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Roles for Histones H4 Serine 1 Phosphorylation in DNA Double Strand Break Repair and Chromatin Compaction: A Dissertation

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ROLES FOR HISTONES H4 SERINE 1 PHOSPHORYLATION IN DNA DOUBLE STRAND BREAK REPAIR AND CHROMATIN COMPACTION

A Dissertation Presented

By

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ABSTRACT

The study of DNA templated events is not complete without considering the chromatin environment. Histone modifications help to regulate gene expression, chromatin compaction and DNA replication. Because DNA damage repair must occur within the context of chromatin, many remodeling enzymes and histone modifications work in concert to enable access to the DNA and aid in restoration of chromatin after repair is complete. CK2 has recently been identified as a histone modifying enzyme. In this study we identify CK2 as a histone H3 tail kinase *in vitro*, identify the phospho-acceptor site *in vitro*, and characterize the modification *in vivo* in *S. cerevisiae*. We also characterize the DNA damage phenotype of a strain lacking a single catalytic subunit of CK2. We further characterize the CK2- dependent phosphorylation of serine 1 of histone H4 *in vivo*. We find that it is recruited directly to the site of a DSB and this recruitment requires the SIN3/RPD3 histone deacetylase complex. We also characterize the contribution of H4 serine 1 phosphorylation in chromatin compaction by using reconstituted nucleosomal arrays to study folding in the analytical ultracentrifuge.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

2xYT	Rich medium for enhanced bacterial growth for protein expression
5-FOA	5- fluroorotic acid
ac	acetylation
AMP	ampicillin
ATM	ataxia telangiectasia, mutated
ATP	adenosine triphosphate
ATR	ATM related
AUC	analytical ultracentrifuge
bp	base pair
BRCT	BRCA1 C-terminal
CAF-1	chromatin assembly factor- 1
ChIP	chromatin immunoprecipitation
CK2	enzyme also known as CKII or formerly casein kinase II
CPT	camptothecin
Da	dalton
DNA	deoxyribonucleic acid
DSB	double strand break
dsDNA	double stranded DNA
DTT	dithiothreitol
E Buffer	extraction buffer
EDTA	ethylene diamine tetraacetic acid
EGTA	ethylene glycol tetraacetic acid
EM	electron microscopy
FACT	facilitates chromatin transcription
FHA	fork head associated
γ-H2AX	H2AX (or H2A in *S. cerevisiae*) phosphorylated on S139 (S129 in *S. cerevisiae*)
GST	Glutathione-S-Transferase
H4S1E	recombinant Xenopus H4 with serine 1 mutated to glutamic acid
HAT	histone acetyltransferase
HDAC	histone deacetylase
HIR	histone regulatory complex
HTP-C	histone H2A phosphatase complex
HML	hidden mat left
HMR	hidden mat right
HMTase	histone methyl transferase
HP1	heterochromatin protein 1
HR	homologous recombination
HU	hydroxyurea
IgG	immunoglobin G
IP	immunoprecipitate
IPTG	isopropyl-β-D-thiogalactoside
IR	gamma irradiation
KOAc	potassium acetate

LB	Luria-Bertani
me	methylation
me3	trimethylthation
MEF	mouse embryonic fibroblasts
mL	milliliter
MgOAc	magnesium acetate
MNase	micrococcal nuclease
MMS	methyl methanesulfonate
MMTV	mouse mammary tumor virus
MW	molecular weight
NHEJ	nonhomologous end joining
MRN	Mre11, Rad50, Nbs1
MRX	Mrell, Rad50, Xrs2
NETN	20mM Tris-HCL pH8.0, 100mM NaCl, 1mM EDTA, 0.5% NP-40 (v/v)
nm	nanometer
OD_{600}	optical density at 600 nm
PAGE	polyacrylamide gel electropherisis
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PEG	polyethylene glycol
PEV	position effect variegation
PMSF	phenylmethanesulphonylfluoride
PNK	polynucleotide kinase
phos	phosphorylation
PVDF	Polyvinylidene Fluoride
R	ratio of octamer: nucleosome positioning sequence
RCAF	replication coupled assembly factor
rDNA	ribosomal DNA
recon	reconstitution (of array from purified components)
RNA	ribonucleic acid
RNAPII	RNA polymerase II
RPA	replication protein A
RPM	rotations per minute
RSC	remodels the structure of chromatin
S	sedimentation coefficient in Svedburgs
SAGA	Spt-Ada-Gcn5- acetyltransferase
SD	synthetically defined
SDS	sodium dodecyl sulfate
SE	chromatin array reconstituted with xH4 with E inserted at second position
shRNA	short hairpin RNA
SIE	strand invastion/ extension
SNF	sucrose non-fermenting
SS	chromatin array with xH4 with S inserted at second position
SSBR	single strand break repair
ssDNA	single stranded DNA
sup	supernatant

