4.1b and c). However, it remains possible that Gcn5’s histone acetyltransferase activity may play a direct role in recruitment through acetylation of H3, as seen in human cells\textsuperscript{88}, particularly because histone acetylation by Gcn5 is known to stabilize the interaction of SWI/SNF with DNA\textsuperscript{160,161}.

Similarly to Gcn5, removal of the NuA4 acetyltransferase resulted in a defect in DNA resection and SWI/SNF recruitment. However, disruption of NuA4 by either Esa1 depletion or inactivation of the Yng2 subunit produced only minor effects on the recruitment of INO80 and SWR-C subunits to a DSB, which may be due to the concurrent defects in end processing. On the other hand, the drastic effect on SWI/SNF recruitment is likely due to histone H4 or H2A acetylation by NuA4. Indeed, SWI/SNF is likely to interact with one or more of these residues directly, as several \textit{in vitro} studies have shown interaction between SWI/SNF and NuA4 acetylated nucleosomes\textsuperscript{160,168,169}. Moreover, deletion of the Swi2 bromodomain significantly impacted SWI/SNF’s ability to bind and remodel a NuA4 acetylated nucleosomal template \textit{in vitro}\textsuperscript{169}. In future
studies it will be interesting to investigate which of the acetylated residues SWI/SNF specifically interacts with during the DNA damage response.

In all experiments where SWI/SNF recruitment was affected, we also saw a defect in histone H2A.X phosphorylation surrounding a DSB. By removing the bromodomain of Swi2, we were able to show that this effect is directly linked to SWI/SNF recruitment to the break site. A similar effect is observed in human cells\textsuperscript{88,170} demonstrating a conserved role for SWI/SNF in promoting this important histone mark during the DNA damage response. We propose a model where, in response to a DSB, acetylation by NuA4 and Gcn5 allows for increased DNA end processing and thus increased recruitment of NuA4 and other chromatin modifying enzymes. Acetylation also promotes SWI/SNF recruitment to the break site, in turn promoting the phosphorylation of H2A.X by the

Figure 4.5. A model for the interaction of NuA4 acetylation, SWI/SNF DSB recruitment, and γH2AX during DSB damage response. See text for details.
checkpoint kinases Mec1 and Tel1 (Figure 4.5). How SWI/SNF promotes γH2AX formation or maintenance is not yet known but promises to be an interesting topic for future studies.

**Methods**

_Yeast strains_

All strains are derivatives of JKM139128, and were generated by one-step PCR disruption and confirmed by PCR analysis. Full genotypes are available in this appendix. All strains were grown at 30ºC as previously described\textsuperscript{106}, with the exception that yng2Δ cultures was grown overnight in YPD (1% yeast extract, 2% bactopeptone, 2% glucose), then washed and resuspended in warm YPRs (1% yeast extract, 2% bactopeptone, 2% raffinose, 0.2% sucrose (made and added fresh)) for 3-4 hours pre-induction. G2/M arrest was achieved using 30 mg/ml nocodazole for 4-5 hours. Degradation of Esa1 was achieved by addition of 1-naphthaleneacetic acid (NAA; Sigma) to a final concentration of 500 µm; an equal volume of solvent (100% ethanol) was added to control cultures. Cultures were concurrently transferred to a 22ºC water bath.

_Chromatin Immunoprecipitation_

Lysates were prepared as previously described\textsuperscript{106}. Briefly, mid-log phase cells were cross-linked with formaldehyde for 15 minutes, lysed, and sonicated to obtain an average DNA fragment size of 500 bp. Immunoprecipitations (IPs) were performed by adding 1-2 µl of antibody to diluted chromatin lysate and incubated overnight at 4ºC. 1%
sarkosyl was also added to SWI/SNF IPs. Sepharose protein A beads (50% slurry; Rockland) were added for 2 hours at 4°C, washed, and eluted at 65°C. IP and 10% input DNAs were purified and quantified by qPCR. Primer sequences are available in the appendix. Fold enrichment represents the ratio of IP to input DNA at the break region, normalized to the same ratio obtained for the *ACT1 ORF*. These values were corrected for DSB induction and normalized to pre-induction (0 h) values. Error bars indicate s.e.m from at least two independent biological replicates.

