Linker Histone Functions of HMO1- Implications for DNA repair

Arvind Panday
Louisiana State University and Agricultural and Mechanical College, apanda2@lsu.edu
LINKER HISTONE FUNCTIONS OF HMO1- IMPLICATIONS FOR DNA REPAIR

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy in

The Department of Biological Sciences

by
Arvind Panday
B.S., University of Delhi, 2009
M.S., Madurai Kamaraj University, 2011
December 2016
Dedicated to my parents, Dr. Anne Grove and Dr. Shrikant Ekbote
ACKNOWLEDGMENTS

First and foremost, I would like to thank my supervisor, Dr. Anne Grove, for the patient guidance, encouragement and advice she has provided throughout my time as her student. I am forever indebted to her. I have been extremely lucky to have a supervisor who cared so much about my work. She has been supportive and has given me the freedom to pursue various projects without objection. Dr. Grove provided me the freedom to work on my own. This helped me to develop my skill to work independently. The door to Dr. Grove’s office was always open whenever I had a question about my research. She is my primary resource for getting my publications. Words cannot express my gratitude for everything that she has done for me. Dr. Grove was and remains my best role model for a scientist, and mentor.

I would like to acknowledge my dissertation committee members, Dr. David Donze, Dr. Craig M Hart and the dean’s representative Dr. Bin Li for providing their valuable time and critical feedback. Thanks Dr. Donze for his expertise with Yeast biology and generously donating Yeast strains. A special thank you for all the fruitful discussions. Thanks Dr. Hart for his excellent skills to listen intently and have contribution in my thesis.

I am thankful to my best and true friends who have supported me: Himanshu Batra, Jitender Bisht, Dhirendra Singh. I have enjoyed many useful scientific discussions with them.

I wish to thank my sister Manju and her husband Amit, and my Niece Baua who have brought great joy to my life. I am indebted to my parents and Dr. Shrikant Ekbote to made me into who I am.
I thank all the friends that I made at Dr. Grove’s laboratory, Ambuj, Kavitha, Ashish, Anuja, Dinesh, Afsana, Smitha and Nabanita for providing cheerful ambience to work. Last but not the least, I want to thank my favorite facility in lab, sound system to increase my work efficiency.
# TABLE OF CONTENTS

ACKNOWLEDGMENTS ........................................................................................................ iii

LIST OF TABLES ................................................................................................................ viii

LIST OF FIGURES ............................................................................................................... ix

ABSTRACT ........................................................................................................................... xii

CHAPTER 1. INTRODUCTION .......................................................................................... 1
Nucleosome structure and organization ......................................................................... 1
Linker histone binding to the nucleosome ........................................................................ 4
Diversity of linker histones ................................................................................................. 6
Yeast linker histone Hho1p ................................................................................................. 7
Chromatin represses transcription ..................................................................................... 9
Chromatin represses DNA repair ....................................................................................... 10
High mobility group (HMGB) proteins ............................................................................. 13
Structure of HMGB proteins ............................................................................................. 15
Binding of HMGB proteins to nucleosomes ..................................................................... 16
Yeast HMGB protein Hmo1p and its structure ................................................................. 17
Hmo1p is a component of the Pol I transcription machinery and equivalent of UBF .... 19
Hmo1p regulates Pol II transcription ................................................................................. 22
Hmo1p stabilizes noncanonical chromatin structures ..................................................... 24
Hmo1p (and its C-terminal tail) and DNA double-strand break (DSB) repair ................. 26
Interplay between Hho1p and Hmo1p ............................................................................. 28
References ......................................................................................................................... 29

CHAPTER 2. YEAST HIGH MOBILITY GROUP PROTEIN HMO1P STABILIZES
CHROMATIN AND IS EVICTED DURING REPAIR OF DNA DOUBLE STRAND
BREAKS ............................................................................................................................. 45
Introduction ....................................................................................................................... 45
Materials and methods .................................................................................................... 48
Strain construction ............................................................................................................ 48
ChIP and PCR analysis .................................................................................................... 48
Survival following DSB induction .................................................................................... 50
DNA end resection .......................................................................................................... 52
Results ............................................................................................................................... 53
Efficient DSB repair in absence of Hmo1p ..................................................................... 53
Hmo1p localizes to the MAT locus and is evicted during DSB repair ............................... 56
Rapid kinetics of H2A phosphorylation and dephosphorylation in absence of Hmo1p... 60
H2A phosphorylation correlates with Arp5 recruitment ................................................. 62
Rapid H3 eviction and redeposition in hmo1Δ strains ..................................................... 65
DNA end resection and Rad51 recruitment .................................................................... 68
APPENDIX: COPYRIGHT PERMISSIONS .................................................. 133
VITA ........................................................................................................ 140
LIST OF TABLES

Table 2.1 Strains and their genotype .................................................................49

Table 2.2 Sequences of primers used for ChIP, DNA resection, and gene expression...51
LIST OF FIGURES

Figure 1.1. Assembly of nucleosome core particle .................................................3
Figure 1.2. Histone H1 associates with linker DNA ...............................................4
Figure 1.3. Domain organization of H1, Hho1p, and Hmo1p ...............................6
Figure 1.4. Proposed interaction of H1 and HMGB proteins with nucleosomes ....7
Figure 1.5 Chromatin as a barrier to transcription and DNA repair ....................12
Figure 1.6. Model of Hmo1p and its interaction with DNA ............................14
Figure 1.7 Hmo1p-mediated stabilization of genomic DNA .................................21

Figure 2.1. Survival of WT and hmo1Δ strains following induction of DNA double strand breaks .................................................................55
Figure 2.2 Fidelity assay .........................................................................................55
Figure 2.3. Survival of DDY3kuΔ and DDY3kuΔhmo1Δ strains following induction of DNA double strand breaks ...............................................................56
Figure 2.4. Survival of WT and hmo1Δ strains following induction of DNA double strand breaks .................................................................57
Figure 2.5. Efficiency of DSB induction .................................................................57
Figure 2.6. Hmo1p eviction from the vicinity of DSB ............................................59
Figure 2.7. Hmo1p localization at POL5 ................................................................60
Figure 2.8. Role of Hmo1p in MATa transcription ..............................................61
Figure 2.9. Survival after exposure to hydroxyurea (HU) ......................................61
Figure 2.10. H2A phosphorylation .......................................................................63
Figure 2.11 Arp5 localization ..............................................................................64
Figure 2.12. Detection of γ-H2A 3.1 kb downstream of DSB .............................66
Figure 2.13 H3 localization ..................................................................................66
Figure 2.14. Quantitative analysis of H3 localization determined by ChIP ........67
Figure 2.15. Quantification of DNA resection by qPCR .........................................................69
Figure 2.16 Rad51 recruitment to DSB .................................................................................69
Figure 2.17. Rad51 localization .........................................................................................71
Figure 2.18 Ku recruitment to DSB ..................................................................................72
Figure 2.19 Survival assay .................................................................................................72
Figure 2.20 Effect of Hmo1p C-terminal tail on H2A phosphorylation .................................73
Figure 2.21. Effect of Hmo1p C-terminal tail on Arp5 recruitment and H3 eviction ...... 74
Figure 2.22. Effect of Hmo1p C-terminal tail on Rad51 recruitment .................................74

Fig. 3.1 Resistance of chromatin to nuclease digestion requires linker histone H1 or Hmo1p containing its lysine-rich extension .................................................................92
Fig. 3.2 Effect of Hho1p on resistance of chromatin to nuclease digestion and on cellular content of Hmo1p ........................................................................................................94
Fig. 3.3 Resistance of chromatin to MNase digestion monitored at specific loci ..........95
Fig. 3.4 Effect of Hmo1p or Hho1p on binding of the other protein .................................97
Fig. 3.5 Equivalent binding of Hmo1p to MAT in different growth phases .........................98
Fig. 3.6 Effect of linker histone H1 on MNase sensitivity of chromatin isolated from synchronized cells and on growth rate .................................................................99
Fig. 3.7 Both Hmo1p and Hmo1p deleted for its C-terminal tail compete with H1 for binding to chromatin ........................................................................................................100
Fig. 3.8 Dynamic chromatin environment in hmo1Δ that leads to faster chromatin remodeling and DSB repair is restored to wild-type levels by expression of H1.102
Fig. 3.9 Chromatin state in hmo1Δ that leads to faster DNA end resection and faster Rad51 recruitment after DNA double-strand break is reversed on expression of H1 .................................................................104
Fig. 4.1 Plasmid map of pMV1328 .....................................................................................116
Fig 4.2 Hmo1p requirement for efficient dsDNA breaks repair depends on the types of DSB .......................................................................................................................117
Fig 4.3 Hmo1p requirement for repair accuracy depends on the types of DSB ........118
Fig 4.4 Truncated Hmo1p affects repair efficiency and repair accuracy ...............119
Fig 4.5 Hmo1p and Ku function in separate pathways to repair overhang DSB ........120
Fig 4.6 Hmo1p prevents caffeine sensitive resection .....................................122
Fig 4.7 Caffeine rescued hmo1Δ phenotype ..................................................123
Fig 5.1 Hmo1p is a hybrid of HMGB and linker histone H1 ..............................127
ABSTRACT

The DNA of eukaryotic cells does not exist in free linear strands; it is tightly packaged and wrapped around nuclear proteins in order to be accommodated it inside the nucleus. The basal repeating unit of chromatin, termed the nucleosome, provides the first level of compaction of DNA into the nucleus. Nucleosomes are interconnected by linker DNA and associated linker histones to form 30 nm fibers. The highly diverse linker histones are critical for compaction and stabilization of higher order chromatin structure by binding DNA entering and exiting the nucleosome. The lysine-rich C-terminal domain (CTD) of metazoan H1 is crucial for such stabilization. This study concerns the functions of *Saccharomyces cerevisiae* Hmo1p, an high mobility group (HMGB) family protein unique in containing a terminal lysine-rich domain and functions in stabilizing genomic DNA.

My study suggests that Hmo1p shares with mammalian linker histone H1 the ability to stabilize chromatin, as evidenced by the absence of Hmo1p or deletion of the Hmo1p CTD creating a more dynamic chromatin environment that is more sensitive to nuclease digestion and in which chromatin remodeling events associated with DNA double strand break repair occur faster; such chromatin stabilization requires the lysine-rich extension of Hmo1p. Further, my data indicates that Hmo1p functions in the DNA damage response by directing lesions towards the error-free pathway. My results suggest that Hmo1p controls DNA end resection and favors the classical non-homologous end joining (NHEJ) over alternate end Joining (A-EJ) that is error-prone process. In all, my study identifies a novel linker histone function of Hmo1p in *Saccharomyces cerevisiae* with the ability to stabilize genomic DNA, and appears to go beyond conventional linker histone function.
CHAPTER 1

INTRODUCTION

The DNA of all eukaryotic cells is tightly packaged into chromatin, a nucleoprotein complex consisting of DNA associated with histone and non-histone proteins. The nucleosome is the fundamental unit of chromatin in which ~146 base pairs of DNA wrap around the histone octamer composed of two copies each of H2A, H2B, H3, and H4. Higher order organization of nucleosome core particles is controlled by association of the intervening linker DNA with either linker histone H1 or with high mobility group (HMGB) proteins. While H1 is thought to stabilize the nucleosome by preventing DNA unwrapping, the DNA bending imposed by HMGB may propagate into the nucleosome to destabilize chromatin. For metazoan H1, chromatin compaction requires its lysine-rich C-terminal domain, a domain that is buried between globular domains in the previously characterized yeast linker histone Hho1p. Yeast Hmo1p, an HMGB family protein unique in containing a lysine-rich C-terminal domain and in stabilizing genomic DNA. On rDNA and genes encoding ribosomal proteins, Hmo1p appears to exert its role primarily by stabilizing nucleosome-free regions or “fragile” nucleosomes. Thus, Hmo1p appears to have evolved a unique function involving both the ability to stabilize conventional nucleosome arrays as well as DNA regions characterized by low nucleosome density or the presence of noncanonical nucleosomes. This dissertation presents work carried out to elucidate the function of *Saccharomyces cerevisiae* HMGB protein Hmo1p in terms of chromatin compaction and in modulating chromatin stability and dynamics during DNA repair.
Nucleosome structure and organization

The static structure of the nucleosome core particle has been determined at high resolution, and the folding of a four-nucleosome array has also been reported (1,2). The nucleosome core particle consists of a histone octamer composed of two H2A/H2B heterodimers and an (H3/H4)\textsubscript{2} heterotetramer about which ~146 bp of DNA is wrapped about 1.7 times in a left-handed supercoil (Figure 1.1). Thanks to identification of DNA sequences that preferentially associate with core histones, it has been possible to achieve high-resolution structural information (3,4). In vitro, nucleosome formation at a specific sequence is directed by intrinsic properties of the DNA and it is nucleated by association of the (H3/H4)\textsubscript{2} tetramer, which marks the initial point of DNA bending and therefore defines the dyad axis (Figure 1.1A); binding of (H3/H4)\textsubscript{2} is followed by deposition of two H2A/H2B dimers (5-7). In vivo, nucleosome assembly is catalyzed by chaperones (8-10). The histone octamer makes numerous direct contacts to DNA, most in the DNA minor grooves, and the resulting DNA structure deviates significantly from canonical B-form. Adjacent nucleosomes are connected by linker DNA of variable length to generate nucleosomal arrays (Figure 1.2); the N-terminal tails of core histones extend away from the nucleosome core particle, and these positively charged extensions have been implicated in contacts to the DNA and to neighboring nucleosomes and to other chromatin associated proteins.

Higher order levels of organization in which nucleosomal arrays associate with other proteins remain poorly understood. Interactions between nucleosomes promote the folding of the nucleosomal array into a more compact 30 nm fiber, for example by
interaction of the H4 N-terminal tail with an acidic patch formed at the H2A/H2B interface on a neighboring nucleosome (11). Linker histone H1 plays an indispensable role in

![Figure 1.1. Assembly of nucleosome core particle. (A) Association of the (H3/H4)_2 tetramer with DNA nucleates nucleosome assembly and defines the dyad axis. (B) H2A/H2B dimer. (C) Two H2A/H2B dimers are deposited to generate the nucleosome 1 core particle. H3 N-terminal tails emerge near the DNA entry/exit points. Histones H2A and H2B are shown in blue and green, respectively; H3 is shown in red and H4 is depicted in orange. Based on PDB 1KX5.](image)

stabilizing the 30 nm fiber in which nucleosomes are clustered tightly together, decreasing internucleosomal distance and fixing the entry/exit angle of DNA (12-16). This compaction is affected by nucleosomal repeat length as repeat length must be sufficient to accommodate H1 binding; for nucleosomal arrays with shorter repeat lengths (167 bp in this study), internucleosome interactions drive folding of a more compact fiber that is less affected by linker histone binding (17). However, in proliferating cells, evidence of 30 nm fibers is lacking, and chromosome organization is instead thought to involve a zig-zag geometry and long-range looping that is modulated by the density of linker histones (18-22). This organization is thought to involve formation of topologically associated domains by formation of loops within higher-order chromatin structures; precisely how H1 mechanistically participates is unresolved.
Figure 1.2. Histone H1 associates with linker DNA. (A) The globular domain of histone H1 (purple) binds the nucleosome at the dyad. The structure of a dinucleosome is depicted; color code for core histones as in Figure 1. (B) Four-way junction DNA mimics the DNA configuration at the nucleosome dyad, perhaps explaining the preferred binding of H1 to such junctions. Dinucleosome represents the asymmetric unit in the structure of a tetranucleosome with one linker DNA trimmed for clarity (PDB 1ZBB) (2). The H1 globular domain and its localization relative to the dyad is based on the structure of the chicken H5 globular domain in complex with a nucleosome (PDB 4QLC) (30). Representation of four-way junction is based on PDB 3CRX.

Linker histone binding to the nucleosome

Histone H1 binds linker DNA where it enters and exits the nucleosome (Figure 1.2) (23-25). Unlike core histones, which have residency times on a scale of hours, linker histones are quite mobile with residency times measured in minutes (26,27). Metazoan linker histones have a tripartite structure. They interact with about 20 bp of DNA (either asymmetrically by preferentially binding one linker segment or by protecting 10 bp of entering and exiting DNA) to create the chromatosome, consisting of ~167 bp of DNA, the core histone octamer and one molecule of H1. The ~20-35 amino acid N-terminal domain is followed by the highly conserved central globular domain of ~75 amino acids and a long C-terminal domain (CTD; ~100 amino acids; Figure 1.3). The globular domain
adopts a winged-helix DNA-binding motif (28); its interaction with DNA at the nucleosomal entry/exit points gives rise to protection of the additional ~20 bp (23,29). The structure of the chicken linker histone H5 in complex with a nucleosome reveals binding of the globular domain on the nucleosome dyad axis, interacting with both DNA linkers, whereas the Drosophila linker histone H1 binds off-dyad (29,30); this suggests that interaction with linker histones in different binding modes might differentially control higher-order chromatin organization. In general, linker histones bind preferentially to four-way DNA junctions compared to linear DNA (31), and binding to the nucleosome at the DNA entry/exit points is thought to reflect this preference for a specific DNA geometry (Figure 2).

Notably, the regions flanking the globular domain, particularly the lysine-rich CTD, are required for formation of higher-order structures (Figure 1.4) (32,33). The low-complexity sequence of the CTD, which includes ~40% lysine and a significant content of alanine and proline, results in the domain remaining unstructured in aqueous solution due to charge repulsion, but acquiring a kinked helix conformation when bound to DNA (34,35). Interactions with the CTD promote formation of higher order chromatin structures as well as increasing the residence time (23,32,36). Modeling suggests that a highly charged CTD compacts chromatin more effectively, resulting in silencing, whereas less-charged CTDs promote a chromatin folding in which the genome is more accessible (37). The N-terminus, which is also unstructured, affects positioning and DNA binding affinity (38,39). While H1 binding modes may be distinct for different H1 isoforms, current data support a mode of binding in which the H1 globular domain binds near the dyad axis with the CTD mainly contacting one linker DNA such that linker DNA is organized into a stem-
like structure (Figure 1.4) (29,33). In this configuration, one H1 has been proposed to link three nucleosomes and to prevent association of additional H1 protomers, likely due to electrostatic repulsion.

![Domain organization of H1, Hho1p, and Hmo1p](image)

Figure 1.3. Domain organization of H1, Hho1p, and Hmo1p. Metazoan H1 typically contains a 40-50 amino acid linker, followed by a globular domain of ~80 amino acids (orange) and a long CTD characterized by S/TPXK-like repeats. Hho1p contains a lysine-rich N-terminal segment followed by a globular domain with similarity to that of H1 (orange). Another lysine-rich segment connects this globular domain to the second globular domain (gray). Hmo1p contains box A (red), which has little similarity to consensus HMG domains, followed by a lysine-rich linker, the box B domain (green), and a lysine-rich CTD. Mammalian HMGB proteins have a similar domain organization as Hmo1p, except that the CTD is acidic.

**Diversity of linker histones**

The H1 family of linker histones is the most divergent class of histone proteins (40). For example, while the sequence of core histone H4 is 92% identical between yeast and human, the level of sequence identity between human H1 and yeast Hho1p is only 31%. In addition, multiple different H1 subtypes exist in most eukaryotes. Some are constitutively expressed in all cells, while others are developmentally regulated, restricted to specific cell types, or induced at certain stages of differentiation. Covalent modifications contribute further to functional diversity (14-16). Although the sequence of the winged helix motif is relatively well conserved, the CTDs are extremely variable, both in length and amino acid composition. Considering the role of the CTD in folding of nucleosomal
arrays, different H1 isoforms are likely to exert different effects on chromatin organization. That the lysine-rich CTD is key to organization of genomic DNA is reflected in Euglenozoan protists, such as the kinetoplastids, which possess small linker histones that lack the winged helix motif entirely and are similar to the basic CTD of metazoan histone H1 (41), although the amino acid composition may differ from that of the metazoan proteins. Such single-domain H1 proteins likely compact DNA by mechanisms that are distinct from those employed by metazoan H1. By contrast, *Gallus gallus* (chicken) erythrocyte linker histone H5 shares greater sequence homology (66%) to the human histone H1.0, while the CTD is quite divergent (42).

**Yeast linker histone Hho1p**

In contrast to higher eukaryotes, less is known about linker histone function in *S. cerevisiae*. Sequencing of the yeast genome showed the existence of an unusual linker histone H1 named Hho1p, characterized by having two globular domains, one of which
exhibits significant homology to the globular domain of metazoan H1 (Figure 1.3) (43). A short basic tail precedes the H1-like globular domain, and the second globular domain follows a lysine-rich linker. No other linker histones have been reported that contain two globular domains. While the first globular domain closely resembles the winged helix-turn-helix motif characteristic of metazoan H1, the second globular domain is unstructured under physiological conditions, but adopts a winged helix fold in presence of high concentrations of tetrahedral anions (44). Only the first globular domain can associate with nucleosomes to protect additional DNA from nuclease digestion in vitro whereas the second domain exhibits the greatest affinity for four-way junction DNA (45,46). Four-way junction DNA may mimic the DNA conformation at nucleosomal entry/exit points (Figure 1.2), and the ability of Hho1p to bind two four-way junction structures simultaneously has been reported, raising the possibility that Hho1p may bridge two adjacent nucleosomes (47); however, direct evidence for such binding has not been demonstrated.