Su(var)	suppression of variegation
SWI	mating type SWItching
TAP	tandem affinity purification
TBST	Tris buffered saline with tween
TE	10mM Tris 1mM EDTA pH 8.0
TEV	tobacco etch virus
ts	temperature sensitive
ub	ubiquitylation
UIM	ubiquitin interacting motif
UV	ultraviolet
WCE	whole cell extract
WT	wild type
YEPD	Rich media containing 2% glucose (yeast extract, peptone media)
YEPR	Rich media containing 2% raffinose

CHAPTER I

INTRODUCTION

As all DNA-templated events in eukaryotic organisms must occur within the context of chromatin, it is no wonder that the study of chromatin comprises such a vast field of research. The cell has a truly elegant way of organizing its DNA, such that transcription, replication and repair can occur within this compact structure. This introductory chapter will outline the complexity of chromatin structure as well as the mechanisms the cell employs to deal with this complexity, especially within the context of repairing a DNA double strand break.

Chromatin Organization

All eukaryotes maintain their genome as a nucleoprotein complex, which consists of DNA wrapped around four histone proteins. The basic repeating unit of chromatin is the nucleosome. The central core of the nucleosome consists of two copies each of four histone proteins. Two copies of H3 and H4 join to form a histone tetramer. In addition to the tetramer are two histone H2A/ H2B dimers which form the histone octamer. Around the octamer, 147 base pairs of DNA wraps approximately 1.7 times in a left-handed superhelix to form the nucleosome (Luger at al., 1997a).

The crystal structure of the nucleosome has provided much insight into the organization of chromatin. Each of the four histones contain a globular histone-fold domain, which is a structural domain involved in dimer-tetramer interfaces within the nucleosome. Each histone also contains an N- terminal domain, called the histone “tail” which extrudes from the nucleosome surface. The histone tails do not contribute to the structure of the individual

nucleosomes but help contribute to the structure of chromatin as a whole. Histones H2A and H2B contain C-terminal domains which also extrude from the surface of the nucleosome.

Nucleosomes, which are separated from each other by linker DNA, form a nucleosomal array, also considered to be the primary structure of chromatin. This array has a 10nm diameter as visualized with EM, and assumes the shape of the canonical "beads-on-a-string" nucleosome fiber *in vitro* (Thoma et al., 1979). The nucleosomal array then undergoes further levels of compaction, with linker histones and non-histone proteins to form secondary and tertiary structures (Lu et al., 2006).

Histones H3, H4, H2A and H2B are the four main "replicative" histones. They are expressed during S-phase for incorporation into the newly synthesized DNA directly at the replication fork. There are also variants of the histones, which are expressed throughout the cell cycle and appear to have specific functions outside the role of the canonical histones. Histone H2A has the most variants of the four histones, including H2A.X, H2A.Z, macroH2A and H2ABbd. Histone H2A in *S. cerevisiae* and *S. pombe* resembles mammalian H2A.X. H2A.X has an extended C-terminus which has an important serine residue which is phosphorylated in response to DNA double strand breaks (Reviewed in Ismail and Hendzel, 2008).

Histone H3 also has variants which are independent of replication. Histone H3.3 is expressed throughout the cell cycle. H3 in *S. cerevisiae* most resembles mammalian H3.3. The centromere- specific H3 variant is CENP-A in mammals, or Cse4p in *S. cerevisiae*. There is also a testis-specific H3t in mammals. (Reviewed in Loyola and Almouzni, 2007)

In order for the cell to be able to contend with nucleosomal DNA, two types of chromatin specific enzymes are employed: ATP-dependent chromatin remodeling enzymes and histone modifying enzymes. The ATP-dependent remodeling enzymes use the energy from ATP

hydrolysis to disrupt histone-DNA contacts. They can do so by creating helical torsion of the DNA, mobilizing nucleosomes (i.e. sliding), and aiding histone dimer exchange (Smith and Peterson 2005, van Vugt et al., 2007). ATP-dependent remodeling enzymes are essential for all DNA-templated events such as transcription (Sudarsanam et al., 2000), sister chromatid cohesion (Huang and Laurent, 2004), DNA replication (Papamichos-Chronakis and Peterson, 2008), and DNA repair (Bao and Shen, 2007).