*Western blotting*

Whole cell extracts were prepared by TCA extraction and proteins were separated by SDS-PAGE, blotted onto nitrocellulose membrane (GE) and probed with α-AID (BioRois) antibody using standard methods.

*Antibodies*

Rabbit polyclonal antibodies Arp5 (ab12099), Yaf9 (ab4468), Eaf3 (ab4467), and H2A-S129phos (γH2AX; ab15083) are commercially available from Abcam, as is the AID antibody from BioRois (APC004Am). Anti-Snf6 and anti-Swi2, and Anti-Sth1 antibodies were kind gifts from J. Reese (Pennsylvania State University) and B. Cairns (University of Utah), respectively.
## Supplementary Information

### Table 4.S1.
Percent unbroken DNA at the HO cut site in *wt* and *gcn5Δ* strains, at indicated time points after addition of galactose. ± s.e.m. shown.

<table>
<thead>
<tr>
<th></th>
<th><em>wt</em></th>
<th><em>gcn5Δ</em></th>
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<tr>
<td>0 h</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>2 h</td>
<td>2.22 ± 0.78</td>
<td>2.09 ± 0.22</td>
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<tr>
<td>4 h</td>
<td>1.63 ± 0.64</td>
<td>1.10 ± 0.32</td>
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</tbody>
</table>

### Table 4.S2.
Percent unbroken DNA at the HO cut site in cells treated with ethanol (+EtOH) or synthetic auxin (+NAA), at indicated time points after addition of galactose. ± s.e.m. shown.

<table>
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<th>+EtOH</th>
<th>+NAA</th>
</tr>
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<tbody>
<tr>
<td>0 h</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>4 h</td>
<td>21.8 ± 4.9</td>
<td>23.5 ± 7.3</td>
</tr>
<tr>
<td>6 h</td>
<td>16.6 ± 1.7</td>
<td>19.3 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>wt</td>
<td>yng2Δ</td>
</tr>
<tr>
<td>-------</td>
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<td>--------</td>
</tr>
<tr>
<td>0 h</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>2 h</td>
<td>10.6 ± 1.7</td>
<td>18.8 ± 2.7</td>
</tr>
<tr>
<td>4 h</td>
<td>2.3 ± 0.6</td>
<td>8.7 ± 0.8</td>
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</tbody>
</table>

**Table 4.S3.** Percent unbroken DNA at the HO cut site in asynchronous *wt* and *yng2Δ* strains, at indicated time points after addition of galactose. ± s.e.m. shown.

<table>
<thead>
<tr>
<th></th>
<th>wt</th>
<th>swi2Δbr</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 h</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>2 h</td>
<td>3.26 ± 0.16</td>
<td>2.78 ± 0.95</td>
</tr>
<tr>
<td>4 h</td>
<td>2.42 ± 0.40</td>
<td>2.04 ± 0.66</td>
</tr>
</tbody>
</table>

**Table 4.S4.** Percent unbroken DNA at the HO cut site in *wt* and *swi2Δbr* strains, at indicated time points after addition of galactose. ± s.e.m. shown.
CHAPTER V

Summary and Conclusions
We are still working to understand the complexity that is the chromatin response to a DNA double-strand break. Every year research identifies an increasing number of chromatin modifications that occur in response to a DSB, but how these modifications function in the DNA damage response is not always clear. Moreover, while many ATP-dependent chromatin remodeling enzymes have been implicated in DNA damage repair, the specifics of their recruitment or functional roles have often remained elusive. In addition, it is becoming increasingly clear that not only the ability to properly carry out repair, but also chromatin remodeling activities specifically are important for human health, namely in preventing disease development such as cancer\textsuperscript{171,172}. Thus understanding these aspects of damage repair is critical. The work presented here has focused on addressing some of the areas that are still unknown, namely how chromatin remodeling enzymes are recruited to DSBs, and their function once there.