Micrococcal nuclease (MNase) digests accessible linker DNA to produce DNA fragments corresponding to the chromatosome, whereas digestion of nucleosomal arrays depleted of H1 is faster and generates shorter ~146 bp fragments corresponding to the nucleosome core particle (48). In contrast to the nuclease sensitivity that results from eliminating H1, the absence of Hho1p does not result in significant reorganization of nucleosomes or a change in the chromatin structure during vegetative growth, perhaps due to absence of a terminal lysine-rich domain (43,49). The proposed binding mode for Hho1p in which its globular domains simultaneous engage adjacent nucleosomes would be expected to generate a different type of nucleosome compaction compared to H1, for which one H1 has been proposed to link three nucleosomes to generate a zig-zag pattern,
consistent with differential sensitivity to MNase of H1- or Hho1p-containing chromatin (33,47).

Genome-wide, Hho1p binding was shown to be variable and to be concentrated at rDNA, where it has been implicated in repressing expression of Pol II-transcribed reporter genes embedded in the rDNA, suggesting a role in rDNA compaction (50). A general role for Hho1p in formation of DNA loops and for DNA compaction during stationary phase was also reported (51,52). By contrast, Hho1p has also been demonstrated to prevent establishment of silent chromatin, perhaps by modifying the barriers that separate transcriptionally active chromatin from heterochromatin (52-55). Thus, both H1 and Hho1p have been implicated in long-range DNA looping and DNA compaction, however, the molecular mechanisms by which these proteins exert such functions are likely to differ.

**Chromatin regulates transcription**

In general, transcriptional repression correlates with chromatin condensation. Even nucleosomal arrays are repressive to transcription as nucleosomes prevent transcription factors from accessing their cognate DNA (Figure 1.5). Promoters are therefore typically depleted of nucleosomes compared to the transcribed regions. Such nucleosome-free regions are found just upstream of the transcriptional start site, while the +1 nucleosome, which is found downstream of the start site, is localized strongly to this position. In yeast, nucleosome-free regions are typically maintained by transcription factors (56,57).

Genome-wide profiling has demonstrated not only absence of nucleosomes from active gene promoters, but also a more extensive absence of linker histones, both upstream and downstream of the transcriptional start site (58). An early instructive
example of transcriptional repression by H1 in *Xenopus* shows that reduced H1 expression leads to upregulation of 5S rRNA expression (59). More recent studies have suggested gene-specific transcriptional regulation by H1 as opposed to global effects and that H1 subtypes have an uneven distribution across the genome (60,61). For example, chromatin immunoprecipitation (ChIP) showed a relative depletion of H1.2 and H1.4 in actively transcribed chromatin, whereas all somatic subtypes were detected in heterochromatin (62). Consistent with this observation, H1.2 was reported to be overexpressed in cancer cells where it is recruited to target genes by association with trimethylated H3 lysine 27 (H3K27me3) and contributes to establishment of silent chromatin by a mechanism that requires its CTD (63). In heterochromatin, methylated H1 is implicated in recruitment of factors such as heterochromatin protein 1 (HP1) (64,65). Consistent with the ability of H1 to organize linker DNA into a stem-like structure (Figure 1.4), H1 has been proposed to repress transcription by limiting nucleosome unwrapping as opposed to physically blocking transcription factor binding (66). This is consistent with the genome-wide analyses that point to extensive H1 displacement in transcriptionally active genes (58,67). Such displacement may be aided by chaperones (68).

**Chromatin regulates DNA repair**

Genome integrity is continuously challenged by both endogenous and environmental agents that induce DNA damage. Such damage occurs in the context of chromatin, and higher order chromatin structure is generally repressive for DNA repair. Consistent with the ability of H1 to organize linker DNA into a stem-like structure (Figure 1.5). The “access-repair-restore” model describes sequential events involved in DNA repair in terms of detection of the lesion, chromatin remodeling to allow access to the
repair machinery, the actual repair event, and finally restoration of the original chromatin state (69,70). In this scenario, chromatin is viewed as a barrier that needs to be dismantled for DNA repair to proceed (Figure 1.5). However, the picture is more complex, and emerging evidence has pointed to a role for the nucleosome in recruiting DNA repair proteins (71).

Among the various DNA lesions, double strand breaks (DSBs) are particularly genotoxic, and continuous DNA damage without efficient DSB repair may result in tumorigenesis and ageing. The primary DSB repair pathways include homologous recombination (HR) and non-homologous end joining (NHEJ). Homologous recombination relies on DNA homology between sister chromatids and precisely repairs DSBs, while NHEJ is a more error prone process that uses no or very limited sequence homology to rejoin two DNA ends (72). DNA repair proteins such as Ku and Rad51p play a critical role in DSB repair. Rad51p promotes homologous recombination to repair DSB lesions, however, the chromatosome inhibits homologous pairing. To overcome this barrier and to aid Rad51p-mediated homologous pairing, Rad54p, a member of the ATP-dependent nucleosome remodeling factor family, is required (73,74). The linker histone functions as a negative regulator to suppress inappropriate DNA recombination, which may cause chromosomal aberrations. In S. cerevisiae, Hho1p was also reported to suppress homologous recombination (75,76).

To efficiently repair DSBs by Rad51p and Rad54p-mediated homologous recombination, linker histone H1 is evicted by a histone chaperone, Nap1p, suggesting that eviction of H1 promotes repair by homologous recombination (77). By contrast, Ku, which is integral to DSB repair by non-homologous end-joining, has been reported to
readily displace H1 from DNA ends (78). Monoubiquitylation of H1 at DSB sites has also been recently implicated in recruitment of repair factors, adding to the collection of histone marks that contribute to repair events (79).

Figure 1.5 Chromatin as a barrier to transcription and DNA repair. (A) Condensed chromatin prevents the binding of RNA polymerase and associated transcriptional factors. Chromatin remodelers unmask the transcriptionally active site and allow the recruitment of transcriptional machinery. (B) Chromatin represses the binding of DNA repair protein to the DNA DSB site, posttranslational histone modification and chromatin remodelers open the chromatin to expose damaged site and facilitate the recruitment of DNA repair proteins.
**High mobility group (HMGB) proteins**

In eukaryotes, high mobility group (HMG) proteins are abundant nuclear proteins that make up a significant fraction of DNA-binding non-histone proteins. The HMGB protein family is the largest family of HMG proteins and a major nucleosome-binding constituent of the metazoan nucleus (80). In addition to roles in DNA-dependent events, HMGB proteins sense cellular stress and function as extracellular cytokines, contributing to inflammatory and immune responses (81). The HMGB subfamily is divided into two classes, sequence-specific transcription factors that are expressed in a few cells and non-sequence-specific chromatin-associated proteins, which are abundant constituents of all eukaryotic nuclei. Transcription factors such as lymphoid enhancer-binding factor 1 (LEF-1) and sex determining region Y (SRY) usually contain a single 80 amino acid HMG box in which three α-helices create an L-shaped motif (Figure 1.6B) (82). Most non-sequence-specific chromatin-associated HMGB proteins, e.g. mammalian HMGB1-4, possess two HMGB domains and bind preferentially to non B-form DNA structures such as four-way junctions and DNA modified by the anticancer agent cisplatin (83,84). Exceptions have been described, for example in *S. cerevisiae* and *Drosophila melanogaster*, which encode single HMG-box proteins (NHP6A/B and HMGD, respectively) that bind DNA without sequence preference (85,86).

The HMG-box serves as the primary site of binding to DNA and chromatin. The interaction of HMGB proteins with DNA is very dynamic; HMGB proteins bind transiently to B-form DNA and bend their DNA targets, and the mode of interaction of HMGB proteins with chromatin has therefore been characterized as a “hit and run” (83,87).
The energy required for DNA bending derives from the extensive contacts of HMG boxes with the minor groove of DNA. Since the energetic cost of bending DNA is lessened in distorted or pre-bent DNA, HMGB1 proteins associate with high affinity to such distorted DNA structures (83,88-90). The functional consequences of HMGB1 binding to damaged DNA have been alternately suggested to be a shielding of the lesion from the repair machinery or enhanced recognition of the damaged site (89,91,92). For example, HMGB1 has been reported to sensitize cells to cisplatin by impeding repair, perhaps

![Figure 1.6. Model of Hmo1p and its interaction with DNA. (A) Hmo1p was modeled using Swiss Model in automated mode using human HMGB1 (PDB 2YRQ) as template. HMGB1 is shown in blue and the Hmo1p model is overlaid with box A and box B domains in red and green, respectively. Predicted Hmo1p intercalating residues Leu55 from box A and Phe114 from box B are shown in stick representation. Ser138 is in the position occupied by DNA-intercalating Ile in HMGB1 box B. Helices are identified with Roman numerals. The Hmo1p C-terminal extension (black) is inferred to interact with box A. (B) HMGB1 box B (blue) overlaid with Hmo1p box B (green), showing interaction of helix III in the DNA minor groove and intercalation of Phe between DNA bases. HMGB1 box B-DNA is based on PDB 2GZK.](image)

influenced by cellular redox state (93,94); conversely, human HMGB1 has been reported to facilitate nucleotide excision repair (NER) by recruiting the NER protein XPA to interstrand crosslinks (95). Interestingly, the recruitment of Xeroderma pigmentosum complementation group A (XPA) to non-damaged sites was increased in HMGB1-
depleted cells, suggesting that HMGB1 not only promotes NER, but also facilitates specificity of XPA-mediated damage recognition.

**Structure of HMGB proteins**

The canonical HMGB proteins have a molecular mass of ~25 kDa, containing two similar HMG domains, box A and B, and a C-terminal tail of ~30 acidic amino acids (Figure 1.3). Despite their similarity, box A differs from box B in the relative orientations of helices I and II and in the trajectory of the helix I-II loop, and the two domains have distinct electrostatic surface potentials in their DNA binding regions. The concave sides of both domains bind in the minor groove of DNA using van der Waals and electrostatic interactions to induce a bend towards the major groove. The A domain has a greater preference for distorted DNA (96,97), whereas the B domain binds less selectively to distorted DNA structures, but can introduce an approximately right-angled bend into linear DNA (98). Partial DNA intercalation of hydrophobic residues located toward the N-terminus of helix I and II introduces a kink into the bound DNA and thus enhances the bend associated with widening of the minor groove (Figure 1.6B). The bends induced by either domain likely reinforce each other (99,100). For HMGB1, acetylation of lysine residues in the box A domain occurs *in vivo*, and it has been reported that substitution of these lysine residues compromise preferred binding to both four-way junction DNA and constrained minicircles (97).

An important feature of HMGB1 and HMGB2 is the presence of a long acidic C-terminal ‘tail’ consisting of ~30 (HMG1) or ~20 (HMG2) acidic residues. The acidic tail primarily interacts with box B, but functions to lower the DNA-binding affinity of both domains (84,101,102). Further, the tail is required for preferential binding to DNA.
minicircles relative to linear DNA (103). A dynamic assembly has been proposed in which the acidic tail transiently brings the two HMG-box domains together. On account of the ability to bend DNA, HMGB proteins are generally referred-to as architectural, creating nucleoprotein complexes in which the modified DNA structure promotes association of additional proteins. In this capacity, HMGB proteins participate in numerous DNA-dependent functions, ranging from DNA replication to gene transcription.

**Binding of HMGB proteins to nucleosomes**

Consistent with preferred binding to four-way junction DNA in their open square conformation (104), HMGB proteins bind nucleosomes at the DNA entry/exit points (Figure 1.2). The DNA bending and underwinding that results from HMGB1 binding is transmitted to the nucleosome core (Figure 1.4). This may affect contacts between DNA and core histones and prime the nucleosome core for binding of transcription factors or chromatin remodeling complexes, thus HMGB binding is generally associated with more dynamic chromatin and facilitated transcription. HMGB1 binding in the vicinity of the DNA entry/exit points on the nucleosome may be facilitated by interactions between its acidic tail and the N-terminal tail of histone H3, which exits near the DNA entry/exit points of the nucleosome and contacts the linker DNA (Figure 1.1) (102,105-109). Binding of the HMG boxes to DNA frees the acidic tail from intramolecular interactions with the DNA-binding surfaces, allowing it to interact with H3. A predicted consequence of this interaction is enhanced DNA binding by HMGB1 (110).

Although H1 has higher affinity for reconstituted dinucleosomes compared to HMGB1, binding of HMGB1 displaces the linker histone, perhaps aided by its preferred binding to constrained DNA conformations (111-113). *In vitro*, an interaction between the
acidic tail of HMGB1 and linker histone H1 has also been reported, suggesting that interaction with H1 may increase the DNA binding affinity of HMGB1 by preventing interaction between the acidic tail and the HMG domains, thereby facilitating replacement of H1 for HMGB1 (114). This enhanced binding of HMGB1 in turn affects chromatin remodeling, for example by facilitating the binding of the imitation switch (ISWI)-containing remodeling factors ATP-utilizing chromatin remodeling and assembly factor (ACF) and chromatin remodeling and assembly complex (CHRAC) to chromatin (115).

Yeast HMGB protein Hmo1p and its structure

*S. cerevisiae* expresses several HMGB proteins, of which Hmo1p and Hmo2p contain two globular HMG-like domains. HMO2 (also known as NHP10), which is unique in exhibiting a preferred binding to DNA ends, is a component of the INO80 chromatin remodeling complex that is recruited to DNA damage sites (116,117). Hmo1p was first identified by its co-purification with an unidentified DNA helicase (118). Hmo1p has also been identified in closely related species such as *Saccharomyces kluyveri* (119). In addition, an Hmo1p counterpart is encoded in the *Schizosaccharomyces pombe* genome (120). According to the literature summarized in the *Saccharomyces* Genome Database (121), Hmo1p has been reported to exhibit physical or genetic interactions with a total of 290 genes or gene products.

Hmo1p has two globular domains named box A and box B of about 80 amino acids each, similar to mammalian HMGB (Figure 1.1). Box A, which has only limited similarity to consensus HMG domains, functions as a dimerization domain; it has low affinity for DNA, but exhibits some structural specificity including preferred binding to four-way junction DNA, whereas the canonical box B has higher affinity for DNA, but lower
structural specificity (120,122). The Hmo1p box A domain contributes to DNA bending; in contrast, the box B domain contributes most of the DNA binding affinity, but fails to bend linear DNA (123). There is no high-resolution structural information available for Hmo1p, however, a structure-based model predicts that both box A and box B domains adopt the HMG fold (Figure 1.6A). Alignment with the human HMGB1 used as a template for modeling predicts that Leu55 at the end of helix II corresponds to the HMGB1 box A intercalating residue (Phe in HMGB1); due to poor sequence conservation at the start of box A, it cannot be predicted with confidence if a potential intercalating residue is present at the end of helix I (Phe at the end of HMGB1 box A helix II is the only intercalating residue in this domain). For HMGB1 box B, Phe and Ile, respectively, are the DNA intercalating residues found at the ends of helices I and II; the corresponding residues in Hmo1p box B are Phe114 and Ser138 (which is not predicted to intercalate between DNA bases).

In addition to the A and B domains, Hmo1p has a C-terminal domain that is characterized by a stretch of basic amino acids; this is in marked contrast to mammalian HMGB protein in which the C-terminal extension is acidic. Deletion of the lysine-rich extension does not reduce affinity for linear DNA, arguing against a direct interaction between the CTD and this type of DNA substrate. Instead, interactions between box A and the C-terminal extension were reported to induce a conformation that is required for in-phase DNA bending in vitro (123,124).

Deletion of the HMO1 gene in yeast is not lethal, but results in a severe growth defect and reduced plasmid stability (118,125). Inactivation of HMO1 is synthetically lethal with fpr1 deletion that also results in a plasmid loss phenotype; fpr1 encodes the peptidyl-
prolyl cis-trans isomerase FKBP12, and over-production of Hmo1p in cells deleted for
fpr1 is toxic. FKBP12 disrupts self-association of Hmo1p, suggesting that the toxicity
could be due either to uncontrolled accumulation of Hmo1p at certain target DNA sites or
to sequestering of unbound Hmo1p (126). FKBP12 is otherwise best known as the
receptor for the immunosuppressive drugs FK506 and rapamycin, and binding to either
drug is toxic due to inhibition of signal transduction (127).

Hmo1p is a component of the Pol I transcription machinery and equivalent to UBF

Transcription by RNA polymerase (Pol) I of rRNA genes has been suggested to
be the rate-limiting step in ribosome production (128,129). Intricate networks adapt rRNA
production to metabolic rates as ribosome production must keep up with cellular
demands. The synthesis of rRNA, which accounts for at least ~ 80% of total transcriptional
activity during normal growth, is in most cases thought to be regulated based on control
of active genes as opposed to epigenetic mechanisms that change the ratio of active to
silenced genes (130,131). Distinct nuclear compartments, the nucleoli, form around the
rDNA, and nucleolar structure and cell cycle progression is dependent on rDNA
transcription (132,133).

In S. cerevisiae, approximately 150 rDNA repeats are arranged head-to-tail on
chromosome XII. Each repeat encodes 35S rRNA synthesized by RNA Pol I and the Pol
III-transcribed 5S rRNA. In exponentially growing yeast cells, more than half of the rDNA
is transcriptionally silenced (134-137). The remaining fraction constitutes active rRNA
genes that are largely depleted of nucleosomes, but instead loaded with RNA Pol I and
Hmo1p, with Hmo1p stabilizing the open chromatin state in absence of RNA Pol I
transcription (135,138). While an initial analysis suggested that Hmo1p was bound
throughout the rDNA (139), a more stringent approach revealed preferred Hmo1p binding to Pol I-transcribed regions of the rDNA and that Hmo1p remained bound in absence of Pol I (138,140). A mechanism by which Hmo1p may secure a nucleosome-free region of rDNA involves dimerization of Hmo1p through its box A domains to stabilize DNA bridges and loops (Figure 1.7) (141). However, the looped DNA structure formed by Hmo1p is dynamic and is predicted to be easily disrupted by the force generated by a transcribing RNA polymerase (141). Hmo1p has also been implicated in resumption of RNA Pol I transcription elongation and reopening of rDNA chromatin after DNA repair; UV light-induced DNA lesions block transcription and lead to a special chromatin structure at the rDNA locus characterized by dissociation of RNA Pol I and loading of histones downstream of the lesion, but retention of Hmo1p (142).

Upon nutrient limitation, rDNA transcription is downregulated, and this correlates with a reduction in nucleolar size, a process that is dependent on condensins and involves a compaction of the rDNA (143). It was recently reported that Hmo1p is also involved in such contraction of the nucleolus and that its binding is increased across the 35S rRNA gene in response to starvation (144). As noted above, several previous studies have shown Hmo1p binding either across the rDNA or with preferred association to transcribed regions, depending on method of detection, ChIP protocol, and normalization strategy (138-140,145). In contrast, Wang et al. (144) report limited Hmo1p binding to 35S rRNA genes in log-phase cells (4 hour growth following inoculation of cultures) and an ~6-fold enrichment during nutrient limitation (24 hour growth); whether the failure to detect Hmo1p binding in log-phase cells is due to variations in ChIP protocol or to the genetic background is not clear.
Hmo1p preferentially associates with the transcribed region of the 35S rDNA locus and it promotes rRNA production both as a component of the RNA Pol I transcription apparatus and by facilitating rRNA maturation (139,140,145,146). Overproduction of Hmo1p suppresses the severe growth phenotype caused by a deletion of the gene encoding Rpa49p, a conserved subunit of RNA Pol I and the homolog of human PAF53.

Figure 1.7 Hmo1p-mediated stabilization of genomic DNA. (A) On nucleosome-free DNA, Hmo1p promotes formation of loops and bridges that depend on dimerization of the box A domains (141). Such topological domains may also be mediated by the concerted action of Hmo1p and Top2 (185). (B) A possible nucleosome-stabilizing binding mode for Hmo1p is illustrated in which the structure-specific box A domain binds near the dyad and DNA bending by Hmo1p is prevented due to the lysine-rich CTD contacting linker DNA.

and rpa49Δhmo1Δ double mutants are inviable, indicating that Hmo1p is a component of the Pol I transcription machinery (146). Rrn3p is required for initiation by yeast RNA Pol I, and it is subsequently released during elongation in a process that requires Rpa49p and the presence of another transcribing RNA polymerase (147). Absence of Rpa49p also leads to decreased density of transcribing RNA polymerases on a given gene which result in compromised assembly of the nucleolus (148). The increased distance between transcribing polymerases in the rpa49Δ mutant would also be expected to result in topological constraints due to positive DNA supercoiling accumulating in front of a
polymerase and negative supercoiling developing in its wake; consistent with an \textit{rpa49}\textsuperscript{Δ} mutant accumulating torsional stress, \textit{rpa49}\textsuperscript{Δ} is lethal when the type I topoisomerase Top3 is inactivated (146). Since Hmo1p bends and loops DNA, it may counteract the torsional stress imposed by transcribing Pol I, thereby alleviating the \textit{rpa49}\textsuperscript{Δ} phenotype. Alternatively, or in addition, absence of Hmo1p-mediated DNA looping on rDNA not associated with transcribing RNA polymerase may lead to a nucleosome deposition that is inhibitory to transcription (135).

In mammals, RNA Pol I requires upstream binding factor (UBF) for initiation and elongation (149-151). UBF contains six HMG boxes and binds throughout the rRNA gene locus (152). However, yeast lacks UBF, and Hmo1p has been proposed to be a functional analog of UBF and to be important for maximal Pol I transcription (146). Comparable function of UBF and Hmo1p is supported by the observation that both proteins are highly enriched in the nucleolus and localized throughout the transcribed rDNA region and that both proteins contain HMG domains that may promote DNA bending and DNA looping (139,140,152,153). Further support for overlapping function of Hmo1p and UBF was provided by the observation that expression of human UBF1 or \textit{S. pombe} Hmo1p also suppress the \textit{rpa49}\textsuperscript{Δ} growth phenotype (120).

\textbf{Hmo1p regulates Pol II transcription}

In \textit{S. cerevisiae}, the ribosome is made up of four rRNAs (5S, 5.8S, 18S, and 25S) and 79 ribosomal proteins (RP) expressed from 138 genes (154). RP gene transcription constitutes up to 50\% of RNA Pol II mediated transcription, and it is coordinately regulated in response to environmental conditions (155). In prokaryotes, ensuring production of stoichiometric levels of ribosomal proteins is simple because RP genes form operons,
whereas in eukaryotes such regulation is more complicated as each RP gene yields a monocistronic mRNA. A number of transcription factors have been reported to contribute to regulation of RP gene activity, including Rap1p, which binds the majority of RP genes and forms nucleosome-free regions in target promoters (145,156). Hmo1p binds RP gene promoters with variable occupancy and has been implicated in pre-initiation complex (PIC) assembly by covering a nucleosome-free region and in recruitment of the transcription factor forkhead like (Fhl1p) (139,140,145,157,158).

Pol II transcription requires basal transcription factors including TFIID, which contains the TATA box-binding protein (TBP) and TBP associated factors (TAFs). TAF1 N-terminal domain (TAND) inhibits binding of TBP to the TATA element (159); it has been reported that Hmo1p interacts with TBP and TAND and that HMO1 deletion decreases transcription of TAND-dependent genes, suggesting that Hmo1p prevents inhibitory TBP-TAND interactions. In addition, an interaction between Hmo1p and TFIID was suggested by the observation that HMO1 deletion causes an upstream shift in transcription start sites of genes under control of Hmo1p-enriched promoters, but not of genes driven by promoters with limited Hmo1p occupancy (158). This shift in transcriptional start site was subsequently linked to the ability of Hmo1p to mask a nucleosome-free region to prevent inappropriate PIC assembly (157). This nucleosome-free region was later reported to exhibit sensitivity to micrococcal nuclease and to contain unstable or “fragile” nucleosomes, perhaps rendered unstable through the action of the essential multifunctional transcription factor Rap1p (160).

Fhl1p (Forkhead like) is a transcription factor with sequence similarity to the forkhead (FH) winged helix DNA binding domain. On RP genes, Fhl1p has been reported
to remain bound and to recruit either the co-activator Ifh1p (Interacts with forkhead) or co-repressor co-repressor with Fhl1 (Crf1p). During vigorous growth, Fhl1p and Rap1p recruit Ifh1p, which results in maximal transcription (161-163). In addition, Sfp1p (Split finger protein) has been reported to be required for maximal transcription from RP promoters (164). During stress and nutrient starvation, Ifh1p dissociates from RP promoters and Fhl1p recruits Crf1p while Sfp1p translocates to the cytoplasm, events that lead to downregulation of RP gene transcription (161,162,164,165). Dissociation of Hmo1p was also reported under conditions of RP gene repression, leading to an upstream shift of the +1 nucleosome, suggesting that Hmo1p is important for placement of the +1 nucleosomes in either a repressive or active position (166).

Little is known about the role of Hmo1p in regulation of Pol II-transcribed genes other than the RP genes. Excess Hmo1p represses the HMO1 promoter, however, the underlying mechanism is unknown (125). Given the self-association of Hmo1p, it is tempting to speculate that excess Hmo1p promotes an accretion of Hmo1p on the HMO1 promoter that adversely affects binding of either transcription factors or RNA Pol II.

Regulation of gene transcription involves ATP-dependent chromatin-remodeling complexes that either slide or evict nucleosomes or alter their composition (167). The conserved SWI/SNF complex, for instance, is critical for modulation of gene expression during a variety of cellular processes. Among the HMGB proteins Hmo1p and NHP6A/B, all stimulate the sliding activity of switch/sucrose non-fermentable (SWI/SNF), but only Hmo1p promotes SWI/SNF binding to the nucleosome, histone octamer transfer, and exposure of nucleosomal DNA. Notably, the stimulatory effect requires the Hmo1p CTD and the presence of linker DNA, as no binding of Hmo1p to nucleosomes devoid of linker
DNA could be detected (168). Based on these observations, Hmo1p appears to recruit SWI/SNF to nucleosomes by a mechanism that requires changes in DNA topology.

**Hmo1p stabilizes noncanonical chromatin structures**

On rDNA and on ribosomal protein gene promoters, Hmo1p appears to exert an effect in large part through its association with nucleosome-free DNA or DNA associated with “fragile” nucleosomes. The potential instability of DNA containing repetitive sequence elements, such as that characterizing the rDNA array, necessitates protective measures. In humans, long CAG repeat tracts underlie hereditary neurodegenerative diseases including Huntington disease, as they have a propensity to expand. The length of CAG repeat tracts correlates with their instability; duplex DNA exhibits unusual flexibility and unwound DNA may engage in intramolecular base pairing to form hairpin structures that hinder DNA replication (169). When embedded in the yeast chromosome, CAG repeat tracts are bound and stabilized by Hmo1p, which establishes a noncanonical chromatin organization (170). The length of CAG repeat tract chromatin that is protected from nuclease digestion is shorter than that protected by a nucleosome, raising the possibility that tetramer cores of histones associate with the DNA and that Hmo1p may have replaced H2A and H2B, perhaps serving as a linker between tetramer cores.

Recombination events and genomic instability may also be triggered by clashes between replication and transcription (171). Dedicated topoisomerases such as Top2p relieve the topological constraints that result when a replication fork encounters transcription and promote fork progression. In S-phase, intergenic regions close to some transcribed genes exhibit low nucleosome density, but accumulate both Hmo1p and Top2p; together, Top2p and Hmo1p appear to suppress chromosome fragility at the M-
G1 transition (172). Top2p binding occurs independently of Hmo1p, while a function of Hmo1p may be to maintain the low nucleosome density required to facilitate the Top2p-mediated DNA looping, which promotes formation of topological domains and gene transcription.

DNA damage may lead to events ranging from mutagenesis to chromosomal rearrangements. The DNA damage response (DDR) allows for a delay of cell cycle progression to ensure DNA repair and replication of the genome by high-fidelity polymerases and to mediate fork restart (173). The error-free mode involves a recombination event in which the newly synthesized strand is used as template for replication of the damaged strand, whereas the error-prone mode relies on trans-lesion synthesis. This pathway choice is important for genome integrity. Among the myriad of events associated with replication, DNA topological changes include the sister chromatid bridges that form when replication forks pass through transcriptionally active chromatin loops (172). The association of Hmo1p with such junctions has also been implicated as one of the mechanisms by which Hmo1p promotes the error-free DNA damage tolerance pathway by facilitating template switching (174), and it is consistent with the preferred binding of Hmo1p to four-way DNA junctions compared to linear DNA (122). These functions of Hmo1p require its C-terminal extension (174), shown to be required for DNA bending and bridging (123,124).

**Hmo1p (and its C-terminal tail) and DNA double-strand break (DSB) repair**

Absence of Hmo1p does not affect the bead and string pattern of nucleosomes, but makes the chromatin hypersensitive to nucleases (118,175), suggesting that Hmo1p stabilizes chromatin. Genome-wide analysis of Hmo1p binding revealed extensive yet
variable association across the genome, with particular enrichment at genes encoding ribosomal proteins and rRNA (139). The coverage of Hmo1p-binding would be consistent with an effect on chromatin stabilization that is detectable by analysis of bulk chromatin.

DSBs are induced in the context of chromatin. The presence of sister chromatids allows repair by homologous recombination, whereas non-homologous end-joining operates without involving a separate copy of the DNA duplex. A number of histone modification and chromatin remodeling events precede DNA repair pathway choice and render the chromatin template accessible to repair proteins (176). In yeast, a DSB may be site-specifically created at the mating type locus MAT by inducing expression of HO endonuclease. In chapters 2 and 3, I will present an investigation of the role of Hmo1p and its basic extension in DNA DSB repair and associated chromatin remodeling.

Replication-independent endogenous DNA DSBs occur spontaneously and are not pathological lesions in that they do not induce mutation or cell death. Instead, they may possess important biological functions, perhaps in relieving topological stress that might otherwise result in uncontrolled DNA breakage. They have also been reported to occur non-randomly, for example, with an increased frequency in heterochromatin (178). Such breaks are repaired either by Ku- or Rad51p-dependent pathways, as evidenced by increased levels of such breaks in cells deleted for either Ku or Rad51p (179). Conversely, deletion of Hmo1p resulted in reduced break levels, an outcome that would be consistent with absence of Hmo1p facilitating chromatin remodeling events required for their elimination.

While the exact nature of Hmo1p interaction with nucleosomes remains unknown, the preferred binding of Hmo1p to four-way DNA junctions is consistent with binding at
the dyad, as reported for H1, which likewise binds preferentially to DNA junctions (Figure 1.2) (33). Based on the inference that DNA bending and underwinding by mammalian HMGB may facilitate nucleosome unwrapping, Hmo1p might likewise be expected to destabilize nucleosomes; however, the observation that interactions between the Hmo1p box A domain with the CTD are required for DNA bending offers an alternative scenario (108,123,124). It is conceivable that the Hmo1p lysine-rich domain contacts DNA directly when Hmo1p associates with nucleosomes, a circumstance in which DNA-bending by Hmo1p would likely be attenuated (Figure 1.7), thus allowing Hmo1p to stabilize the nucleosome.

**Interplay between Hho1p and Hmo1p**

Both genes encoding core histones and the *hho1* gene are transcribed in S-phase, suggesting that Hho1p acts in concert with the core histones (180). Consistent with this observation, Hho1p binds the DNA entry/exit points of nucleosomes (49). However, during vegetative growth the absence of Hho1p does not affect global chromatin structure as evidenced by changes in MNase sensitivity (49,175). Further, its deletion does not result in growth or mating defects, significant global changes in gene expression, or a change in average nucleosome distance (53). Phenotypes associated with *hho1* deletion are subtle and have pointed to roles for Hho1p in suppressing homologous recombination and suppressing the formation of silent chromatin (perhaps by affecting function of the Sir complex) and in promoting formation of chromatin loops and in chromatin compaction during stationary phase (51,52,54,75). Disruption of *hho1* does result in a substantial increase in the levels of its own transcript, suggesting a feedback system for *hho1* gene
regulation (53). Curiously, a feedback mechanism for regulation of the HMO1 promoter was also suggested by the increased HMO1 promoter activity in an hmo1Δ strain (125).

Another commonality between Hho1p and Hmo1p is their preferred binding to rDNA. For Hho1p, this localization to rDNA is associated with repression of recombination and with efficient transcriptional silencing by compaction of rDNA chromatin (50, 76), whereas functions of Hmo1p range from rDNA compaction during starvation to participating as a component of the Pol I transcription machinery as discussed above (139, 140, 142, 144-146). The inter-dependence of Hmo1p and HHO1 is described in detail in chapter 3.

REFERENCES:


and suppresses tumorigenesis caused by hyperactive JAK-STAT signaling. *Epigenetics Chromatin, 7*, 16.


homologous recombination by RAD51 and RAD54 in higher-ordered chromatin containing histone H1. *Sci Rep*, 4, 4863


126. Dolinski, K.J. and Heitman, J. (1999) Hmo1p, a high mobility group 1/2 homolog, genetically and physically interacts with the yeast FKBP12 prolyl isomerase. Genetics, 151, 935-944.


CHAPTER 2
YEAST HIGH MOBILITY GROUP PROTEIN HMO1 STABILIZES CHROMATIN AND IS EVICTED DURING REPAIR OF DNA DOUBLE STRAND BREAKS

Introduction

Packaging of eukaryotic DNA into nucleosomes organizes the genome, but reduces accessibility of proteins, which are required for cellular processes such as repair of damaged DNA, replication, or transcription. To overcome this nucleosome barrier, cells have evolved mechanisms to open chromatin structures, such as the recruitment of ATP-dependent chromatin remodeling complexes. These complexes change the packaging state of chromatin by moving, destabilizing, ejecting or restructuring the nucleosome (1,2).

DNA damage and repair occurs in the context of chromatin. DNA double-strand breaks (DSBs) arise due to either exogenous factors, for example ionizing radiation, or endogenous events such as stalled replication forks. Unrepaired DSBs promote genome instability that may lead to tumorigenesis or cell death, and efficient repair is therefore essential (3). The two major DSB repair pathways are homologous recombination (HR) and nonhomologous end-joining (NHEJ). HR relies on homologous sequences to maintain the fidelity of DNA repair. In eukaryotes, homology recognition and strand exchange is mediated by the recombinase protein Rad51, which is recruited to DSBs after nucleolytic degradation to generate single-stranded 3'-ends (4). NHEJ is considered error-prone. It is initiated by Ku, which binds DNA free ends and arrive early at break

This chapter originally appeared as Panday et al. (2015) Yeast high mobility group protein HMO1 stabilizes chromatin and is evicted during repair of DNA double strand breaks. Reprinted with permission from Nucleic Acids Research 43(12): 5759-5770.
Ku facilitates binding of proteins involved in DNA end-processing and intermolecular end-joining, including Ligase IV, which is required for ligation of broken DSB ends (5).

Chromatin remodeling is an integral part of the DSB response and it is required for the sequential recruitment of DNA repair proteins at the break site. In yeast, one of the earliest events in response to DSB is phosphorylation of histone H2A on serine 129, a modification that spreads from the vicinity of the break in both directions, spanning around 50 kb (6,7). H2A is the primary yeast H2A isoform, yet the phosphorylated version is often referred-to as γ-H2AX since the equivalent phosphorylation event in mammalian cells involves the H2A isoform H2AX (which is absent in yeast) (8). This H2A phosphorylation is required for recruitment and retention of both chromatin remodeling complexes and DNA damage response proteins.

Several chromatin remodelers, including INO80, are recruited to the damage site in a γ-H2A-dependent fashion. INO80 is a conserved member of the SWI/SNF family that remodels chromatin by repositioning nucleosomes along the DNA (9). This remodeling complex contains multiple subunits, including the catalytic subunit Ino80 and three actin-related subunits Arp4, Arp5 and Arp8 (10); deletion of Arp5 and Arp8 mimics an ino80Δ phenotype and such mutants are deficient in DSB repair (10-12). INO80 participates in both HR and NHEJ pathways (11-14), and it is involved in HR-mediated recovery of stalled DNA replication forks (15). Nhp10, a high mobility group (HMGB) protein also known as HMO2, binds DNA ends and is present only in the INO80 complex and not in SWR1 or other known chromatin remodeling complexes and it is required for INO80 recruitment to γ-H2A (13,16). Major roles of INO80 include histone displacement and
nucleosome disruption to enable the recruitment of repair proteins; after the completion of DNA repair, histone redeposition restores the chromatin structure (17).

HMGB proteins are non-histone DNA binding proteins with established roles in chromatin organization or dynamics (18). *Saccharomyces cerevisiae* contains 10 HMGB proteins, of which Nhp6 and Hmo1p have been shown to affect chromatin structure. Deletion of the *HMO1* gene makes the chromatin hypersensitive to nuclease (19), which indicates a general role for Hmo1p in stabilizing higher order chromatin structures. In addition, *hmo1Δ* strains exhibit increased mutagenesis frequency (20); it was subsequently suggested that this may be explained by the ability of Hmo1p to prevent lesions from entering error-prone repair pathways (21). Hmo1p has two DNA binding domains, box A and box B, and a lysine-rich C-terminal extension. Hmo1p bends DNA and both box A and the basic C-terminal extension is required for such changes in DNA topology (22-24). The lysine-rich C-terminal extension also confers on Hmo1p the ability to compact DNA, as evidenced by enhanced DNA end-joining (23).

The nuclease-sensitive chromatin phenotype associated with *HMO1* deletion is surprising by comparison to mammalian HMGB proteins, which are thought to promote flexible chromatin structures by competing with histone H1 for binding to linker DNA; by contrast, the role of yeast H1 in chromatin organization appears more limited (18,25). We show here that Hmo1p stabilizes chromatin as evidenced by faster chromatin remodeling in its absence. This stabilization requires the lysine-rich C-terminus. Specifically, H2A phosphorylation, recruitment of INO80 to a DSB site, histone H3 eviction, and DNA resection is more efficient in an *hmo1Δ* strain, and Hmo1p is evicted along with core histones during DSB repair. Furthermore, we show that these events correlate with more
efficient repair by both HR and NHEJ in hmo1Δ strains, that absence of Hmo1p promotes recruitment of Rad51, even in absence of induced DSBs, and that tracking of Ku protein from DNA ends correlates with efficient chromatin remodeling. We suggest that Hmo1p stabilizes higher order chromatin structures, perhaps by its lysine-rich domain promoting DNA compaction, and that its eviction is important for efficient DSB repair.

Materials and methods

Strain construction

Strains are derived from either DDY3 or the donorless JKM179, which lacks HML and HMR loci on chromosome 3 and contains an integrated galactose-inducible HO endonuclease gene (26,27). DDY3 is isogenic to W303-1A. DDY-AB, which encodes a truncated version of Hmo1p deleted for its C-terminal extension, was previously described (28). The DDY1299 derivative of DDY3 in which HMO1 is deleted was also previously described (28); strain JKM179hmo1Δ was created using the same approach, except that the selection marker URA3 was amplified from pRS426 (29). The gene encoding Ku was deleted by amplifying the URA3 marker with primers that include ~80 nt of flanking sequence homologous to the ku gene, followed by transformation of either DDY3 or DDY1299 haploid cells to generate DDY3kuΔ and DDY3hmo1ΔkuΔ, respectively. A strain expressing Hmo1p-FLAG was created from DDY3 using primers amplifying the selection marker kanamycin. All strains are described in Table 2.1.

ChIP and PCR analysis

Chromatin Immunoprecipitation (ChIP) was performed as described (26), with minor modifications. Yeast cells were grown at 30°C in 2% raffinose-containing YP or in synthetic defined (SD) dropout media to an optical density at 600 nm of 1.0. A 100 ml
culture aliquot was removed and utilized as the uninduced sample for the ChIP assay. Galactose was added to the remaining culture to a final concentration of 2% to induce HO, and cells were collected at different time intervals for the ChIP assay. To repress HO expression and prevent further DNA damage, 2% glucose was added and cells were harvested at different time intervals for ChIP assay. Cells were fixed with formaldehyde (37%) diluted to 1.2% in the culture medium and incubated at room temperature for 20 min with gentle shaking. Cells were lysed by vortexing with glass beads for 40 minutes at 4°C using lysis buffer (50 mM HEPES-KOH, pH 7.5, 140 mM NaCl, 1% Triton X-100 and 0.1% sodium deoxycholate) containing protease inhibitors, pepstatin A (1 μg/ml), leupeptin (1 μg/ml) and phenyl methyl sulfonyl fluoride (PMSF (100mM). To shear chromatin into 100–2000 bp fragments (predominant size ~500 bp), the lysate was

Table 2.1 Strains and their genotype

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDY3</td>
<td>MATa ADE2 his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 can1-100</td>
</tr>
<tr>
<td>DDY3hmo1FLAG</td>
<td>MATa ADE2 his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 can1-100 hmo1-FLAG(KANMX4)</td>
</tr>
<tr>
<td>DDY1299</td>
<td>MATa ADE2 his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 can1-100 hmo1Δ::HIS3</td>
</tr>
<tr>
<td>DDY3-AB</td>
<td>MATa ADE2 his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 can1-100 hmo1-tail::URA3</td>
</tr>
<tr>
<td>DDY3kuΔ</td>
<td>MATa ADE2 his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 can1-100 kuΔ::URA3</td>
</tr>
<tr>
<td>DDY3hmo1ΔkuΔ</td>
<td>MATa ADE2 his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 can1-100 hmo1Δ::HIS3 kuΔ::URA3</td>
</tr>
<tr>
<td>JKM179</td>
<td>MATa hoΔ hmlΔ::ADE1 hmrΔ::ADE1 ade1-100 leu2-3,112 lys5 trp1::hisG ura3-52 ade3::GAL::HO</td>
</tr>
<tr>
<td>JKM179hmo1Δ</td>
<td>MATa hoΔ hmlΔ::ADE1 hmrΔ::ADE1 ade1-100 leu2-3,112 lys5 trp1::hisG ura3-52 ade3::GAL::HO hmo1Δ::URA3</td>
</tr>
</tbody>
</table>
sonicated six times for 10 sec each at 25% amplitude while keeping the samples on ice intermittently. Sheared chromatin was then aliquoted for ChIP reactions (100 μl of lysate). To reduce the non-specific binding to Sepharose beads, the lysate was precleared using protein G-Sepharose beads (GE Healthcare). For immunoprecipitation, the following antibodies were used: 5 μl of anti-FLAG (Sigma), 5 μl of antibody against phosphorylated H2A (Ser 129) (Merck Millipore), 2 μl of anti-Rad51 (Santa Cruz Biotechnology), 2 μl of anti-Arp5 (Abcam), 2 μl of anti-H3 (Abcam), and 2 μl of antibody against Ku (30).

Extracted DNA from ChIP samples or input DNA was analyzed by PCR; monitored loci included MAT (72 bp downstream of the DSB), 0.2 kb upstream, 3.1 kb downstream, 9.5 kb downstream, and 29.5 kb upstream of the DSB and at POL5. PCR products were loaded on 1.4% agarose gels containing 0.01% ethidium bromide. Primer sequences are provided in Table 2.2. Signal intensities from PCR data were quantified from the TIFF images by using ImageJ software (31) with some modifications. Images were first transformed to 16-bit-type images, and the threshold function was set to black and white type of image to avoid background interference. The rectangle tool was used to define the area around PCR bands. Fold enrichment was calculated as signal intensity ratio of ChIP/Input DNA. The presence of histone H3 was also determined using quantitative real-time PCR (qRT-PCR). qPCR was conducted using an ABI Prism 7000 sequence detection system and SYBR Green for detection. Data were normalized to input control. Each experiment was repeated three times and average and standard deviations (SD) are reported.