Through the course of the studies presented in Chapter II we have found that several chromatin modifying enzymes share a common pathway of recruitment to DNA DSBs. This pathway is dependent on the repair pathway choice (NHEJ v. HR) that is primarily governed by the cell-cycle phase that damage occurs within. Specifically, NHEJ events in G1 inhibit robust recruitment, whereas HR-specific resection and the Rad51 recombinase are required for high levels of recruitment in G2/M. However, it is
unlikely that Rad51 directly interacts with each of these complexes individually. Firstly, the distribution of these complexes to either side of the break has been limited to 10kb, whereas Rad51 is capable of coating much greater distances\textsuperscript{99}. Secondly, we failed to see an interaction between purified Rad51 and the SWI/SNF complex in an \textit{in vitro} pull-down assay (data not shown). The favored model, therefore, is that one of these complexes might interact with Rad51 (directly or indirectly) and in so doing initiate a cascade of either direct protein recruitment or chromatin modifications that promote the interaction of other complexes with the DSB region, or a combination therein. However, a model that limits the distribution of chromatin modifying enzymes to approximately 10 kb to either side of a DSB has yet to be put forth.

The studies presented here also investigated the chromatin remodeling enzyme Fun30. We showed that the recruitment of this enzyme is also cell-cycle regulated (Figure 2.S2), and another group has shown that this recruitment is also dependent on the resection machinery\textsuperscript{118}. Although we did not pursue experiments to directly show dependence on Rad51 like the other complexes examined, we believe that this enzyme will also display the same recruitment requirements. Data presented in Chapter III goes on to show that Fun30 has a significant impact on the occupancy of several histones surrounding an induced DSB, including H2A.Z. It is interesting to note that Fun30 and H2A.Z interact genetically\textsuperscript{148}. Given that Fun30-deficient strains display a severe resection phenotype, and that H2A.Z (and SWR-C) has also recently been shown to be important for resection via the Exo1 pathway\textsuperscript{102}, it is tempting to think that Fun30 may function in the parallel Sgs1-Dna2 pathway. While the deletion of \textit{FUN30} with either
EXO1 or SGS1 increased the defect in resection even further over deleting FUN30 alone \(^{119,120}\), there was a greater effect in fun30 exo1 mutants, indicating that Fun30 plays a greater role in the Sgs1-mediated resection pathway \(^{120}\). Additionally, overexpression of Exo1 suppressed the resection and damage-sensitivity phenotypes of fun30 cells \(^{120}\).

Furthermore, while the Sgs1/Dna2 complex can resect through a mononucleosome easily in vitro, it is inhibited in resection by an increasing histone nucleosome density on a nucleosomal array \(^{102}\), indicating that it may require the aid of a chromatin remodeling enzyme in vivo. However, the data presented here indicate that Fun30 only has an effect on histone removal very close to the site of a DSB, whereas the resection defect seen in fun30 cells is at greatest at distal locations. It is possible that this initial histone removal gives Sgs1-Dna2 a ‘running start’ to boost resection through chromatin \(^{102}\), or Fun30’s ability to antagonize Rad9 binding \(^{118}\) aids Sgs1-Dna2 activity more than Exo1. These ideas are not mutually exclusive, and Fun30 also promotes resection via the Exo1 pathway, which may also benefit from either of these Fun30 activities. Clearly more study into Fun30’s function in resection is needed to test these hypotheses.

Finally, Chapter IV investigates the interactions among histone acetylation, chromatin remodeling enzyme recruitment, and \(\gamma\)H2AX formation. While these data echo similar interactions found in human cells \(^{88}\), the yeast system is distinct in that we found a role for NuA4-dependent acetylation in recruiting SWI/SNF to a DSB. In addition, we found that Gcn5 activity also promotes the recruitment of chromatin remodeling complexes other than SWI/SNF, something that has not yet been investigated in the mammalian system. Another very important difference between the two systems is
that in human cells recruitment of SWI/SNF and Tip60 (human NuA4) was dependent on the phosphorylation of H2A.X, something that we did not observe in yeast (Figure 2.2). An explanation for this is not readily available, except possibly that our experiments focus on repair by HR by arresting cells in G2/M, where the human study was not synchronized. It remains possible that γH2AX could function in recruiting chromatin remodeling complexes under different conditions. A further direction for yeast studies is identifying the specific histone tail residues that are involved in the recruitment of SWI/SNF to a DSB. This would also help identify which histone acetyltransferase is directly responsible for its recruitment. It is interesting to note that γH2AX levels are significantly higher in G1 arrested cells, where repair is primarily by non-homologous end joining, and chromatin modifying enzyme recruitment to a break is very low (Figure 2.1). This phenomenon suggests the possibility that a different mechanism for γH2AX formation and maintenance exists in that cell phase, although this has yet to be tested. Moreover, whether other chromatin marks are differentially regulated throughout the cell cycle has yet to be explored, but promises to be an interesting topic for future studies.