Survival following DSB induction

Strains of JKM179 background possess a genomic galactose inducible HO
Table 2.2 Sequences of primers used for ChIP, DNA resection, and gene expression.

<table>
<thead>
<tr>
<th>Primers Used</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAT p1</td>
<td>TCCCCATCGTCTTGCTCT</td>
</tr>
<tr>
<td>MAT p2</td>
<td>GCATGGGCAGTTTACCTTTAC</td>
</tr>
<tr>
<td>0.2 kb upstream p1</td>
<td>AAAGAAGAAGTTGCAAAGAAATGTGG</td>
</tr>
<tr>
<td>0.2 kb upstream p2</td>
<td>TGTTGCGGAAAGCTGAAACTAAAAG</td>
</tr>
<tr>
<td>1.6 kb upstream p1</td>
<td>ATGTCTCTGACTTTTTTGACGAGG</td>
</tr>
<tr>
<td>1.6 kb upstream p2</td>
<td>ACGACCTATTTGTAACCAGCAG</td>
</tr>
<tr>
<td>29.8 kb upstream p1</td>
<td>TCGTCGTCGCCATCATTTTC</td>
</tr>
<tr>
<td>29.8 kb upstream p2</td>
<td>GCCCAAGTGGAGAGGTTGC</td>
</tr>
<tr>
<td>3.1 kb downstream p1</td>
<td>CTAATGCTGCAAAAATCCATATGCT</td>
</tr>
<tr>
<td>3.1 kb downstream p2</td>
<td>CTCTATGTTGTTTTTACCTACCGC</td>
</tr>
<tr>
<td>9.5 kb downstream p1</td>
<td>TGGATCATGGACAAGGTCCTAC</td>
</tr>
<tr>
<td>9.5 kb downstream p2</td>
<td>GCCGaaaACATGGGACTCT</td>
</tr>
<tr>
<td>MATα p1</td>
<td>GTGGCATATTACTCCACTTCAAGTAAG</td>
</tr>
<tr>
<td>MATα p2</td>
<td>AACTAGCAAACAAAGGAAAGTC</td>
</tr>
<tr>
<td>MATα p1</td>
<td>AATGGCACGCGGACAAATGC</td>
</tr>
<tr>
<td>MATα p2</td>
<td>AACTAGCAAACAAAGGAAAGTC</td>
</tr>
<tr>
<td>Ho cut site p1</td>
<td>ATGTGAACCGCATGGGCAGT</td>
</tr>
<tr>
<td>HO cut site p2</td>
<td>TGTTGCTCTACTATCTTGCC</td>
</tr>
<tr>
<td>POL5 p1</td>
<td>TCCTTGTTCACCTTTGGTGGA</td>
</tr>
<tr>
<td>POL5 p2</td>
<td>GTGTTCCTAGTCTACCCATCG</td>
</tr>
<tr>
<td>q MATα p1</td>
<td>GCCGAAAACATAAACAGAAGACTCTG</td>
</tr>
<tr>
<td>q MATα p2</td>
<td>CCGTGCTTGGGGTATATTTGATG</td>
</tr>
<tr>
<td>IPP1 Fw</td>
<td>CCCAATCATCCAGACACCAAGAAGG</td>
</tr>
<tr>
<td>IPP1 Re</td>
<td>AGCAATAGTTTCCACATTCTTCAACACATC</td>
</tr>
</tbody>
</table>
endonuclease gene. For DDY3-derived strains, the galactose-inducible *HO* endonuclease gene was furnished on a centromeric plasmid, with *DDY3HMO1FLAG*, *DDY3* and *DDY1299* transformed with plasmid carrying URA3 marker and *DDY3-AB*, *DDY3kuΔ*, and *DDY3hmo1ΔkuΔ* transformed with plasmid carrying the TRP marker. Cells of *JKM179* background were grown at 30°C in raffinose-containing YP media. Transformed strains *DDY3HMO1FLAG*, *DDY3*, and *DDY1299* were grown in SD drop out media minus uracil and *DDY3-AB*, *DDY3kuΔ*, and *DDY3hmo1ΔkuΔ* were grown in SD drop out media minus tryptophan. Cells were grown at 30°C to an optical density at 600 nm of 1.0, at which point 2% galactose was added to induce *HO* and DSB for 4 hours. To monitor survival, 0.1 mL cell culture was plated at 10^{-3} dilution on YPD or SD drop out agar media in replica and incubated at 30°C. Cultures to which no galactose was added were plated as a control. After 48 hours colonies were counted. Each experiment was repeated three times and data reported as mean with standard deviations.

To monitor cell viability, a trypan blue exclusion assay was used. Cells were grown at 30°C to OD<sub>600</sub> of 1.0, at which point 2% galactose was added to induce *HO* for 4 hours. Cells were collected and mixed with 0.4% trypan blue (1:1 vol/vol), placed on a hemocytometer, and immediately examined under an inverted microscope. The fraction of dead cells is reported as the number of blue cells divided by total number of cells. The assay was repeated three times and average and standard deviations (SD) are reported.

**DNA end resection**

Cells were grown at 30°C to an OD<sub>600</sub> of 1.0, and DSB was induced with 2% galactose. Cells were harvested after induction times of 20 minutes, 1 hour, 2 hours, 3 hours and 4 hours. Genomic DNA was extracted by vortexing cells with glass beads and
phenol. Twenty microliters of genomic DNA sample (60 ng in 1X NEB Exonuclease I buffer) was digested with 20 units of *E. coli* exonuclease I at 37°C overnight. The level of DNA resection adjacent to the specific DSB was measured by qPCR using primers annealing 1.6 kb upstream of the DSB. All values were normalized to values for an independent locus on chromosome 5 (*POL5*). The assay was repeated three times and average and standard deviations (SD) are reported.

RESULTS
Efficient DSB repair in absence of Hmo1p

To determine how the presence of Hmo1p affects DSB repair, we monitored cell survival after induction of a DSB. HO endonuclease introduces a single DSB in the mating type (*MAT*) locus, and repair by HR, the pathway of choice, involves one of the homologous silent mating type *HM* cassettes as a donor to create a *MAT* gene of the opposite mating type. A survival assay was performed using *DDY3* (26) and *DDY3hmo1Δ* (28), which possess *HMLα* and *HMRα* loci and preferentially repair DSB by HR. Yeast cells were transformed with plasmid carrying galactose-inducible *H-O*, DSB was induced by galactose, and cells were plated with glucose to allow repair. The survival assay indicated that recovery from DSB induction was ~2-fold more efficient in the *DDY3hmo1Δ* strain compared to the isogenic WT (Fig. 2.1A). No difference in plating efficiency was observed for cells not producing HO. Since Hmo1p was reported not to localize to the *GAL* promoter used to drive expression of *HO* (32), glucose repression of *HO* expression is unlikely to be affected by the absence of Hmo1p. We verified viability of cells by staining with trypan blue; while cells not induced to produce HO were viable, a significant
proportion of dead cells were observed in DDY3 after continuous HO induction, whereas this fraction was significantly reduced in hmo1Δ cells (Fig. 2.1B).

To verify that the HMO1 deletion did not compromise fidelity of HR or change the preferred repair pathway, a fidelity experiment was performed with DDY3 and DDY3hmo1Δ. Result showed efficient mating type switching from MATa to MATa with no evidence of residual MATa cells (Fig. 2.2), indicating that repair proceeded by HR in both strains and not by NHEJ. Sequencing confirmed fidelity of the repair. These results suggest that DSB repair by DDY3hmo1Δ proceeds by HR and without compromising the fidelity.

To rigorously rule out the possibility of repair by NHEJ, the survival assay was repeated with strains in which the gene encoding Ku80, which is indispensable for NHEJ, was inactivated. Data were consistent with the previous result, showing ~3-fold greater survival in the DDY3hmo1ΔkuΔ strain compared to DDY3kuΔ (Fig. 2.3A). That the HMO1 deletion resulted in even greater repair efficiency (survival) in a kuΔ background is intriguing and may reflect that competition between HR and NHEJ protein recruitment to the DSB site is attenuated in absence of Ku. The lower plating efficiency of DDY3kuΔ compared to DDY3 after HO induction is reflected in a greater proportion of inviable cells in the DDY3kuΔ strain (Fig. 3.3B, C).

To assess if the role of Hmo1p is specific to HR, the survival assay was performed with the donorless (HMLα and HMRα deleted) JKM179 (27) and JKM179hmo1Δ, which repair DSB by NHEJ. Again, we found ~2-fold greater survival in JKM179hmo1Δ compared to the isogenic parent JKM179 (Fig. 2.4A) and greater viability in absence of
Figure 2.1. Survival of WT and hmo1Δ strains following induction of DNA double strand breaks. (A) Survival of DDY3 and corresponding hmo1Δ strain. After DSB induction, cells were diluted 10^4-fold and plated and colonies counted. Survival is represented as colonies per OD. Cells not induced to express HO were plated as control (ctrl). (B) Viability of DDY3 and corresponding hmo1Δ strain as determined by trypan blue exclusion.

Figure 2.2 Fidelity assay. PCR product obtained using genomic DNA isolated from DDY3 WT and corresponding hmo1Δ strains using primer specific for MATα and MATα showing gene conversion from MATα to MATα following DNA repair.

Hmo1p (Fig. 2.4B). To ensure that differential survival was not due to different efficiencies of DSB induction in the two strains, qPCR was performed using primers that flank the DSB site, revealing no significant difference in DSB induction between the two strains (Fig. 2.5). Taken together, these data indicate that hmo1Δ strains repair DSB more efficiently than WT via both HR and NHEJ.
Figure 2.3. Survival of DDY3kuΔ and DDY3kuΔhmo1Δ strains following induction of DNA double strand breaks. (A) Survival of DDY3kuΔ and corresponding hmo1Δ strain. After DSB induction, cells were diluted 10⁴-fold and plated and colonies counted. Survival is represented as colonies per OD. Cells not induced to express HO were plated as control (ctrl). (B) Survival of DDY3 and corresponding kuΔ strain. After DSB induction, cells were diluted 10⁴-fold and plated and colonies counted. Survival is represented as colonies per OD. Cells not induced to express HO were plated as control (ctrl). (C) Viability of DDY3 and corresponding kuΔ strain as determined by trypan blue exclusion. Three independent experiments were performed. Error bars represent standard deviation.

Hmo1p localizes to the MAT locus and is evicted during DSB repair

The observation that Hmo1p affects the efficiency of DSB repair by both HR and NHEJ suggests that the effect is repair pathway independent, perhaps affecting upstream events such as chromatin remodeling. To address if Hmo1p localizes directly to the MAT locus, we used a Flag-tagged Hmo1p strain (DDY3 background) and performed chromatin immunoprecipitation (ChIP) to monitor localization of Hmo1p at MAT, 0.2 kb upstream, 9.5 kb downstream and 29.8 kb upstream from the DSB site during DSB induction and repair. Hmo1p was found to localize evenly throughout the locus (Fig. 2.6; 0 hr). By comparison, genome-wide analysis of Hmo1p localization revealed that Hmo1p binding is variable throughout the genome, with particular enrichment of Hmo1p at ribosomal protein promoters (as high as ~11-fold above background), and 2.7-fold above
Figure 2.4. Survival of WT and hmo1Δ strains following induction of DNA double strand breaks. (A) Survival of JKM179 and corresponding hmo1Δ strain. After DSB induction, cells were diluted 10^4-fold and plated and colonies counted. Survival is represented as colonies per OD. Cells not induced to express HO were plated as control (ctrl) (B) Viability of JKM179 and corresponding hmo1Δ strain as determined by trypan blue exclusion. Three independent experiments were performed. Error bars represent standard deviation.

Figure 2.5. Efficiency of DSB induction. Kinetics of HO cleavage at MAT was monitored as a reduction in qPCR signals amplified with primer pairs flanking the MAT locus using genomic DNA isolated from DDY3 and corresponding hmo1Δ strain (left) and JKM179 and corresponding hmo1Δ strain (right). qPCR signal of no damage control sample was used to calculate percent cut. Experiment was repeated three times. Error bars represent standard deviation.
background at the MAT locus (32). When DSB was induced continuously for 4 hours, we observed gradual loss of Hmo1p at all monitored locations beginning after 2 hours of DSB induction, with complete disappearance after 4 hours, even at the most distant site 29.8 kb downstream (Fig. 2.6A, B).

To determine Hmo1p localization during DNA repair, DSB was induced for 1 hr., following which further DNA damage was prevented by the addition of glucose. We found significant loss of Hmo1p after 2 hours of repair, especially at sites proximal to the DSB (Fig. 2.6C, D). To verify that differential Hmo1p localization is specific to the DSB site, we monitored Hmo1p localization at the POL5 gene as a control (Hmo1p occupancy at the POL5 gene promoter was previously reported to be 1.5-fold above background (32); as expected, we observed no change in Hmo1p binding to POL5 during DSB induction and repair at the MAT locus (Fig. 2.7). Evidently, Hmo1p localizes to the MAT locus, and it is selectively evicted from sites proximal to the DSB during DNA repair. These results are consistent with Hmo1p directly affecting DNA repair as a component of chromatin, as opposed to exerting indirect control over factors involved in repair, and they implicate Hmo1p eviction as a necessary step in DNA repair. Since Hmo1p occupancy is variable across the genome, we reasoned that Hmo1p localization to the MAT locus might serve a regulatory role. We therefore compared MATa transcription in DDY3 and DDY3hmo1Δ strains. Relative to a control locus (IPP1 at which Hmo1p localization was reported to be below background levels (32), transcript levels were reduced ~50% in the hmo1Δ strain (Fig. 2.8). Considering that Hmo1p is localized throughout the genome, we also wondered if increased survival following DSB induction in the hmo1Δ strain is unique to DSBs
induced at MAT. To explore this question, we monitored survival of wild-type and hmo1Δ strains after exposure to hydroxyurea (HU).

Figure 2.6. Hmo1p eviction from the vicinity of DSB. (A) ChIP showing Hmo1p localization at indicated loci relative to the DSB site during continuous damage induced by galactose. IC, input control; No, no antibody; IP, immunoprecipitation with antibody to FLAG-tagged Hmo1p. (B) Densitometric semi-quantitative analysis of ChIP data shown in (A), obtained with ImageJ software. (C) ChIP showing Hmo1p localization at indicated loci relative to the DSB site after DNA damage (galactose) and during repair (glucose). (D) Densitometric analysis of ChIP data shown in (C). Fold enrichment= ChIP/ Input DNA. Three independent experiments were performed. Error bars represent standard deviation.

HU stalls replication forks, an event that may lead to fork collapse and induction of DNA double strand breaks, particularly after prolonged exposure to HU (33). We found that the hmo1Δ strain exhibits an increased resistance to HU compared to the isogenic wild type strain after an 8 hour exposure to HU (Fig. 2.9). This is consistent with the increased survival after HO-induced DSB at MAT, and it is consistent with the interpretation that the effect of Hmo1p is global.
Figure 2.7. Hmo1p localization at POL5. (A) ChIP with DDY3 using antibody to FLAG-tagged Hmo1p showing Hmo1p localization at POL5 during DNA damage (galactose) and repair (glucose). IC, input control; No, no antibody; IP, immunoprecipitation with antibody to FLAG-tagged Hmo1p. (B) Densitometric analysis of ChIP data shown in (A), obtained with ImageJ software. Fold enrichment = ChIP/Input DNA. Three independent experiments were performed. Error bars represent standard deviation.

Rapid kinetics of H2A phosphorylation and dephosphorylation in absence of Hmo1p

DSBs elicit a DNA-damage response that includes a rapid phosphorylation of histone H2A isoforms that spreads about 50 kb on either side of the DSB (6,7). To assess if this event is affected by Hmo1p, we performed ChIP with DDY3 and DDY3hmo1Δ using a previously characterized antibody against γ-H2A that is specific to the phosphorylated histone variant (Ser129) (34). We monitored γ-H2A appearance at MAT and 29.8 kb upstream of the damaged site. After 20 minutes of DSB induction, we found a higher level of H2A phosphorylation at these loci in the hmo1Δ strain compared to DDY3, and this difference was also seen after 1 hour of DNA damage (Fig.2.10 A, B).
Figure 2.8. Role of Hmo1p in MATa transcription. (A)-(B) PCR product amplified using cDNA generated from total RNA extracted from DDY3 and corresponding hmo1Δ strain. (A) MATa. (B) IPP1 (inorganic pyrophosphatase). (C) Densitometric analysis of gene expression. Relative transcript level = MATa gene expression/IPP1 gene expression. Three independent experiments were performed. Error bars represent standard deviation.

Figure 2.9. Survival after exposure to hydroxyurea (HU). (A) DDY3 and corresponding hmo1Δ strain. (B) JKM179 and corresponding hmo1Δ strain. After exposure to HU for 8 h, cells were washed, diluted 104-fold and plated and colonies counted. Survival is represented as colonies per OD. Each experiment was repeated three times and data reported as mean with standard deviations.
Restoring chromatin after DNA repair by γ-H2A dephosphorylation is an important step that in yeast involves removal of γ-H2A followed by dephosphorylation (35). We analyzed the γ-H2A removal event by ChIP by adding glucose after 1 hour of DNA damage to suppress further DSB induction. One hour after glucose addition, we observed appreciably reduced γ-H2A in DDY3hmo1Δ compared to WT and this difference was consistent after 2 hours of DNA repair (Fig. 2.10 A, B).

To verify that these events are DNA repair pathway independent, we also performed the ChIP assay using JKM179 and the corresponding hmo1Δ strain, using the same time intervals and loci to monitor H2A phosphorylation. Again, we observed more efficient γ-H2A accumulation in the hmo1Δ strain, followed by its more efficient disappearance during repair (Fig. 2.10 C, D). Thus, these results reveal that the kinetics of both γ-H2A accumulation and removal are more rapid in an hmo1Δ strain compared to the isogenic WT parent strain, and that these events are independent of the repair pathway (HR or NHEJ).

**H2A phosphorylation correlates with Arp5 recruitment**

The chromatin remodeling complex INO80 is recruited to DSB sites in a γ-H2A-dependent process (11-14). Furthermore, association of INO80 with the MAT locus prior to DSB induction has been reported; while γ-H2A-dependent accumulation of INO80 was observed downstream of MAT after DSB induction, a pre-existing pool at the MAT locus that is involved in MAT transcription is associated with histone displacement, whereas the newly recruited pool was proposed to have a role in strand invasion (14). We performed ChIP assay with DDY3 and DDY3hmo1Δ using antibody against Arp5, a conserved subunit of INO80 in yeast and mammals. Prior to DSB induction, we observed the
Figure 2.10. H2A phosphorylation. (A) ChIP with DDY3 WT and corresponding hmo1Δ strain using antibody to phosphorylated H2A at MAT and at 29.5 kb upstream of DSB during DNA damage (galactose) and repair (glucose). IC, input control; No, no antibody; IP, immunoprecipitation with antibody to -H2AX. (B) Densitometric analysis of ChIP data shown in (A), obtained with ImageJ software. (C) ChIP with JKM179 WT and corresponding hmo1Δ strain using antibody to phosphorylated H2A at MAT and at 29.5 kb upstream of DSB during DNA damage (galactose) and repair (glucose). (D) Densitometric analysis of ChIP data shown in (C). Fold enrichment=ChIP/Input DNA. Three independent experiments were performed. Error bars represent standard deviation.

expected pre-existing pool of Arp5 at the MAT locus in both DDY3 and DDY3hmo1Δ strains, whereas no Arp5 was detectable 3.1 kb downstream (Fig. 2.11A, B; 0 min). After inducing DSB for 20 min, the pre-existing pool of Arp5 at the MAT locus was reduced in DDY3hmo1Δ while no change was seen in DDY3; after 2 hours of damage, the pre-existing Arp5 pool was completely lost in DDY3hmo1Δ, whereas complete loss of Arp5 in DDY3 was seen only after 4 hours of damage (Fig. 2.11A, B). Furthermore, after 2 hours of DSB induction, more efficient accumulation of Arp5 was observed 3.1 kb downstream.
of the DSB site in DDY3hmo1Δ compared to WT (Fig. 2.11A, B). These data show that the pre-existing pool of INO80 at the MAT locus is more rapidly displaced in the hmo1Δ strain after DSB induction, followed by its accumulation downstream of the break site. DNA repair would be expected to result in a reappearance of Arp5 at MAT and disappearance downstream. DNA repair after 4 hours of DSB induction indeed resulted in the expected restoration of the INO80 localization observed prior to DNA damage (Fig. 2.11A, B).

Figure 2.11 Arp5 localization. (A) ChIP with DDY3 WT and corresponding hmo1Δ strain using antibody to Arp5 at MAT and 3.1 kb downstream of DSB during DNA damage (galactose) and repair (glucose). IC, input control; No, no antibody; IP, immunoprecipitation with antibody to Arp5. (B) Densitometric analysis of ChIP data shown in (A), obtained with ImageJ software. (C) ChIP with JKM179 WT and corresponding hmo1Δ strain using antibody to Arp5 at MAT and 3.1 kb downstream of DSB during DNA damage (galactose) and repair (glucose). (D) Densitometric analysis of ChIP data shown in (C). Fold enrichment=ChIP/Input DNA. Three independent experiments were performed. Error bars represent standard deviation.
We also examined these events in the JKM179 background, observing the same pattern of more rapid Arp5 displacement at MAT and enhanced accumulation downstream in the hmo1Δ strain, followed by restoration of Arp5 localization after DNA repair (Fig. 2.11C, D). Thus, irrespective of DNA repair pathway, the pre-existing INO80 pool was displaced faster from the break site in the hmo1Δ strains, and the γ-H2A-dependent INO80 accumulation downstream was more efficient in hmo1Δ. The enhanced INO80 recruitment in hmo1Δ would be consistent with the more efficient DSB repair in hmo1Δ strains by either HR or NHEJ.

Recruitment of INO80 to DSB sites depends on the presence of γ-H2A. However, the DNA end resection that is a prerequisite for repair by HR should result in disruption of nucleosomes, including the loss of γ-H2A. We verified that γ-H2A is present 3.1 kb downstream of the DSB site in both DDY3 and the corresponding hmo1Δ strain after 2-4 h of DSB induction, consistent with the observed INO80 recruitment (Fig. 2.12).

**Rapid H3 eviction and redeposition in hmo1Δ strains**

ChIP assay with DDY3 and DDY3hmo1Δ using antibody against histone H3 was used to monitor H3 at MAT and 0.2 kb upstream from the DSB. H3 disappearance 0.2 kb upstream of the DSB was evident in both strains after 1 hour of DNA damage and became more prominent at both sites after further DNA damage, with more efficient H3 eviction in the hmo1Δ strain (Fig. 2.13 A, B). DNA repair (addition of glucose) resulted in redeposition of H3 in both strains (Fig. 2.13 A, B). In the JKM179 background, H3 eviction was also more efficient in the hmo1Δ strain after 2 hours of DSB induction, and redeposition after repair occurred more efficiently (Fig. 2.13 C, D). H3 eviction has been reported to parallel
Figure 2.12. Detection of γ-H2A 3.1 kb downstream of DSB. (A) ChIP with DDY3 WT and corresponding hmo1Δ strain using antibody to phosphorylated H2A. IC, input control; No, no antibody; IP, immunoprecipitation with antibody to γ-H2AX. (B) Densitometric analysis of ChIP data. Fold enrichment = ChIP/Input DNA. Three independent experiments were performed. Error bars represent standard deviation.

Figure 2.13 H3 localization. (A) ChIP with DDY3 WT and corresponding hmo1Δ strain using antibody to H3 at MAT and 0.2 kb upstream of DSB during DNA damage (galactose) and repair (glucose). IC, input control; No, no antibody; IP, immunoprecipitation with antibody to H3. (B) Densitometric analysis of ChIP data shown in (A), obtained with ImageJ software. (C) ChIP with JKM179WT and corresponding hmo1Δ strain using antibody to H3 at MAT and 0.2 kb upstream of DSB during DNA damage (galactose) and repair (glucose). (D) Densitometric analysis of ChIP data shown in (C). Fold
enrichment=ChIP/Input DNA. Three independent experiments were performed. Error bars represent standard deviation.

DNA end resection, and it has been suggested that input control DNA used for normalization may be lost as a consequence of such resection (17). We therefore verified H3 occupancy using qRT-PCR (Fig. 2.14).

Figure 2.14. Quantitative analysis of H3 localization determined by ChIP followed by qRT-PCR. ChIP with DDY3 WT and corresponding hmo1Δ strain using antibody to H3 at MAT and 0.2 kb upstream of DSB during DNA damage (galactose) and repair (glucose). Data is normalized by using corresponding input at each time point. Error bars represent standard deviation.

While the semi-quantitative assessment reveals some variability by comparison, the qRT-PCR analysis confirmed H3 eviction after DSB induction and redeposition following repair. Since amplification of input control DNA is constant after DSB induction (as determined by qRT-PCR), the reduced H3 occupancy observed may reflect a combination of nucleosome remodeling and displacement due to DNA resection.
DNA end resection and Rad51 recruitment

In yeast, the MRX complex (Mre11-Rad50-Xrs2) initiates DNA end resection in concert with the Sae2 endonuclease to generate short 3'-ended ssDNA overhangs. Such ssDNA ends limit Ku binding and promote more extensive resection by Exo1 and the helicase/endonuclease complex consisting of Sgs1-Top3-Rmi1 and Dna2. This extensive resection has been reported to depend on nucleosome remodeling by Fun30 (36,37). To assess if DNA end resection is affected by Hmo1p, we performed a DNA resection assay in which genomic DNA isolated at various times after induction of DSB was incubated with *E. coli* Exo I to degrade single-stranded overhangs. As shown in Fig. 2.15, DNA resection is slower in *DDY3* compared to the corresponding *hmo1Δ* strain, as measured by qRT-PCR using primers that anneal 1.6 kb from the DSB site.

The homologous recombination protein Rad51 is recruited after 3'-end processing to initiate the homology search (38). We monitored Rad51 recruitment to the *MAT* locus in *DDY3* and *DDY3hmo1Δ* by ChIP, observing enhanced Rad51 recruitment in the *hmo1Δ* strain, consistent with faster resection (Fig. 2.16 A, B). However, in absence of DSB induction, we also observed increased Rad51 localization in the *hmo1Δ* strain at *MAT*, 0.2 kb upstream, 9.5 kb downstream, and 29.8 kb upstream as well as at the unrelated *POL5* locus (Fig. 2.17). Evidently, Rad51 association with undamaged DNA is greater in *hmo1Δ* and its recruitment to a DSB site is enhanced in absence of Hmo1p.

Tracking of Ku from DNA ends correlates with histone eviction

The Ku heterodimer binds free DNA ends and plays an important role in NHEJ-mediated
Figure 2.15. Quantification of DNA resection by qPCR. PCR products were amplified after Exonuclease I treatment of genomic DNA isolated at the indicated times following DSB induction using primers that anneal 1.6 kb upstream of the DSB. All values were normalized to that for an independent locus (POL5). DNA resection was measured in DDY3 and the corresponding hmo1Δ strain.

Figure 2.16 Rad51 recruitment to DSB. (A) ChIP with DDY3 WT and corresponding hmo1Δ strain using antibody to Rad51 at MAT during DNA damage induced by galactose. IC, input control; No, no antibody; IP, immunoprecipitation with antibody to Rad51. (B) Densitometric analysis of ChIP data shown in (A), obtained with ImageJ software. Fold enrichment=ChIP/Input DNA. Three independent experiments were performed. Error bars represent standard deviation.
DNA repair (5). ChIP assay with JKM179 and JKM179hmo1Δ using antibodies against Ku showed the expected accumulation of Ku at the break site within the MAT locus in JKM179, followed by its rapid disappearance after DNA repair (Fig. 2.18A, left panel). In contrast, Ku was essentially undetectable at the MAT locus in the hmo1Δ strain. Since Ku translocates from DNA ends (39), we reasoned that the absence of Ku from the break site in hmo1Δ might be a consequence of more efficient tracking. Indeed, we found that Ku was enriched 0.2 kb upstream of the break site in hmo1Δ, whereas this tracking event was less efficient in WT (Fig. 2.18 A, B). After DNA repair, accumulation of Ku at the 0.2 kb upstream site was reduced after 1 hour and it was undetectable after 2 hours in both strains (Fig. 2.18 A, B). These data suggest that tracking of Ku from DNA ends correlates with histone eviction and that both events are faster in hmo1Δ than in WT.

**Truncation of the Hmo1p C-terminal tail phenocopies Hmo1p deficiency**

The C-terminal tail of Hmo1p is indispensable for DNA bending (23,24). To address if this architectural function of Hmo1p is required for the more stable chromatin structure characteristic of WT strains, we compared DDY3 with the AB strain (28), which expresses Hmo1p truncated for its C-terminal tail. Survival after induction of DSBs showed ~2-fold increase in the AB strain, which indicates that it repairs DSBs more efficiently than WT (Fig. 2.19). Consistent with more efficient repair, ChIP experiments showed more rapid H2A phosphorylation and dephosphorylation in the AB strain, both at MAT and 29.5 kb upstream (Fig. 2.20 A, B).

As observed for DDY3hmo1Δ, recruitment of INO80 (Arp5) occurred more efficiently downstream of the break site in AB upon induction of DNA damage (Fig. 2.21 A, B). Furthermore, after addition of glucose, rapid loss of Arp5 was observed.
downstream of MAT and a faster accumulation of Arp5 was seen at MAT in the AB strain (Fig. 2.21A, B). These events correlated with faster H3 eviction in AB 0.2 kb upstream of the break site and with faster redeposition following repair (Fig. 2.21C, D).

Figure 2.17. Rad51 localization. (A) ChIP with DDY3 WT and corresponding hmo1Δ strain using antibody to Rad51 at MAT, 0.2 kb upstream, 9.5 kb downstream, 29.8 kb upstream from DSB and at POL5 during growth in raffinose (no DSB induction). IC, input control; No, no antibody; IP, immunoprecipitation with antibody to Rad51. (B) Densitometric analysis of ChIP data shown in (A), obtained with ImageJ software. Fold enrichment = ChIP/Input DNA. Three independent experiments were performed. Error bars represent standard deviation.
Figure 2.18 Ku recruitment to DSB. (A) ChIP with DDY3 WT and corresponding hmo1Δ strain using antibody to Rad51 at MAT during DNA damage induced by galactose. IC, input control; No, no antibody; IP, immunoprecipitation with antibody to Rad51. (B) Densitometric analysis of ChIP data shown in (A), obtained with ImageJ software. (A) ChIP with JKM179 WT and corresponding hmo1Δ strain using antibody to Ku at MAT and 0.2 kb upstream of DSB during DNA damage (galactose) and repair (glucose). (B) Densitometric analysis of ChIP data shown in (A). Fold enrichment=ChIP/Input DNA. Three independent experiments were performed. Error bars represent standard deviation.

Figure 2.19 Survival assay. Survival of DDY3 and corresponding AB strain expressing Hmo1p deleted for its C-terminal tail following induction of DNA double strand breaks.
After DSB induction, cells were diluted $10^4$-fold and plated and colonies counted. Survival is represented as colonies per OD. Cells not induced to express HO were plated as control (ctrl). Three independent experiments were performed. Error bars represent standard deviation.

Figure 2.20. Effect of Hmo1p C-terminal tail on H2A phosphorylation (A) ChIP with DDY3 WT and AB strain expressing Hmo1p deleted for its C-terminal tail using antibody to phosphorylated H2A at MAT and 29.5 kb upstream of DSB during DNA damage (galactose) and repair (glucose). IC, input control; No, no antibody; IP, immunoprecipitation with antibody to -H2AX. (B) Densitometric analysis of ChIP data shown in (A), obtained with ImageJ software.

Rad51 association with MAT was likewise increased in AB after DSB induction (Fig. 2.22A, B). Thus, these results indicate that the AB strain phenocopies the hmo1Δ strain, featuring a higher efficiency of the chromatin remodeling events that are required for DSB repair.

DISCUSSION
The DNA damage response has to operate in the context of chromatin. After DSB, the first posttranslational modification event is the H2A phosphorylation that spreads bidirectionally and creates a docking site for the chromatin remodeler INO80 and other
Figure 2.21. Effect of Hmo1p C-terminal tail on Arp5 recruitment and H3 eviction. (A) ChIP with *DDY3* WT and *AB* strain expressing Hmo1p deleted for its C-terminal tail using antibody to Arp 5 at *MAT* and 3.1 kb downstream of DSB during DNA damage (galactose) and repair (glucose). IC, input control; No, no antibody; IP, immunoprecipitation with antibody to Arp5. (B) Densitometric analysis of ChIP data shown in (A), obtained with ImageJ software. (C) ChIP with *DDY3* WT and *AB* strain using antibody to H3 at *MAT* and 0.2 kb upstream of DSB during DNA damage (galactose) and repair (glucose). (D) Densitometric analysis of ChIP data shown in (C). Fold enrichment = ChIP/Input DNA. Three independent experiments were performed. Error bars represent standard deviation.

Figure 2.22. Effect of Hmo1p C-terminal tail on Rad51 recruitment. (A) ChIP with *DDY3* WT and *AB* strain using antibody to Rad51 at *MAT* during DNA damage (galactose). (B)
Densitometric analysis of ChIP data shown in (A). Fold enrichment=ChIP/Input DNA. Three independent experiments were performed. Error bars represent standard deviation.

proteins associated with DSB repair. INO80 plays a major role in chromatin dynamics around a DSB and may facilitate eviction of nucleosomes in the immediate vicinity of the DSB to allow DNA resection (11,13). Nucleosome disassembly parallels the extensive DNA end resection, which is facilitated by the chromatin remodeler Fun30 (36,37). After the completion of DNA repair, affected chromatin regions must be restored, events described by the "access-repair-restore" model (40). We report here that these events are modulated by the HMGB protein Hmo1p.

HMGB proteins bend their target DNA sites and serve architectural roles in nucleoprotein complex assembly. Vertebrate HMGB1 is thought to bind nucleosomal linker DNA to relax the chromatin structure and promote access to remodeling complexes and transcription factors (41-44). The yeast homolog Hmo1p also binds DNA with little sequence specificity, bends DNA, and recognizes altered DNA conformations (19,22-24). In contrast to vertebrate homologs, deletion of Hmo1p results in nuclease-sensitive chromatin (19), pointing to a role for Hmo1p in stabilizing chromatin. In addition, Hmo1p accumulates on ribosomal RNA genes, where it appears to prevent chromosome fragility in absence of nucleosomes; at the ribosomal DNA promoter, upstream activating factor (UAF) contains histones H3 and H4, but not H2A and H2B, suggesting that the presence of Hmo1p may prevent fragility (45). At the rDNA locus, Hmo1p not only stabilizes the chromosome structure, but it is associated with the open rDNA to promote transcription of ribosomal genes (46-48). Hmo1p mediates DNA bridging between strands, stabilization
of DNA loops, and DNA compaction by reducing the apparent DNA persistence length, which may contribute to compaction of nucleosome-free DNA (23,49).

Hmo1p is localized throughout the genome, but not uniformly so. At the MAT locus, its presence appears to modulate MAT transcription and perhaps prevent chromosome fragility. A role for Hmo1p in stabilizing the local chromatin structure is supported by the observation that it, like core histones, must be evicted for DSB repair. Furthermore, in the absence of Hmo1p, DNA damage-associated chromatin remodeling events and DNA end resection are faster. That the strain expressing Hmo1p deleted for its C-terminal tail phenocopies hmo1Δ may reflect either that DNA bending is required for the stabilizing effect of Hmo1p on chromatin or that the C-terminus is necessary for its recruitment. The yeast HMGB protein Nhp6A has also been shown to associate with certain chromosomal regions and to stabilize nucleosomes, and DNA bending was shown to be critical for this function (50). We also note that Hmo1p contains a lysine-rich C-terminus, in contrast to vertebrate HMGB1, whose C-terminal tail is acidic. This basic extension is reminiscent of the lysine-rich domain of histone H1, which has been implicated in DNA compaction (51). It is therefore conceivable that the ability of Hmo1p to stabilize chromatin may likewise rely on its lysine-rich extension, particularly in light of the more limited role of histone H1 in yeast compared to vertebrates.

Association of Rad51

The wrapping of DNA about the histone octamer as well as higher order organization protects the DNA from nucleases such as micrococcal nuclease (MNase), and the ability of MNase to digest chromatin DNA has been generally used to identify sensitive sites (52). Such nucleosome fragility has been for example associated with
environmental stress response genes, where it has been suggested to render these
genes more responsive to environmental signals and rapid changes in transcriptional
activity (53,54). Absence of Hmo1p renders chromatin nuclease sensitive, implying
development of fragile chromatin structures.

In the absence of DSB induction, we observed an enrichment of Rad51 at all sites
monitored in the \textit{hmo1Δ} strain in addition to enhanced recruitment after DSB induction.
Rad51 functions in repair of DSBs and stalled replication forks. Events that cause DSB
formation or inhibit replication promote formation of Rad51 repair complexes that may be
detected as foci. Accumulation of Rad51 on undamaged double stranded DNA is usually
prevented by translocases such as Rad54, which prevent toxicity associated with such
binding (55). Accumulation on dsDNA is rendered possible because Rad51 has little
preference for ssDNA compared to dsDNA (56). We speculate that the less-stable
chromatin structure associated with absence of Hmo1p may promote accessibility of
Rad51 to undamaged DNA sites.

The DNA damage-independent Rad51 recruitment is intriguing and may reflect
formation of fragile chromatin regions on removal of Hmo1p that attract surveillance
complexes in preparation for eventual DNA damage. Under non-DNA damage conditions,
Rad9 was shown to interact with fragile genomic regions and was suggested to facilitate
genome surveillance and efficient responses in the event of DNA damage (57). It was
also reported that the level of replication-independent endogenous DSBs was lower in
strains lacking chromatin condensing proteins Hmo1p and Sir2 (Silent Information
Regulator 2), but higher in absence of DNA repair proteins such as Ku and Rad51 (58).
Notably, these authors also reported lower levels of such DSBs in human cells lacking HMGB1.

**Ku tracking from DNA ends correlates with chromatin remodeling**

The Ku heterodimer threads onto free DNA ends by virtue of its toroidal structure and it is a key player in the NHEJ pathway of DSB repair (5). Ku in turn recruits proteins required for end-processing and ligation. However, topologically trapped Ku may interfere with cellular processes, including competition with HR-mediated repair and post-repair recovery (27,59); Ku tracks from DNA ends, and recent evidence suggests that complete removal of Ku from DNA involves ubiquitylation (39,60). Recruitment of yeast Ku to HO-induced strand breaks was previously reported to occur within 15 min of break induction and Ku started to disappear from the DSB site ~1 hour after glucose addition (59). Our results were consistent with these findings. Notably, Ku was nearly undetectable at the MAT locus during DSB induction in the hmo1Δ strain, whereas it only disappeared from the DSB site in the isogenic WT strain after ~1 hour of repair. Instead, Ku appeared faster 0.2 kb upstream from the break site in hmo1Δ compared to WT, suggesting that tracking of Ku from the free DNA end is faster in the hmo1Δ strain, an observation that is consistent with more efficient DNA repair. These data not only suggest that efficient tracking of Ku from DNA ends correlates with efficient repair, but that these events correlate with faster histone eviction.

Taken together, our results show that Hmo1p stabilizes the chromatin structure and that it is evicted along with nucleosomes to facilitate recruitment of proteins involved in DSB repair. Efficient DNA resection requires a nucleosome-free region near the DSB (36,37,61), which is consistent with our observation that HR is more efficient when histone
eviction near the DSB is facilitated by remodeling or end resection events. Likewise, NHEJ is faster under such conditions, as reflected by our observation that Ku tracking from the DSB site correlates with repair efficiency and chromatin remodeling. Since the presence of Hmo1p protects fragile chromosomal regions, the association of Hmo1p with the MAT locus is intriguing. Hmo1p promotes DNA association in vitro (23), it facilitates sister chromatid junction during replication (62), and it has been shown to direct DNA lesions towards HR-mediated repair (21); it is therefore conceivable that a specific function of Hmo1p at MAT is to facilitate HR-dependent mating type switching.

REFERENCES:


CHAPTER 3

THE HIGH MOBILITY GROUP PROTEIN HMO1 FUNCTIONS AS A LINKER HISTONE IN YEAST

Introduction

Genomic DNA is packaged into nucleosomes by association with core histones, which are among the most highly evolutionarily conserved proteins. The linker DNA that separates these nucleosome core particles may associate with histone H1, much more heterogeneous proteins that condense the polynucleosome fiber(1,2). H1 proteins typically contain a short N-terminus, a central globular domain, and a basic C-terminal domain. H1 binds the DNA that enters and exits the nucleosome and bends it as a first step towards formation of a compact structure. This binding is mediated by the globular domain, however, the chromatin compaction function of H1 requires its basic C-terminal extension, which organizes the linker DNA; the C-terminal domain operates as an intrinsically disordered protein with folding coupled to DNA binding (3-8). Interaction of H1 with linker DNA manifests as an increased resistance to digestion by micrococcal nuclease (MNase) (9).

The extensive compaction imposed by nucleosomes and linker histones is generally a barrier to events such as DNA repair and gene transcription, and covalent modification of histones as well as nucleosome remodeling operate together to facilitate access to required DNA-dependent machineries.

This chapter originally appeared as Panday and Grove (2016) The high mobility group protein HMO1 functions as a linker histone in Yeast. Reprinted with permission from Epigenetics and Chromatin 9(1),1.
For example, early events following induction of DNA double strand breaks include phosphorylation of histone H2A (H2AX in mammalian cells), which is associated with recruitment of proteins to repair foci(10). More recently, ubiquitylation of human H1 was also implicated in recruitment of repair factors(11). Consistent with a function in compacting chromatin, binding of histone H1 has been generally associated with repression of transcription and DNA repair(12,13).

Acting in opposition to H1, mammalian high mobility group (HMGB1) proteins contain two HMG domains (box A and box B) followed by an acidic C-terminal extension. With binding sites for H1 and HMGB1 partially overlapping, likely resulting in mutually exclusive interactions with the DNA entry/exit points on the nucleosome, HMGB1 proteins have been shown to induce a less stable chromatin structure(14-17). HMGB proteins are ~10 times less abundant than H1, more mobile, and bind with lower affinity. Like H1, HMGB1 bends DNA, but the acidic C-terminus lowers DNA binding affinity for linear DNA and confers preferred binding to pre-bent or distorted DNA(18,19). The C-terminal extension has also been reported to interact directly with the N-terminal tail of histone H3(20). Exchange of H1 for HMGB1 and vice versa is likely facilitated by the fast on/off rates characteristic of both proteins (14).