The results outlined above that SWI/SNF recruitment is promoted by NuA4 and Gcn5 activities supports the model for DSB recruitment of chromatin modifying enzyme whereby a cascade of events is triggered by one enzyme’s interaction with Rad51. In addition to these data, in a strain lacking the SWR-C (swr1Δ) SWI/SNF recruitment displays a significant defect (Figure A1). Taken together, the data as a whole suggests a model that is in line with the cascade model, but we can now describe this sequence of events with regards specifically to SWI/SNF recruitment. In this model SWR-C, either
itself or through its incorporation of H2A.Z early after DSB formation\textsuperscript{99}, may promote the recruitment of NuA4 to the DSB, and in turn NuA4 histone acetylation promotes SWI/SNF recruitment. There are, of course, several parts of this model that require experimentation to confirm or refute. First is the interaction among SWR-C, H2A.Z, and NuA4: can H2A.Z affect recruitment of NuA4, and would this then be SWR-C dependent? Interestingly, it has been shown that NuA4 acetylation of H2A and H4 promotes the incorporation of H2A.Z by SWR-C\textsuperscript{153}. While this is seemingly in opposition to the model above, this interaction may either be specific to transcription rather than repair, or it could function in a feed-back loop that enhances the high levels of SWR-C and NuA4 found at a DSB. Yet another alternative is that the cell cycle position may regulate it as this was also not controlled for in the original observations.

A second part of this model to be tested is the relationship between SWR-C and SWI/SNF: Is recruitment of SWI/SNF dependent on H2A.Z in any way? And a corollary of that interaction would ask if H2A.Z can affect $\gamma$H2AX levels like SWI/SNF does. Finally, the mechanism of SWR-C recruitment to a DSB needs to be determined. It may yet be possible that SWR-C directly interacts with Rad51. Still another possibility for this interaction is Gcn5. We have already seen that Gcn5 significantly impacts the recruitment of SWR-C to a DSB (Figure 4.1); however, this could be through its effect on resection or via another mechanism entirely.

Experimentally testing all of these interactions could be done by the same chromatin immunoprecipitation methods employed throughout this body of work using various mutated strains, such as \textit{swr1} \textit{A} and \textit{htz1} \textit{A} to start. SWR-C interaction with
Rad51 could be tested via *in vitro* pull-down assays. Still, this model is not comprehensive as the recruitment of RSC and INO80 are not accounted for.

**Figure 5.1.** A proposed model for SWI/SNF, NuA4 and SWR-C recruitment to a DSB. Solid black arrows indicate a known positive interaction. Red solid arrow indicates an unknown interaction proposed by this model. The dotted line indicates a known interaction, but that has not specifically been shown in response to a DSB.

The results presented in this work demonstrate an important lesson for the field of DSB repair: the importance of cell-cycle regulation on obtained data and data interpretation. The majority of previous studies have been done in asynchronously growing cultures, and we have shown here to that the cell-cycle phase during damage and repair has a significant impact on results. We have shown that the levels of both chromatin remodeling enzymes, histone occupancy, and H2A.X phosphorylation all demonstrate cell-cycle dependency. As an unintended consequence of this previously
unknown dependency, an H2A.X mutant that harbored a defect in cell cycle progression led researchers to a misinterpretation of data (see Chapter II). It remains unknown how many other results could be interpreted differently once cell-cycle and/or repair pathway is taken into account. The impact of cell-cycle and pathway choice on results may also explain some of the differences seen between yeast and mammalian systems. We have already seen this lesson taken to heart by several recent studies that performed their experiments within the context of cell cycle.\textsuperscript{108,173–175}