Yeast was long thought to lack histone H1 until sequencing identified Hho1p as having the greatest sequence similarity to H1(21). Hho1p has a different modular organization, with the H1-like globular domain followed by a short basic linker and a second globular domain. Moreover, during vegetative growth the absence of Hho1p does not result in any apparent phenotype or notable change in bulk chromatin structure, as evidenced by changes in MNase sensitivity(21,22). Evidence is also accumulating that
Hho1p has little overall effect on transcription, as inactivation of *hho1* only results in differential expression of <1% of genes (23-25). Roles of Hho1p in transcription may be due to more subtle functions, such as a contribution to silencing and barrier element activity (26,27). Hho1p has also been reported to inhibit repair of DNA double strand breaks by homologous recombination (but not non-homologous end-joining) (28); its roles in homologous recombination have been associated with yeast ageing, a phenotype that may be linked to its contribution to formation of chromatin loops (29).

Yeast contains several HMGB proteins of which the single HMG-domain proteins Nhp6A/B have been associated with changes in gene activity and chromatin structure, but no changes in bulk chromatin structure were seen in *nhp6A/B* mutant strains as measured by sensitivity to MNase digestion (30). By contrast, deletion of Hmo1p was reported to render chromatin hypersensitive to nuclease (31). Genome-wide, the association of Hmo1p with chromatin is variable, being highly enriched at sites such as rDNA and genes encoding ribosomal proteins (32,33), with lower occupancy at other sites; however, nearly 1,000 genes were reported to have Hmo1p occupancy at least 2-fold above background, consistent with the ability to detect changes in MNase sensitivity when examining bulk chromatin (32). Consistent with its abundance at rDNA, Hmo1p has been implicated in rDNA transcription and rRNA processing (34,35). Specialized functions in coordinating expression of rDNA and genes encoding ribosomal protein genes in response to signaling by target of rapamycin (TOR) kinase have also been well established (36-38).

Hmo1p has two globular box A and box B domains, of which only box B is a consensus HMG domain, followed by a C-terminal lysine-rich domain. The presence of a
lysine-rich extension in Hmo1p is unusual for HMGB proteins and likely to result in properties distinct from those characteristic of vertebrate HMGB1 proteins. *In vitro*, both box A and box B contribute to DNA binding(39), whereas the C-terminal domain is required for DNA compaction and in-phase DNA bending as well as for optimizing nuclear import(40-43). We show here that Hmo1p functions as a linker histone as evidenced by the observation that the more dynamic chromatin structure created by *HMO1* deletion is reversed by expression of human H1.

**Materials and Methods**

**Strain and plasmid construction**

*DDY3* is isogenic to *W303-1A*. The *DDY1299* derivative of *DDY3* in which *HMO1* is deleted, strain *HMO1-AB*, which encodes a truncated version of Hmo1p deleted for its C-terminal extension, and strain expressing Hmo1p-FLAG were previously described(44). Strains *APY1* and *APY2* were constructed by transforming *DDY3-HMO1FLAG* and *DDY1299*, respectively, with a KpnI-Pmel digest of plasmid p687 that harbors *URA3* flanked by *hho1* sequence(45). The deletion strains were confirmed by Western blot using anti-Hho1p (ab7183; Abcam). The 2 µm plasmid pH1 containing the gene encoding human histone H1.2 under control of the strong constitutive TEF1 promoter and *LEU2* marker was synthesized by DNA2.0.

**ChIP and qRT-PCR analysis**

Yeast cells were grown at 30°C in 2% raffinose-containing YP or in synthetic defined (SD) dropout media to an optical density at 600 nm of 1.0. DSB was induced by addition of galactose to a final concentration of 2% to induce HO. To repress HO expression, 2% glucose was added(46). Chromatin Immunoprecipitation (ChIP) was performed as
described(46). For comparison of Hmo1p binding to MAT during exponential and stationary phase, cells were incubated in YPD medium and an aliquot (10⁹ cells) removed after 4 d (stationary phase), and the culture reinoculated into prewarmed YPD medium and 10⁹ cells collected after 1h (recovery from quiescence) and 4 h (exponential phase; (25)). For immunoprecipitation, the following antibodies were used: 5 µl of antibody against phosphorylated H2A (Ser129) (07-164; EMD Millipore), 2 µl of anti-Rad51 (y-180; Santa Cruz Biotechnology), 2 µl of anti-Arp5 (ab12099; Abcam), 2 µl of anti-H1.2 (ab4086; Abcam), 2 µl of anti-Hho1p (ab7183; Abcam), and 5 µl of anti-FLAG (F1804; Sigma).

qRT-PCR was conducted using an ABI Viia-7 sequence detection system and SYBR Green for detection. Data were normalized to corresponding input control at each time point. Each experiment was repeated three times and average and standard deviations (SD) are reported.

**Survival following DSB induction**

A DSB was induced at the MAT locus by inducing expression of HO endonuclease by addition of galactose. Survival following DSB induction was performed by plating cells on YPD or SD drop out agar media, as described(46). Cultures to which no galactose was added were plated as a control. Each experiment was repeated three times and data are reported as mean (±SD).

**DNA end resection**

Cells were grown at 30°C to an OD₆₀₀ of 1.0, and DSBs were induced by addition of 2% galactose. Cells were harvested after various induction times, and genomic DNA was extracted by vortexing cells with glass beads and phenol. Twenty microliters of genomic DNA (60 ng in 1X Exonuclease I buffer (New England Biolabs)) was digested with 20
units of *E. coli* exonuclease I at 37°C overnight. The level of DNA resection adjacent to the specific DSB was measured by qPCR using primers annealing 1.6 kb upstream of the DSB. All values were normalized to values for an independent locus on chromosome 5 (*POL5*). The assay was repeated three times and reported as mean (±SD) (46).

**Micrococcal Nuclease (MNase) assay**

Yeast cells were grown at 30°C in 2% glucose-containing YP or in synthetic defined (SD) drop out media to an optical density at 600 nm of 0.8. To synchronize yeast cells in G1 phase for a total of 3 h, α-factor (10 µg mL⁻¹; Zymo Research) was added as described (47). One ml culture was removed to prepare spheroplasts, followed by nuclei isolation by using EZ Nucleosomal DNA Prep Kit (Zymo Research). Nuclei were treated with 0.25 U/µl of micrococcal nuclease. Reactions were stopped after 2, 5, or 10 minutes and pure nucleosome DNA was isolated and subsequently resolved in 2% agarose gels. Nucleosomal DNA was also probed by PCR at loci enriched for Hmo1p (18S rDNA, *MAT*, 0.2 kb upstream of *MAT*) and a locus at which Hmo1p was not detected (*KRE5*). Primer sequences were previously reported (46) or are available on request.

**Western Blot**

Cells were grown at 30°C to an OD₆₀₀ of 0.8. Fifty ml culture was removed to extract protein. Cells were lysed by vortexing with glass beads using lysis buffer (100 mM Tris-HCl, 300 mM NaCl, 2 mM EDTA, 10% glycerol, 5% Triton X-100) containing 100 mM β-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail tablet (Roche). Protein concentration was measured using BCA Protein Assay Kit. Fifteen µg of protein were resolved on 12% SDS-PAGE, and the resolved proteins were transferred to polyvinylidene fluoride membrane. Anti-Histone H1.2 (ab4086;
Abcam), anti-Hho1p (ab7183; Abcam), and anti-FLAG (F1804; Sigma) were added at a 1:1000 dilution, whereas secondary antibody was added at a dilution of 1:5000. As internal loading control, anti-GAPDH (ab9485; Abcam) was added at a 1:5000 dilution. The blots were developed by using CN/DAB substrate kit. The intensity of immunoreactive bands was determined by using image J software. The blots were developed by using CN/DAB substrate kit. The intensity of immunoreactive bands was determined using image J software for densitometric analysis.

Growth Curve

A single colony was inoculated into 6 ml YP or synthetic defined media containing 2% glucose and cultured overnight at 30°C. After overnight incubation or when cell reached log phase, cells were diluted to an OD$_{600}$ of 0.05 in 25 ml culture volume. OD$_{600}$ was recorded at regular intervals.

Results and discussion

The C-terminal domain of Hmo1p is required for chromatin compaction

To address if the C-terminal domain of Hmo1p participates in chromatin compaction in vivo during vegetative growth, as reflected in protection of linker DNA from nuclease digestion, we performed micrococcal nuclease (MNase) digestion of chromatin isolated from wild-type cells, hmo1Δ and HMO1-AB that expresses Hmo1p truncated for its C-terminal tail (44). MNase creates double-stranded cuts between nucleosomes, eventually resulting in predominantly DNA corresponding to the length of a mononucleosome (~146 bp). With time of incubation with MNase, chromatin from wild-type cells was depleted of larger DNA fragments while DNA corresponding to mononucleosomes accumulated (Fig. 3.1a). As expected, chromatin from hmo1Δ cells was
much more sensitive to digestion, and no DNA remained after 10 min incubation (Fig. 3.1b). Notably chromatin from \textit{HMO1-AB} cells was as hypersensitive to nuclease as \textit{hmo1\textDelta} cells (Fig. 3.1c), indicating that the ability to protect linker DNA requires the C-terminal domain of Hmo1p. Vegetatively growing \textit{hho1\textDelta} cells were previously reported not to exhibit enhanced MNase sensitivity (21,22). To verify this phenotype under our experimental conditions and in the \textit{DDY3} genetic background, we created an \textit{hho1\textDelta} strain as well as a strain in which both \textit{HMO1} and \textit{hho1} were inactivated and verified absence of Hho1p by Western blot (Fig. 3.2b). As shown in Fig. 3.2a, inactivation of \textit{hho1} does not result in altered sensitivity to MNase, as

![Fig. 3.1 Resistance of chromatin to nuclease digestion requires linker histone H1 or Hmo1p containing its lysine-rich extension. a–c MNase digestion of chromatin isolated from wild-type cells (\textit{DDY3}), \textit{hmo1\textDelta}, and \textit{HMO1-AB}, respectively. d–f MNase digestion of chromatin isolated from wild-type, \textit{hmo1\textDelta}, and \textit{HMO1-AB} cells expressing human linker histone H1.2 under control of a strong, constitutive promoter. Nuclei were digested with 0.25 U/\mu l MNase for the time indicated. Nucleosomal DNA was purified and resolved by agarose gel electrophoresis and stained with ethidium bromide.](image-url)
expected. However, DNA from cells in which both genes encoding Hmo1p and Hho1p are inactivated were more sensitive to MNase digestion compared to hmo1Δ cells. To address if cellular levels of Hho1p and Hmo1p change on inactivation of genes encoding the other protein, we performed Western blot. As shown in Fig. 3.2d, cellular content of Hmo1p-Flag is unaltered in the hho1Δ strain; this is consistent with genome-wide analysis of gene expression in an hho1Δ strain, in which HMO1 was not differentially expressed (23), and it suggests that the unaltered MNase sensitivity of hho1Δ cells is not due to compensatory HMO1 expression. Conversely, cellular content of Hho1p is not affected on inactivation of HMO1 or in cells expressing Hmo1p-AB (Fig. 3.2c).

Since association of Hmo1p with the yeast genome is variable, with particular enrichment at sites such as rDNA and low or undetectable levels at other loci, we also probed specific DNA sites after MNase digestion using PCR. As shown in Fig. 3.3 a, DNA at MAT and 18S rDNA (both loci at which Hmo1p was detected (32)) was amplified as efficiently from DNA from wild-type DDY3 cells exposed to MNase for 5 min as cells not incubated with MNase (Ctrl). By contrast, MAT DNA cannot be amplified from hmo1Δ cells, whereas amplification of DNA representing 18S rDNA was less efficient in hmo1Δ. At KRE5, where Hmo1p was not abundant, equivalent amplification was observed in DDY3 and hmo1Δ cells. Cells expressing Hmo1p-AB deleted for the C-terminal tail featured the same pattern of DNA amplification as hmo1Δ, validating the interpretation that the C-terminal extension is required for the observed resistance to MNase digestion (Fig. 3.3b). The inability to amplify DNA at the MAT locus after MNase digestion of DNA from hmo1Δ or HMO1-AB was verified using primers that anneal 0.2 kb from the cleavage site for the HO endonuclease within the MAT locus (Fig. 3.3c)
Association of Hho1p with genomic DNA has also been reported to be variable, with enrichment at rDNA (24). Inactivation of hho1 did not affect amplification of DNA from

![Fig. 3. 2 Effect of Hho1p on resistance of chromatin to nuclease digestion and on cellular content of Hmo1p. a MNase digestion of chromatin isolated from DDY3, hho1Δ, and hmo1Δ hho1Δ, respectively. Nuclei were digested with 0.25 U/µl MNase for the time indicated. Nucleosomal DNA was purified and resolved by agarose gel electrophoresis and stained with ethidium bromide. b Western blot of lysates from DDY3, hho1Δ, and hmo1Δ hho1Δ using antibody to Hho1p or GAPDH. GAPDH migrates with a Mw ~36 kDa, while Hho1p migrates with a Mw ~28 kDa. c Western blot of lysates from DDY3, hmo1Δ, and HMO1-AB using antibody to Hho1p or GAPDH. Densitometric analysis of three separate blots from three independent experiments shown below. Relative level = Hho1p/GAPDH. d Western blot of lysates from DDY3 and hho1Δ using antibody to FLAG-tagged Hmo1p or GAPDH. Hmo1p-FLAG migrates with a Mw ~35 kDa. Densitometric analysis of three separate blots from three independent experiments shown below. Relative level = FLAG/GAPDH. Error bars represent standard deviation.]

94
the MAT locus after MNase digestion, whereas no DNA was amplified using DNA from the \textit{hmo1Δ\textit{hho1Δ}} strain (Fig. 3.3d). Amplification of DNA from the KRE5 locus after MNase digestion was equivalent for \textit{DDY3, hho1Δ} and \textit{hmo1Δ\textit{hho1Δ}} (data not shown). By contrast, MNase-digestion of DNA from the \textit{hho1Δ} strain resulted in modestly reduced amplification of 18S rDNA, whereas MNase-digestion of DNA from the \textit{hmo1Δ\textit{hho1Δ}} strain resulted in a failure to amplify 18S rDNA (Fig. 3.3d). The implication of this observation is that both Hmo1p and Hho1p contribute to protection of this locus, a conclusion that is consistent with previous reports that both proteins associate with rDNA (24,33,35,48,49).

To address if absence of either Hho1p or Hmo1p influences binding of the other

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig3.3}
\caption{Resistance of chromatin to MNase digestion monitored at specific loci. a, b Amplification of DNA representing MAT, 18S rDNA, and KRE5 after MNase digestion of chromatin isolated from wild-type cells (DDY3) and \textit{hmo1Δ}(a) or \textit{HMO1-AB}(b). c Amplification of DNA using primers annealing 0.2 kb upstream of the HO cleavage site within the MAT locus from \textit{DDY3, hmo1Δ}, and \textit{HMO1-AB}. d Amplification of DNA representing MAT and 18S rDNA from chromatin isolated from \textit{DDY3, hho1Δ}, and \textit{hmo1Δ\textit{hho1Δ}}. In all panels, Ctrl denotes chromatin from the identified strain not incubated with MNase. Data are representative of three repeats.}
\end{figure}
protein, we performed chromatin immunoprecipitation (ChIP) using antibody to FLAG-tagged Hmo1p or antibody to Hho1p and monitored binding at MAT, 18S rDNA, and KRE5. As shown in Fig. 3.4, Hmo1p was enriched at MAT and 18S rDNA compared to KRE5, where only low levels were detected. Inactivation of hho1p had no effect on Hmo1p binding to MAT and KRE5, whereas modest enrichment was seen at 18S rDNA. By comparison, Hho1p was detected both at MAT and 18S rDNA, with lower levels at KRE5; absence of Hmo1p resulted in a markedly increased association with rDNA, whereas binding to the other loci was unaffected. While Hho1p evidently associates with the MAT locus, this binding did not result in protection of linker DNA from MNase digestion in absence of Hmo1p, nor was it affected by cellular levels of Hmo1p (Fig. 3.3a and 3.4d); by contrast, absence of either Hmo1p or Hho1p results in a reciprocal increase in binding of the other protein at rDNA, and only elimination of both proteins renders this DNA significantly more susceptible to MNase digestion (Fig. 3.3d).

In stationary phase, increased binding of Hho1p was reported to correlate with increased resistance to MNase digestion (25). It is conceivable that such increased Hho1p binding to rDNA is responsible for residual resistance to MNase on inactivation of HMO1. By comparison, analysis of Hmo1p binding to the MAT locus did not reveal markedly different levels of binding in exponential phase, stationary phase cells or cells recovering from quiescence (Fig. 3.5).

**Mammalian histone H1 compacts chromatin in hmo1Δ cells**

Considering the conservation of core histones between species, we reasoned that presence of heterologous histone H1 might confer resistance to MNase on yeast chromatin deleted for Hmo1p. A plasmid from which human histone H1.2 was
constitutively expressed was transformed into wild-type, hmo1Δ and HMO1-AB cells. Expression of human histone H1 restored MNase resistance to chromatin isolated from hmo1Δ cells (Fig. 3.1E), but not to chromatin isolated from HMO1-AB cells (Fig. 3.1F). This suggests that the globular domains of Hmo1p are sufficient for binding to linker DNA, and that Hmo1p-AB can compete with H1 for binding. This is similar to H1, whose globular domain binds linker DNA but does not induce compaction. The hmo1Δ strain has a slow growth phenotype (44). Expression of H1 in hmo1Δ largely restores a normal growth rate, whereas DDY3 expressing H1 grows slowly (Fig. 3.6d-f). We therefore performed the MNase assay on cells synchronized in G1 by addition of alpha factor. Again, hmo1Δ cells were more sensitive to MNase digestion, whereas hmo1Δ cells expressing H1 exhibited a sensitivity to digestion similar to that of wild-type DDY3 (Fig. 3.6a).

Fig. 3.4 Effect of Hmo1p or Hho1p on binding of the other protein. a Chromatin immunoprecipitation (ChIP) with DDY3 and hho1Δ using antibody to FLAG-tagged
Hmo1p, monitoring binding at MAT, 18S rDNA, and KRE5. IC, input control; No, no antibody; IP, immunoprecipitation with anti-FLAG. b qRT-PCR analysis of ChIP data corresponding to (a). c ChIP with DDY3 and hmo1Δ using antibody to Hho1p, monitoring binding at MAT, 18S rDNA, and KRE5. d qRT-PCR analysis of ChIP data corresponding to (c). Data were normalized to corresponding input control at each time point. Fold enrichment = ChIP/Input DNA. Three independent experiments were performed. Error bars represent standard deviation. Asterisks represent statistical significance from DDY3 at the same locus based on Student’s t test (P < 0.05).

Fig. 3. 5 Equivalent binding of Hmo1p to MAT in different growth phases. a, b Quantification by qRT-PCR of ChIP using antibody to FLAG-tagged Hmo1p in DDY3, monitoring binding at the MAT locus. Data were normalized to corresponding input control. Error bars represent standard deviation of three experiments. a Cells recovering from quiescence (Rec) compared to stationary phase (Stat). b Cells in exponential phase (Exp) compared to stationary phase (Stat). Fold enrichment = ChIP/Input DNA. Three independent experiments were performed. Error bars represent standard deviation. c Growth of cells after inoculation of fresh media with stationary phase cells to OD600 ~0.05; cells were harvested for ChIP after 1 h (Rec) or 4 h (Exp)

ChIP using human H1.2 antibody was used to verify H1 binding to chromatin at the mating type locus MAT, 18S rDNA, and KRE5. At the MAT locus, reduced H1 binding was observed in wild-type and HMO1-AB cells compared to hmo1Δ, indicating not only direct binding of H1 to yeast chromatin, but also that both Hmo1p and Hmo1p-AB can compete with H1 for binding (Fig. 3.7a). At 18S rDNA, the binding of H1 observed in hmo1Δ was even more efficiently reduced in presence of Hmo1p and Hmo1p-AB (Fig. 3.7b). At KRE5, binding of H1 was equivalent in wild-type, hmo1ΔHMO1-AB cells (Fig. 3.7b). Western blot using human H1.2 antibody verified equal cellular content of H1 in all strains.
Fig. 3.6 Effect of linker histone H1 on MNase sensitivity of chromatin isolated from synchronized cells and on growth rate. a–c MNase digestion of chromatin isolated from synchronized DDY3, hmo1Δ, and hmo1Δ expressing human linker histone H1. Cells were synchronized in G1 phase for a total of 3 h by the addition of alpha factor. Nuclei were digested with 0.25 U/µl MNase for the time indicated. Nucleosomal DNA was purified and resolved by agarose gel electrophoresis and stained with ethidium bromide. d–f Growth curve for wild-type DDY3, hmo1Δ expressing H1, and DDY3 expressing H1. Cells were grown in synthetic-defined media, and cells were collected at regular intervals to measure OD at 600 nm.

**Presence of either Hmo1p or histone H1 creates a chromatin environment in which repair of DNA double strand breaks occurs with equivalent efficiency**

The DNA damage response takes place within the context of chromatin. Hmo1p is evicted along with core histones for repair of DNA double strand breaks (DSBs) at the MAT locus, suggesting it forms an integral part of the chromatin structure (46). Deletion of Hmo1p also appeared to generate a more accessible chromatin structure, as evidenced by faster chromatin remodeling and more efficient DNA repair in hmo1Δ cells. Notably, Hmo1p-AB phenocopied the HMO1 deletion, leading to the interpretation that
the lysine-rich C-terminus is required to generate the chromatin state characteristic of wild-type cells (46).

Fig. 3.7 Both Hmo1p and Hmo1p deleted for its C-terminal tail compete with H1 for binding to chromatin. a Quantification by qRT-PCR of ChIP using antibody to H1 with DDY3, hmo1Δ, and HMO1-AB strains, monitoring binding at the MAT locus. b qRT-PCR analysis of ChIP using antibody to H1, monitoring binding at 18S rDNA and at KRE5. Data were normalized to corresponding input control at each time point. Three independent experiments were performed. Error bars represent standard deviation. Asterisks represent statistical significance from DDY3 based on Student's t test (P < 0.05). c Western blot using antibody to H1 showing equal protein level of histone H1 after transforming plasmid expressing human H1 under control of a strong, constitutive promoter in DDY3 (DDY3 H1), hmo1Δ(hmo1Δ H1), and HMO1-AB strain (HMO1-AB H1). Non-transformed cells DDY3, hmo1Δ, and HMO1-AB were used as negative control. GAPDH expression levels were assessed in all samples as internal loading control, and the blots are representative of four independent experiments. GAPDH migrates with a Mw ~36 kDa, while H1 migrates with a Mw ~30 kDa (slower than its calculated Mw ~22 kDa). d Densitometric analysis of three separate blots from three independent experiments shown in (c). Relative H1 level = H1/GAPDH. Error bars represent standard deviation.
HO endonuclease introduces a single DSB in the MAT locus; this DSB is repaired by a homologous recombination (HR) event that requires one of the homologous silent mating type HM cassettes as a donor. DSBs were induced in cells harboring HO under control of a galactose-inducible promoter and survival assessed after plating cells with glucose to allow repair (46). While hmo1Δ cells more efficiently recovered from DSB induction compared to wild-type cells, this increased recovery in hmo1Δ was reversed by expression of histone H1, as evidenced by equivalent recovery in DDY3 cells and hmo1Δ expressing H1 (Fig. 3.8A). In contrast, expression of H1 in HMO1-AB cells did not reverse the increased survival of HMO1-AB cells (Fig. 3.8a). The survival of wild-type cells expressing H1 was lower than wild-type cells, perhaps because of overloading cells with chromatin compacting proteins (Fig. 3.8a), an inference supported by the very slow growth observed for H1-expressing wild-type cells (Fig. 3.6f). No differences in plating efficiency were observed for cells not experiencing DSB (Fig. 3.8b). These observations suggest that presence of either Hmo1p or H1 create a chromatin state in which DSB repair occurs with equivalent efficiency.

One of the earliest chromatin modification events in response to DSB is phosphorylation of histone H2A on serine 129, creating what is often referred to as γ-H2AX. This modification provides a docking site for factors such as chromatin remodeling complexes and DNA damage response proteins (50,51). Previously, we reported that H2A phosphorylation and dephosphorylation at the DSB site occur faster in hmo1Δhmo1-AB cells compared to the isogenic wild-type parent strain, suggesting that deletion of Hmo1p or its C-terminal extension results in generation of a more dynamic chromatin environment (46). ChIP using antibody against γ-H2AX that is specific to the
phosphorylated histone variant confirmed an increase in γ-H2AX in hmo1Δ cells 20 min after DSB induction, whereas expression of histone H1 resulted in a level of H2A phosphorylation similar to that observed in wild-type cells. Following DNA repair, dephosphorylation of H2A at the damaged site was modestly faster in hmo1Δ cells, while cells expressing either Hmo1p or H1 show identical levels of H2A dephosphorylation (Fig. 3.8C). This suggests that presence of H1 in hmo1Δ reverses the more dynamic chromatin state characteristic of hmo1Δ and HMO1-AB cells.

Fig.3.8 Dynamic chromatin environment in hmo1Δ that leads to faster chromatin remodeling and DSB repair is restored to wild-type levels by expression of H1. a Survival of DDY3, hmo1Δ, HMO1-AB and the corresponding strains expressing H1. After DSB induction, cells were plated and colonies counted. Three independent experiments were performed. Error bars represent standard deviation. b Cells not induced to express HO were plated as control. c qRT-PCR analysis of ChIP using antibody to phosphorylated H2A, monitoring presence of γ-H2AX at MAT during DNA damage (galactose) and repair (glucose). Data are normalized to corresponding input control at each time point. d qRT-
PCR analysis of ChIP using antibody to Arp5, monitoring presence at MAT (left panel) and 3.1 kb downstream of DSB (right panel) during DNA damage (galactose) and repair (glucose). Data are normalized to corresponding input control at each time point. Three independent experiments were performed. Error bars represent standard deviation. In all panels, asterisks represent statistical significance from DDY3 at the respective time points based on Student’s t test (P < 0.05).

The chromatin remodeling complex INO80 is recruited to DSB sites in a γ-H2AX-dependent manner (52). Association of INO80 with the MAT locus prior to DSB induction was also reported; this pre-existing pool was suggested to be involved in MAT transcription, whereas γ-H2AX-dependent accumulation of INO80 downstream of MAT was suggested to play a role in strand invasion (53). We monitored INO80 localization using antibody to Arp5, a conserved subunit of INO80. Upon induction of DNA damage, INO80 levels were reduced in the vicinity of the break site and instead increased 3.1 kb downstream, both events occurring faster when cells expressed neither Hmo1p, nor H1. This accumulation downstream of the break site was likewise reversed faster in the hmo1Δ strain after DNA repair (Fig. 3.8d).

DNA-end resection is required for repair of DSBs by HR and it involves processing of the ends to yield 3’ single stranded DNA overhangs (54). The resected tail is the substrate for Rad51 (55). We monitored formation of single-stranded DNA overhangs by qRT-PCR (Fig. 3.9a). DNA resection was faster in hmo1Δ compared to wild-type, whereas expression of H1 in hmo1Δ restored the slower rate of DNA end resection. Hmo1p-AB cells expressing H1 retained the faster rate of DNA end resection. When monitoring Rad51 recruitment to the MAT locus by ChIP, we observed enhanced Rad51 recruitment in the hmo1Δ strain, whereas expression of H1 in hmo1Δ reduced Rad51 binding to levels observed in wild-type (Fig. 3.9b).
Fig. 3. 9 Chromatin state in \textit{hmo1}Δ that leads to faster DNA end resection and faster Rad51 recruitment after DNA double-strand break is reversed on expression of H1. 

a. Quantification of DNA resection by qRT-PCR using primers that anneal 1.6 kb upstream of the DSB. PCR products were amplified after exonuclease I treatment of genomic DNA isolated at the indicated times following DSB induction. All values were normalized to that for an independent locus (\textit{POL5}). b qRT-PCR analysis of ChIP using antibody to Rad51, monitoring binding at \textit{MAT} after DSB induction (galactose). Data are normalized to corresponding input control at each time point. Asterisks represent statistical significance from \textit{DDY3} at the respective time points based on Student’s t test (P < 0.05). Three independent experiments were performed.

**Conclusion**

Mammalian HMGB proteins compete with histone H1 for binding to linker DNA to create a less stable chromatin environment. Yeast Hmo1p is unique among HMGB proteins in containing a lysine-rich extension, a feature also characteristic of linker histones. Our data suggest that this extension confers on Hmo1p several properties of a linker histone, including resistance to MNase digestion and the generation of a chromatin environment in which events associated with DSB repair occur more slowly. This is also consistent with reported functions of other proteins containing lysine-rich repeats in condensing DNA, including the H1 proteins from protozoa that lack the globular domain as well as bacterial histone-like proteins (56,57).
While vertebrate histone H1 has been shown to stabilize chromatin, the closest H1 homolog in S. cerevisiae, Hho1p, does not compact genomic DNA during vegetative growth as determined by resistance to MNase digestion (6,21,22). In stationary phase, however, increased binding of Hho1p was shown to correlate with increased resistance to MNase digestion (25). By comparison, analysis of Hmo1p binding to the MAT locus did not reveal markedly different levels of binding in stationary phase cells ((46) and data not shown). It is conceivable that an increased level of DNA compaction and protection is necessary during stationary phase to resist environmental stress, and that Hho1p contributes to this. It is also intriguing that deletion of both Hmo1p and Hho1p is required for significantly increased MNase sensitivity at rDNA, while the absence of Hho1p had no effect at MAT and KRE5.

We propose that Hmo1p functions as linker histone during vegetative growth, promoting a chromatin state that is also induced on expression of human H1 in hmo1Δ cells. The equivalent phenotypes of hmo1Δ and Hmo1p-AB expressing Hmo1p deleted for its lysine-rich C-terminus are reversed only on expression of H1 in hmo1Δ. This indicates that the globular domains of Hmo1p compete with H1 for binding to linker DNA, and that the lysine-rich extension is essential for chromatin compaction. Thus, both yeast Hmo1p and H1 from higher eukaryotes possess globular domains with affinity for linker DNA connected to a lysine-rich C-terminal extension that is required for chromatin compaction.

References:


CHAPTER 4

CONTROL OF DNA END RESECTION BY YEAST HMO1

Introduction

DNA double strand breaks (DSBs) are cytotoxic lesions that may be caused either by exogenous factors like free radicals or as a result of metabolic processes such as DNA replication. Persistent DSBs threaten genomic integrity and may induce chromosomal rearrangements and lead to cell death or tumorigenesis. By contrast, programmed DSBs, such as those involved in meiotic recombination, and in V(D)J recombination are critical for genetic diversity and for lymphogenesis (1,2).

Regardless of origin, DSBs are repaired by distinct pathways that are conserved from yeast to humans. Since DSBs occur in the context of chromatin, repair pathway choice is preceded by chromatin remodeling events that allow access to repair machineries. Homologous recombination (HR) requires the presence of an intact homologous donor sequence that is used as a template to repair the chromosomal break. HR is initiated by 5' to 3' resection of one DNA strand to generate a 3'-single-stranded segment that searches for homology within the template duplex after associating with Rad51. In contrast to HR, non-homologous end-joining (NHEJ) is a process by which the broken DNA ends are joined directly without the aid of an intact template. The classical NHEJ involves DNA end-binding by the Ku70/Ku80 heterodimer that makes bridge between broken DNA ends and protects the ends from degradation and extensive resection. Ku heterodimer forms a toroidal or ring-like structure that binds DNA by sliding a DSB end through its opening and functions as docking site to recruit NHEJ repair factors (3-5). DSB ends are processed by MRX, consisting of meiotic recombination 11 (Mre11),
radiation sensitive 50 (Rad50), and x-ray sensitive 2 (Xrs2), and DNA polymerase Pol4 to create compatible, ligatable ends, following which ends are ligated by the dedicated NHEJ ligase IV, composed of DNA ligase4(Dnl4), ligase interacting factor1(Lif1), and non-homologous end-joining defective1(Nej1) (6,7). Overhang polarity makes a significant difference in terms of factor recruitment and repair efficiency, with DSBs containing 5'-overhangs repaired more efficiently than DSBs with 3' overhangs, but with a greater probability of induced mutation.

The choice between HR and NHEJ is in large part determined by the extent of DNA end resection, as extensive resection is inhibitory to NHEJ and channels the lesion towards the HR repair pathway. In yeast, end resection is initiated by the combined action of the endonuclease sporulation in the absence of spo eleven (Sae2) and the MRX complex, followed by more extensive resection by the helicase-nuclease complex Sgs1-Dna2 and the exonuclease Exo1. The endonuclease activity of Mre11, which is promoted by Sae2, nicks the 5'-terminated DNA strand in the vicinity of the DSB end, following which its 3' to 5' exonuclease activity degrades the DNA towards the DSB end. This generates a 3'-tailed substrate for Sgs1/Dna2 and/or Exo1, which degrade the DNA in the opposite direction to generate more extensive resection (8-10). Ku prevents the Exo1- and Sgs1/Dna2-dependent resection(11).

An alternative end-joining (A-EJ) pathway functions as a “plan B”, both for classical NHEJ and for HR, operating on DNA ends that cannot be processed by the initially chosen repair pathway. Although NHEJ is important in DSB repair, a residual end joining activity has been seen in the absence of YKU80 function (12). A-EJ is a Ku or ligase IV independent end joining process. This pathway is poorly characterized and is also
referred to as a backup NHEJ or microhomology-mediated end joining. It uses microhomologies distant from the DSB, suggesting that A-EJ is initiated by DNA end resection, an inference supported by the involvement of Sae2 and Mre11 (13). Since Ku inhibits end resection, it also inhibits A-EJ. A-EJ is a highly error-prone process and often associated with deletions at the repair junctions.

In chapters 2 and 3 I reported that the yeast high mobility group (HMGB) protein Hmo1p stabilizes chromatin and that absence of Hmo1p creates a chromatin environment in which DSB repair and associated events including DNA end resection occur faster. The reported contribution of Hmo1p in controlling resection raises the possibility of its direct role in repair pathway choice. Using a plasmid end-joining assay as a read-out for end-joining to separate roles of Hmo1p in the repair process from its effects on chromatin structure, we report here that transformation of an hmo1Δ strain with linear plasmid DNA results in significantly reduced transformation efficiency, suggesting that Hmo1p promotes DNA end-joining. We propose that Hmo1p controls DNA resection, thereby favoring the more efficient classical NHEJ over A-EJ. In absence of Ku, HMO1 deletion further reduces repair efficiency, suggesting that excessive DNA resection is inhibitory for A-EJ.

**Materials and Methods**

**Strain construction**

*DDY3* is isogenic to *W303-1A*. The *DDY1299* derivative of *DDY3* in which *HMO1* is deleted, strain *HMO1-AB*, which encodes a truncated version of Hmo1p deleted for its C-terminal extension, and strain expressing Hmo1p-FLAG were previously described (14). The gene encoding Ku80 was deleted by amplifying the URA3 marker with primers that
include ~80 nt of flanking sequence homologous to the *ku80* gene, followed by transformation of either *DDY3* or *DDY3 hmo1Δ* haploid cells to generate *kuΔ* and *hmo1Δ kuΔ*, respectively.

**Yeast High Efficiency Transformation**

Cells were grown in YPD at 30°C with constant shaking to an optical density at 600 nm of 0.8, and the pelleted cells were washed with 1X phosphate-buffered saline (PBS) and resuspended in 1X Tris, EDTA, and lithium acetate buffer (TEL) and were left on nutator overnight at room temperature. The next day cells were pelleted and resuspended in 100 μl of 1X TEL per 10 ml culture incubated at room temperature for 30 minutes. Hundred microliter of competent cells, 10 μl of carrier DNA and 1 μg of the plasmid DNA was mixed well in an eppendorf tube and incubated again for 30 minutes. Seven hundred microliter of 40% polyethylene glycol (PEG) /TEL was added to each tube, mixed well and incubated at room temperature for 60 minutes without shaking. Eighty eight microliter of dimethyl sulfoxide (DMSO) was added to each tube, mixed and the cells were subjected to heat shock at 42°C for 45 minutes. The cells were spun gently at 8000 rpm for 30 seconds, then pellets were washed with 300 μl of water and resuspended in 400 μl of water. Two hundred microliter was plated on SD drop out media lacking leucine.

**DNA end resection**

To induce DSB in *DDY3*, the galactose-inducible HO endonuclease gene was furnished on a centromeric plasmid and transformed in *DDY3* with plasmid carrying URA3 marker. Cells were grown in SD drop out media at 30°C to an OD<sub>600</sub> of 1.0, and DSB was induced with 2% galactose. Cells were harvested after DSB induction times of 20 minutes, 1 hour, 2 hours, 3 hours and 4 hours. Genomic DNA was extracted by vortexing cells with
glass beads and phenol. Twenty microliters of genomic DNA sample (60 ng in 1X NEB Exonuclease I buffer) was digested with 20 units of \textit{E. coli} exonuclease I at 37°C overnight. The level of DNA resection adjacent to the specific DSB was measured by qPCR using primers annealing 1.6 kb upstream of the DSB. All values were normalized to values for an independent locus on chromosome 5 (\textit{POL5}). The assay was repeated three times and average and standard deviations (SD) are reported.

\textbf{Plasmid end-joining/ NHEJ repair assay}

In this assay, cells are transfected with linearized plasmid, and recovery of transformants depends on recircularization of the plasmid by the NHEJ pathway. pMV1328 plasmid (Fig. 4.1) was linearized by digesting it with Ncol (generates cohesive end) or NruI (generates blunt end), which both cut within the \textit{KanMX6} coding sequence (having no homology in the yeast genome); reactions were quenched by a phenol-chloroform extraction and DNA precipitated by using ethanol and sodium acetate. Both linearized and circular plasmids were used to transform yeast cells. After 3-4 days of transformation at least 50 colonies were counted for each transformation. Plasmid repair efficiency is the ratio of the number of stable transformants obtained when cells are transformed with linearized versus circular plasmid DNA. Data were normalized to the repair efficiency of wild type. To calculate repair accuracy, Leu\textsuperscript{+} transformants were replica plated on YPD agar medium containing 0.3 mg/ml G418 to test KanMX function and select for intact KanMX6 region after NHEJ.

\textbf{Statistics}

All experiments were independently performed at least three times. Error bars represent standard errors. Two tailed student t-test was used to calculate the P values.
Results and discussion

Inefficient repair efficiency and repair accuracy in the absence of Hmo1p

We used the plasmid end-joining assay to test the requirement of Hmo1p for NHEJ. DNA DSB was introduced into the pMV1328 plasmid by using NcoI to create 5’ overhang. NcoI creates DSB in KanMX6 coding sequence that does not have homology in the yeast genome. With no homologous sequence with which to initiate repair by homologous recombination, the double strand break in the plasmid DNA must be repaired by non-homologous end-joining to allow cells to survive in the selection media. We found that the hmo1Δ mutant shows significantly reduced repair efficiency compared to WT (Fig 4.2A). Then we evaluated if the broken end structure affects the NHEJ repair efficiency of cells in the absence of Hmo1p. We used the restriction enzyme NruI to create DSB with blunt ends in the KanMX6 coding sequence. Interestingly, we found no significant difference
(P>0.05) in the repair efficiency compared to the wild-type (Fig 4.2B). It shows that the requirement of Hmo1p for efficient NHEJ repair depends on the type of DSB cut.

Fig 4.2 Hmo1p requirement for efficient dsDNA breaks repair depends on the types of DSB. (A) Normalized repair efficiency of the NcoI-linearized plasmid. Ncol produces a unique DSB in pMV1328 with 5’ end overhangs. (B) Normalized repair efficiencies of the NruI-linearized plasmid. NruI produces a unique DSB in pMV1328 with blunt DNA ends. Three independent experiments were performed. Error bars represent standard deviation. Asterisk indicates a significant difference from wild type (P<0.05).

To quantify the proportion of mutagenic ligation events test Leu+ transformants were replica plated on G418 plate. Since the cut was induced in the coding sequence of KanMX6 gene, a functional gene will be inherited only if the end joining is accurate. We found that hmo1Δ has reduced repair accuracy compared to WT when cells repair cohesive DSB but not with blunt end DNA repair (Fig. 4.3).

**Requirement of Hmo1p-CTD for efficient NHEJ**

The C-terminal tail of Hmo1p is indispensable for DNA bending and stabilizes chromatin structure (15-17). To test if the Hmo1p-CTD is required for efficient NHEJ, we
created the HMO1-AB (from now onwards for the rest of this chapter, I will refer to it as AB), which encodes a truncated version of Hmo1p deleted for its C-terminal extension and used this strain for plasmid end joining assay. We created cohesive and blunt end cut by using NcoI and Nrul and transformed linear DNA into WT and AB cells. We found that AB behaves like hmo1Δ and the cohesive end cut repair efficiency in AB is lower than WT. Furthermore, repair accuracy percentage in AB is less than WT (Fig. 4.4). It suggests that the C-terminal domain of Hmo1p is required for efficient NHEJ and for fidelity.

**Role of Ku and Hmo1p in plasmid DSB repair is independent of each other**

It is well reported that Ku is indispensable in NHEJ repair of DNA with overhangs and consistent with this we found that deletion of Ku significantly reduced NHEJ repair efficiency (Fig. 4.5). Survival in the absence of Ku is a result of error prone A-EJ. We showed that the absence of Hmo1p reduces the repair efficiency and repair accuracy.
Therefore, we were interested to find the \textit{hmo1\Delta ku\Delta} double mutant repair efficiency and to see the interdependence of Ku and Hmo1p in repair pathway. Interestingly, \textit{hmo1\Delta ku\Delta} double mutant exhibited further reduction in NHEJ cohesive cut repair efficiency, indicating that Hmo1p functions in a Ku-independent repair pathway, or A-EJ (Fig 4.5 On the other hand, when we used NruI to generate blunt end DSB, repair efficiency in \textit{hmo1\Delta ku\Delta} and \textit{Ku\Delta} was similar to the WT (Fig 4.5).

![Graph A](image1)

![Graph B](image2)

\textbf{Fig 4.4} Truncated Hmo1p affects repair efficiency and repair accuracy (A) Normalized repair efficiency of the NcoI-linearized plasmid. NcoI produces a unique DSB in pMV1328 with 5' end overhangs. (B) Normalized repair accuracy of the NcoI linearized plasmid. Repair accuracy was calculated by replica plating the transformed cells on G418 plate. Three independent experiments were performed. Error bars represent standard deviation. Asterisks indicate a significant difference from wild type (P<0.05).