Several previous studies have indicated that many of the complexes studied here, such as RSC, SWR-C, and INO80, have functional roles in early stages of DSB repair\textsuperscript{95,97,176}, and so to find that the bulk of recruitment occurs downstream of resection is surprising. This finding challenges the commonly held “access-repair-restore” model where chromatin remodeling is required to enable access to DNA before repair events can occur. The single-strand annealing DSB repair pathway shares many of the same initial processing steps with HR, such as extensive resection. However, it is interesting to note that this repair occurs normally in the absence of INO80, NuA4, SWI/SNF, or RSC\textsuperscript{98,177–179}. Importantly, this repair pathway does not require Rad51, as invasion of a DNA duplex is not required – only annealing of resected single-stranded DNA\textsuperscript{112,180}. Although our results do not preclude the action of remodeling enzymes in early events of HR, since these functions may only require relatively low levels of protein, they do raise intriguing questions about what these enzymes are doing later in the process that requires such high abundance.
However, it is important to keep in mind that the strains used in this work harbor an unrepairable break because they lack the homologous donor loci. Indeed, if the break were repairable, repair products would be detectable only an hour after break induction\textsuperscript{181}. In truth, in these and other studies using the donor-less strains, the break locus has already been relocated to the nuclear periphery by the start of these time courses (2 h)\textsuperscript{182}.

An enticing possibility for the role of these complexes in repair is to reposition chromosomes within the nucleus, such as to aid in homology search or relocation to the nuclear periphery. Several lines of evidence exist to support these enzymes’ involvement in chromosomal mobility. Firstly, INO80, SWR-C and H2A.Z have recently been shown to be necessary for an increase in chromosomal movements after DSB formation\textsuperscript{107,108}. Secondly, increased chromosomal movements following a DSB was also shown to be dependent on Rad51\textsuperscript{109,110}. Thirdly, the localization of unrepaired DSBs to the nuclear periphery is dependent on Rad51 and the histone variant H2A.Z\textsuperscript{99,182}, which is incorporated by SWR-C. Lastly, a very recent study has shown that binding to the nuclear envelope protein Mps3 is S and G2 specific and requires the chromatin remodeling enzymes INO80 and SWR-C\textsuperscript{108}. These data are compelling; however, more work is needed to determine the roles of the other complexes – namely RSC, SWI/SNF, and NuA4 – that accumulate at a break after initial steps of repair occur.

While these studies have advanced our knowledge in the field of chromatin remodeling activity in response to a DNA DSB, there remain a multitude of questions to be answered. How does Rad51 function to recruit several chromatin remodeling
enzymes to a DSB? How is the differential formation of \( \gamma \text{H2AX} \) regulated? Why are so many chromatin regulators recruited the break? Do they perform unique or redundant roles in repair? Once a functional role in DNA damage repair is established for any complex, it will then be important to tease apart which subunits contribute to that activity. For example, three of INO80’s unique subunits, Nhp10, Arp5, and Arp8, have very different effects on the complex’s activity \textit{in vitro}\textsuperscript{183}, so what contributions do they make during DSB repair?

Continued research into these questions and more are needed to characterize the mechanisms of chromatin remodeling during the DNA damage response. As we understand how these mechanisms affect human disease it will be necessary to define specific biochemical steps that these enzymes affect, and possibly they will then be targets for therapeutics in cancer and other ailments.
Figure A1. SWR-C promotes SWI/SNF recruitment to a DSB. Isogenic, donorless wild-type (wt) and swr1Δ cultures were arrested in G2/M with nocodazole and assayed by ChIP for levels of γH2AX surrounding an induced DSB. Data shown represent two biological replicates; error bars represent s.e.m.
**Table A1.** Genotypes of yeast strains used during these studies.

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Table A2. Primer sequences used for ChIP-qPCR in these studies.

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Bibliography


57. Granata, M. *et al.* Dynamics of Rad9 chromatin binding and checkpoint function are mediated by its dimerization and are cell cycle-regulated by CDK1 activity. *PLoS Genet.* **6**, (2010).


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