\textbf{Hmo1p controls caffeine sensitive resection}

Caffeine is reported to reduce resection by inducing degradation of Sae2 (a nuclease
that plays role in early step of resection) and rapid turnover of Dna2 (a helicase/nuclease that facilitates extensive resection) (18). We reported that the absence of Hmo1p promotes DNA resection in vivo so we suspected that this excessive resection phenotype might be the cause of reduced NHEJ repair efficiency in hmo1Δ. To address this, we did plasmid end joining assay using Ncol to generate cohesive end DSB and we selected transformants on agar plate lacking leucine and containing 0.5 mM caffeine. We found that repair efficiency of hmo1Δ in the presence of caffeine is higher than without caffeine and thus hmo1Δ phenotype is rescued by caffeine (Fig 4.6 and 4.7). Thus absence of Hmo1p promotes too extensive resection that is incompatible with A-EJ that leads to the low repair efficiency in hmo1ΔkuΔ double mutant than in kuΔ single mutant (Fig. 4.5).
However, that effect on repair efficiency was not seen by caffeine when the cells repair blunt end DSB (Fig.4.7). To make sure that the rescued phenotype of hmo1Δ is due to the caffeine limiting resection, we studied the DNA end resection in vivo in the presence of caffeine. DSB was induced by HO endonuclease that generates 5’ end overhangs. We found that the presence of caffeine restored the kinetics of WT resection in hmo1Δ (Fig 4.6). It shows that the absence of Hmo1p promotes the caffeine-sensitive Sae2-/Dna2-mediated DNA end resection that is inhibitory to NHEJ repair pathway and the presence of caffeine restores the WT phenotype by promoting the proteosomal degradation of Sae2 and Dna2. Caffeine has no effect on blunt DSB repair efficiency, indicating that Sae2/Dna2 are not involved in blunt end DSB repair. An implication of this would be that MRX only nicks DSB with sticky ends and is unlikely to be required for DSB repair.

**Conclusion**

A-EJ is initiated by DNA end resection to use micro-homologies of 2-8 nucleotides that are distant from the break site. Our data suggest that Hmo1p prevents homology-mediated A-EJ repair by preventing resection and directing repairs towards NHEJ. Since the hmo1Δ repair phenotype is rescued by caffeine, it indicates that Hmo1p controls the caffeine-sensitive aspect of resection by modulating the resection initiation activity of Sae2 and/or Dna2. On the other hand, Ku inhibits late resection activity of Exo1 and Sgs1/Dna2 (11,19). Since hmo1Δ expressing Ku shows a significant repair phenotype, it is more likely that Hmo1p is interfering with Sae2 activity. In this way, Ku and Hmo1p promote NHEJ repair but they act on resection differently and function in different repair pathways. Excessive resection in hmo1Δ interferes with classical NHEJ, perhaps by creating a tailed substrate (Created by MRX/ Sae2) with lower affinity for Ku but higher
affinity for Exo1 and/or Sgs1/Dna2, reducing repair efficiency. Hmo1p deletion reduces the accuracy of end joining. It indicates that Hmo1p is required to avoid A-EJ, which is error prone and leads to the mutagenic ligation events. Since the repair efficiency is lower in \textit{hmo1Δ kuΔ} than \textit{kuΔ}, the implication is therefore also that the extensive resection that would be characteristic of the double mutant (no inhibition of any of the resection nucleases) reduces A-EJ. Further, our results suggest that Hmo1p is a functional part of NHEJ and like Ku complex, is required for accurate ligation.

![Fig 4.6](image)

Fig 4.6 Hmo1p prevents caffeine sensitive resection (A) Normalized repair efficiency of the Ncol-linearized plasmid. Ncol produces a unique DSB in pMV1328 with 5' end overhangs. \textit{hmo1Δ} cells were plated on the 0.5 mM caffeine-containing SD plates. (B) Quantification of DNA resection by qPCR. PCR products were amplified after Exonuclease I treatment of genomic DNA isolated at the indicated times following DSB induction using primers that anneal 1.6 kb upstream of the DSB. All values were normalized to that for an independent locus (\textit{POL5}). Three independent experiments were performed. Error bars represent standard deviation.
Fig 4.7 Caffeine rescued \( hmo1\Delta \) phenotype (A) Repair efficiency of the NcoI-linearized plasmid. NcoI produces a unique DSB in pMV1328 with 5’ end overhangs. \( hmo1\Delta \ k\Delta\) cells were plated on the 0.5 mM caffeine containing SD plates. (B) Repair efficiency of the NruI-linearized plasmid. NruI produces a unique DSB in pMV1328 with blunt end. 0.5 mM caffeine was used to plate the cells. Three independent experiments were performed. Error bars represent standard deviation.

When transformed linearized plasmid has overhang end, caffeine restores the WT NHEJ repair efficiency in \( hmo1\Delta \). However, it is not true with blunt end DSB repair. MRX recognizes overhang polarity and only nicks DSBs with sticky ends. Probably, MRX stays put and prevents resection starting at the DSB ends. In WT, this might prevent Ku from binding and mediating NHEJ. In \( k\Delta\), it may prevent the resection needed to unmask microhomologies. If blunt-ended DNA is not a substrate for MRX, it would explain why neither Hmo1p or caffeine have an effect, as no resection can take place anyway, provided Ku is protecting the ends from Exo1 and/or Sgs1/Dna2.

References:


CHAPTER 5

SUMMARY AND CONCLUSIONS

In eukaryotes, the basic thread of life is in a complex of DNA and protein called chromatin. The basic repeating unit of chromatin, termed the nucleosome, is formed upon wrapping of ~146 base pairs of DNA around an octamer of core histones (two copies of each histone protein H2A, H2B, H3, and H4). Core histones are small basic proteins, which are highly conserved through evolution. The interaction between DNA and histones are non-specific and include non-polar electrostatic interactions between the positively charged amino groups of the histones and the negatively charged DNA phosphate backbone. Nucleosomes are interconnected by linker DNA that may associate with either linker histone H1 or with non-histone proteins such as the high mobility group (HMGB) proteins and give rise 30 nm diameter "beads-on-a-string" structure. Linker histone stabilizes a higher order 30 nm chromatin by interacting with 20 base pair of DNA as it enters and exits the nucleosome and form chromatosome. To fit the ~2 m of human DNA inside the micron sized nucleus, 30 nm fiber DNA must be further condensed to metaphase chromosome structure.

Unlike core histones, linker histones are a highly diverse group of histones and in human beings at least eleven different subtypes have been reported. Linker histone H1 is characterized by a lysine rich long C-terminus that is indispensable for chromatin condensation (1). Yeast HMGB family protein Hmo1p is unique in containing a lysine-rich C-terminal domain, in marked contrast to mammalian HMGB proteins that have acidic tails. Further, the presence of globular domain in Hmo1p makes it hybrid of HMGB and linker histone H1 (Fig. 5.1).
Hmo1p is a hybrid of HMGB and linker histone H1. Like HMGB, Hmo1p has two globular domain, and like linker histone H1, Hmo1p has a basic C-terminal domain.

Hmo1p stabilizes “fragile” nucleosomes or nucleosome-free regions of the genome (2). In this dissertation, I have elucidated the linker histone function of Hmo1p and its role in various types of DNA DSB repair including homologous recombination, non-homologous end joining and alternate end joining. I present data that supports potential of Hmo1p to stabilize genomic DNA that appears to go beyond conventional linker histone function.

The second chapter in this dissertation describes the role of Hmo1p and Hmo1p-CTD in chromatin stability as evidenced by the eviction of Hmo1p during DSB repair, resulting in faster DNA damage associated chromatin remodeling events within cells. The DNA damage response has to operate in the context of chromatin. ChIP assay showed that the kinetics of H2A phosphorylation, recruitment of INO80 to a DSB site, and nucleosome disassembly in the form of histone H3 eviction is faster during DSB repair in an hmo1Δ strain. Further, DNA end resection and the recruitment of repair proteins Rad51 and
tracking of Ku protein is more efficient in an $hmo1\Delta$ strain during DSB repair. Consistently, these DSB associated chromatin events associated with more efficient repair by both HR and NHEJ in $hmo1\Delta$ strains. Interesting ChIP data showed enrichment of Rad51 at all sites monitored in the $hmo1\Delta$ strain, in addition to enhanced recruitment after DSB induction.

I propose that yeast HMGB protein Hmo1p stabilizes chromatin and as a result of this the DSB repair and associated chromatin remodeling events are more efficient in the absence of Hmo1p. I speculate that the less-stable chromatin structure associated with absence of Hmo1p may promote accessibility of Rad51 to undamaged DNA sites. The strain expressing Hmo1p deleted for its C-terminal tail phenocopies $hmo1\Delta$. It indicates that the C-terminus is necessary for Hmo1p recruitment or to stabilize the chromatin that we discussed in chapter 3.

The third chapter addresses the Hmo1p function as a linker histone in yeast and the role of Hmo1p C-terminal tail in DNA compaction. By using ChIP, we showed that DSB associated chromatin remodeling events occurred more rapidly in the absence of Hmo1p and this phenotype is reversed by the expression of human linker histone H1. Further the DSB repair efficiency and DNA end resection in $hmo1\Delta$ was reversed by expression of histone H1, as evidenced by equivalent recovery and equivalent resection in wild type cells and $hmo1\Delta$ expressing H1. MNase digestion showed that the chromatin from $HMO1-AB$ cells (cell that express Hmo1p without CTD) was as hypersensitive to nuclease as $hmo1\Delta$ cells, indicating that the ability to protect linker DNA requires the C-terminal domain of Hmo1p.
I suggest that Hmo1p function as linker histone during vegetative growth generating a chromatin environment that is similar upon expression of human linker histone in \textit{hmo1Δ} cells. The globular domains of Hmo1p are responsible for binding to linker DNA, and the lysine-rich extension is essential for chromatin compaction. In this way, yeast Hmo1p and H1 from higher eukaryotes possess globular domains with affinity for linker DNA connected to a lysine-rich C-terminal extension that is required for chromatin compaction.

The fourth chapter deals with the role of Hmo1p in controlling resection and its direct role in repair pathway choice. Using a plasmid end joining assay, we separated the role of Hmo1p in repair processes at both chromatin and non-chromatin levels. Transformation of an \textit{hmo1Δ} strain with linear plasmid DNA results in significantly reduced transformation efficiency, suggesting the DNA end-joining role of Hmo1p. DNA end resection is inhibitory for classical non-homologous end joining but resection promotes error-prone alternate end joining. I propose that Hmo1p prevents homology mediated error prone A-EJ repair by preventing caffeine sensitive aspect of early resection and directing repairs towards NHEJ.

Taken together, emerging data suggest that yeast linker histone function is a division of labor between Hho1p and Hmo1p, in which Hho1p may have acquired more specialized functions due to its unusual domain organization, and that the terminal lysine-rich extension of Hmo1p has endowed it with the ability to stabilize both noncanonical and conventional nucleosome arrays.

**FUTURE DIRECTION**

Hmo1p is a yeast linker histone and like linker histone, it stabilizes nucleosome. Hmo1p binding to four-way DNA junctions is consistent with the binding of H1 at the nucleosome
dyad (3). However, establishing the nature of Hmo1p interaction with reconstituted nucleosomes will be important to shed further light on the mechanism by which Hmo1p executes such H1-like nucleosome stabilization. Further, the comparison of the hydroxyl radical footprinting cleavage pattern of dinucleosomes without Hmo1p and with Hmo1p would provide interesting information on the interaction of Hmo1p with nucleosomal DNA at very high resolution.

My preliminary data shows that after DNA DSB at the MAT locus, Hmo1p is not only evicted from the site proximal to the break, but also from its own promoter that is located on different chromosome. In the absence of DSB, Hmo1p associates with its own promoter and HMO1 promoter activity is upregulated in the absence of Hmo1p (4). It would be of great interest to find the cause behind the Hmo1p eviction from its own promoter. It may be possible that some post translational modification of Hmo1p causes its release from its own promoter, so Hmo1p mass spectrometry would be of my great interest.

TOR (target of rapamycin) is a central regulator of ribosome biogenesis and controls Hmo1p expression and HMO1 promoter activity (4). TOR responds to rapamycin to generate responses similar to those elicited by starvation and environmental stress such as hypoxia and DNA damage to reduce cell growth (5,6). We hypothesize that Hmo1p eviction from its own promoter is a result of cell stress and it would be of great interest to see the Hmo1p binding to its own promoter after rapamycin treatment. Overall, I established the role of Hmo1p in chromatin stability and DNA repair, however it would be interesting to study the HMO1 gene regulation during stress condition.
In eukaryotes, HMGB proteins are abundant nuclear proteins, one molecule of HMGB1 is present per every 10-20 nucleosomes. Sequence specific HMGB protein LEF-1 acts as transcriptional factor and involved in Wnt signaling pathway. Non-sequence specific HMGB protein HMGB1 facilitate DNA repair by recruiting NER proteins. HMGB protein HMO2 also known as NHP10 is a subunit of INO80 chromatin remodeling complex binds DNA ends, protecting them from exonucleatic cleavage. HMO2 binds DNA with both blunt and cohesive ends, however, the sequence of a single stranded overhang significantly affects binding.

The evolutionarily variable linker histones are critical for stabilization of nucleosomes by binding DNA entering and exiting the core particle and by facilitating higher order organization. The lysine-rich CTD of metazoan H1 is crucial for such stabilization. The lysine-rich C-terminal domain of HMO1 is unique for HMGB proteins and in marked contrast to mammalian HMGB proteins (and even yeast HMO2) that have acidic tails. Absence of HMO1 or deletion of the HMO1 CTD makes chromatin hypersensitive to nuclease and it facilitates chromatin remodeling events associated with DSB repair phenotypes that are complemented by expression of human H1 in the hmo1Δstrain pointing to a role for HMO1 in chromatin stabilization. However, the ability of HMO1 to stabilize genomic DNA appears to go beyond conventional linker histone function. HMO1 plays a role in transcription by both RNA Pol I and Pol II, and it functions in the DNA damage response by directing lesions towards the error-free pathway. In these circumstances, HMO1 is required for stabilization of nucleosome-free DNA or DNA associated with “fragile” nucleosomes.
References:


APPENDIX: COPYRIGHT PERMISSIONS

9/30/2016 RE: Permission to reuse Article in Thesis - Arvind Panday

RE: Permission to reuse Article in Thesis

Dear Arvind,


Thank you for your recent email requesting permission to reuse all or part of your article in a thesis/dissertation. As part of your copyright agreement with Oxford University Press you have retained the right, after publication, to use all or part of the article and abstract, in the preparation of derivative works, extension of the article into a book length work, in a thesis/dissertation, or in another works collection, provided that a full acknowledgement is made to the original publication in the journal. As a result, you should not require direct permission from Oxford University Press to reuse your article.

Authors of Oxford Open articles are entitled to deposit their original version or the version of record in institutional and/or centrally organized repositories and can make this publicly available immediately upon publication, provided that the journal and OUP are attributed as the original place of publication and that correct citation details are given. Authors should also deposit the URL of their published article, in addition to the PDF version. The journal strongly encourages Oxford Open authors to deposit the version of record instead of the original version. This will guarantee that the definitive version is readily

133
available to those accessing your article from such repositories, and means that your article is more likely to be cited correctly.

For full details of our publication and rights policy please see the attached link to our website:


If you have any other queries, please feel free to contact us.

Kind regards,

Louise

Miss Louise Eyre | Permissions Assistant | Rights Department
Academic and Journals Divisions | Global Business Development
Oxford University Press | Great Clarendon Street | Oxford | OX2 6DP
Tel: +44(0)1865 354454 | Email: louise.eyre@oup.com | www.oxfordjournals.org

http://www.biomedcentral.com/about/policies/license-agreement

License agreement

In submitting an article to any of the journals published by BioMed Central I certify that;

1. I am authorized by my co-authors to enter into these arrangements.

2. I warrant, on behalf of myself and my co-authors, that:

   - the article is original, has not been formally published in any other peer-reviewed journal, is not under consideration by any other journal and does not infringe any existing copyright or any other third party rights;
- I am/we are the sole author(s) of the article and have full authority to enter into this agreement and in granting rights to BioMed Central are not in breach of any other obligation;
- the article contains nothing that is unlawful, libelous, or which would, if published, constitute a breach of contract or of confidence or of commitment given to secrecy;
- I/we have taken due care to ensure the integrity of the article. To my/our - and currently accepted scientific - knowledge all statements contained in it purporting to be facts are true and any formula or instruction contained in the article will not, if followed accurately, cause any injury, illness or damage to the user.

3. I, and all co-authors, agree that the article, if editorially accepted for publication, shall be licensed under the Creative Commons Attribution License 4.0. In line with BioMed Central's Open Data Policy, data included in the article shall be made available under the Creative Commons 1.0 Public Domain Dedication waiver, unless otherwise stated. If the law requires that the article be published in the public domain, I/we will notify BioMed Central at the time of submission, and in such cases not only the data but also the article shall be released under the Creative Commons 1.0 Public Domain Dedication waiver. For the avoidance of doubt it is stated that sections 1 and 2 of this license agreement shall apply and prevail regardless of whether the article is published under Creative Commons Attribution License 4.0 or the Creative Commons 1.0 Public Domain Dedication waiver.

[End of BioMed Central's license agreement]
Explanatory notes regarding BioMed Central's license agreement

As an aid to our authors, the following paragraphs provide some brief explanations concerning the Creative Commons licenses that apply to the articles published in BioMed Central-published journals and the rationale for why we have chosen these licenses.

The Creative Commons Attribution License (CC BY), of which CC BY 4.0 is the most recent version, was developed to facilitate open access as defined in the founding documents of the movement, such as the 2003 Berlin Declaration. Open access content has to be freely available online, and through licensing their work under CC BY authors grant users the right to unrestricted dissemination and re-use of the work, with only the one proviso that proper attribution is given to authors. This liberal licensing is best suited to facilitate the transfer and growth of scientific knowledge. The Open Access Scholarly Publishers Association (OASPA) therefore strongly recommends the use of CC BY for the open access publication of research literature, and many research funders worldwide either recommend or mandate that research they have supported be published under CC BY. Examples for such policies include funders as diverse as the Wellcome Trust, the Australian Governments, the European Commission’s Horizon 2020 framework programme, or the Bill & Melinda Gates Foundation.

The default use of the Creative Commons 1.0 Public Domain Dedication waiver (CC0 or CC zero) for data published within articles follows the same logic, facilitating maximum benefit and the widest possible re-use of knowledge. It is also the case that in some jurisdictions copyright does not apply to data. CC0 waives all potential copy rights, to the extent legally possible, as well as the attribution requirement. The waiver applies to data, not to the presentation of data. If, for instance, a table or figure displaying research data
is reproduced, CC BY and the requirement to attribute applies. Increasingly, however, new insights are possible through the use of big data techniques, such as data mining that harness the entire corpus of digital data. In such cases attribution is often technically infeasible due to the sheer mass of the data mined, making CC0 the most suitable licensing tool for research outputs generated from such innovative techniques.

It is important to differentiate between legal requirements and community norms. It is first and foremost a community norm, not a law that within the scientific community attribution mostly takes the form of citation. It is also a community norm that researchers are expected to refer to their sources, which usually takes the form of citation. Across all cases of research reuse (including data, code, etc), community norms will apply as is appropriate for the situation: researchers will cite their sources where it is feasible, regardless of the applicable license. CC0 therefore covers those instances that lie beyond long-established community norms. The overall effect, then, of CC0 for data is to enable further use, without any loss of citations. For further explanation, we recommend you refer to our Open Data page. In the following, we provide the licenses’ summaries as they can be found on the Creative Commons website.

The Creative Commons Attribution License 4.0 provides the following summary (where ‘you’ equals ‘the user ’):

**You are free to:**

Share — copy and redistribute the material in any medium or format.

Adapt — remix, transform, and build upon the material for any purpose, even commercially. The licensor cannot revoke these freedoms as long as you follow the license terms.
Under the following terms:

- Attribution—you must give appropriate credit, provide a link to the license, and indicate if changes were made. You may do so in any reasonable manner, but not in any way that suggests the licensor endorses you or your use.

- No additional restrictions—you may not apply legal terms or technological measures that legally restrict others from doing anything the license permits.

Notices

You do not have to comply with the license for elements of the material in the public domain or where your use is permitted by an applicable exception or limitation.

No warranties are given. The license may not give you all of the permissions necessary for your intended use. For example, other rights such as publicity, privacy, or moral rights may limit how you use the material.

Please note: For the terms set in italics in the summary above further details are provided on the Creative Commons web page from which the summary is taken (http://creativecommons.org/licenses/by/4.0/).

The Creative Commons 1.0 Public Domain Dedication waiver provides the following summary:

No copyright

The person who associated a work with this deed has dedicated the work to the public domain by waiving all of his or her rights to the work worldwide under copyright law, including all related and neighboring rights, to the extent allowed by law.

You can copy, modify, distribute and perform the work, even for commercial purposes, all without asking permission. See other information below.
Other information

- In no way are the patent or trademark rights of any person affected by CC0, nor are the
  rights that other persons may have in the work or in how the work is used, such as publicity
  or privacy rights.
- Unless expressly stated otherwise, the person who associated a work with this deed
  makes no warranties about the work, and disclaims liability for all uses of the work, to the
  fullest extent permitted by applicable law.
- When using or citing the work, you should not imply endorsement by the author or the
  affirmer.
VITA

Arvind Panday is the Son of Mr. R.S. Panday and Mrs. Urmila Panday. He was born on February 19, 1988 in New Delhi, India. He received his bachelor degree in Zoology (Hons.) from University of Delhi in 2009 and his master degree in Genomics from Madurai Kamaraj University in 2011. As a project student and research assistant he worked in Indian Institute of Technology (IIT) and All India Institute of Medical Sciences (AIIMS) respectively.

In August 2013, he joined the Department of Biological Sciences, Louisiana State University, Baton Rouge, to pursue his doctorate in biological Sciences under the guidance of Dr. Anne Grove. There he studied the role of Hmo1p protein in DNA repair and associated chromatin remodeling in Yeast. During his course of graduate career, he taught introductory biology labs as a teaching assistant to the department. After this scholastic period, he has decided to pursue a career in research and academia.