The histone modifying enzymes, on the other hand, add post-translational modifications, such as phosphorylation, acetylation or methylation to the histone proteins. Many of the histone modifications characterized thus far occur on the tails which extrude form the nucleosome surface, although the importance of modifications within the nucleosome core is also being elucidated.

The Role of Histone Modifications in DNA-Templated Events: A Brief Primer

The role of histone modifications in every DNA-templated event has been studied. Histone modifications have been implicated in replication, transcription, heterochromatin formation, chromatin compaction and DNA damage repair (Peterson and Laniel, 2004). These modifications are believed to recruit effector proteins to chromatin, alter chromatin structure, and alter histone-DNA contacts.

Modifications of Newly Synthesized Histones

The replicative histones, which are synthesized during S-phase of the cell cycle, have characteristic modifications upon synthesis. Histone H4 is acetylated on lysines 5 and 12 (Sobel

at al., 1995). After H4 has been shuttled into the nucleus with the help of the histone chaperones, it is incorporated into chromatin during DNA replication. It is within chromatin that the acetylation marks are removed (Jackson et al., 1976). Acetylation of lysine residues that are native to newly synthesized H3 differ amongst organisms. In budding yeast lysines 9, 14, 23 and 27 are all acetylated, but the removal of these acetyl groups upon incorporation into chromatin is unknown (Kuo et al., 1996). Recently, acetylation of K56 of H3 has been shown to be a modification of newly synthesized histones (Masumoto et al., 2005; Recht et al., 2006).

Histone Modifications and Heterochromatin Formation

The study of Position Effect Variegation (PEV) has provided an elegant model to elucidate the contribution of histone modifications to heterochromatin formation and show the complexity of the downstream events regulated by histone modifications. PEV is the inactivation of a gene in a subset of cells due to its proximity to heterochromatin. Genetic screens for suppressors and enhancers of PEV have helped to elucidate the players involved. Such studies identified the histone methyltransferase (HMTase) Su(var)3-9 which methylates histone H3 at lysine 9 (H3K9me) (Tschiersch et al., 1994; Rea et al., 2000), and HP1 (James et al., 1989). HP1 contains a chromodomain, which specifically recognizes H3K9me (Lachner et al, 2001). H3K9me and HP1, with the help of the RNAi machinery, work in concert to promote the formation and spreading of heterochromatin.

A study from the Martienssen and Grewal labs helped to define how the HMTase is directed to methylate histones that will establish heterochromatin. They found in *S. pombe* RNAi mutants (*ago1*$^{-}$, *dcr1*$^{-}$, and *rdp1*$^{-}$) that H3K9me levels were greatly reduced within the centromeric repeats compared with wild type (WT) (Volpe et al., 2002). In fact, a known marker

for euchromatin, H3K4me, was increased within the centromere. Their observations led to the hypothesis that antisense transcripts within heterochromatic regions recruit the H3K9 HMTase. Once this region is methylated, HP1 is recruited via its chromodomain. HP1 is then able to self-associate with adjacent HP1 molecules and stimulate the spreading of heterochromatin. HP1 spreading enriches the HMTase, which leads to further histone methylation, and further HP1 recruitment (reviewed in Shilatifard 2006). A similar phenomenon occurs in budding yeast. The HDAC, Sir2p, is necessary for chromatin silencing by deacetylating chromatin regions, which then creates a binding region for Sir4p, another member of the silencing complex (Moazed, 2001). In both cases, a histone modifying enzyme helps to create a local chromatin environment to recruit downstream silencing proteins.

Studies in *S. cerevisiae* have shown the importance of a modification within the histone fold domain in silencing, H3 Lysine 79 methylation (H3K79me). The responsible HMTase is Dot1p (Ng et al., 2002) which has been implicated as a high copy disruptor of telomeric silencing (Singer et al., 1998). Deletion of Dot1p also disrupts telomeric silencing (Singer et al., 1998). Mutation of K79 of H3 causes loss of silencing at telomeres, showing that the effect of Dot1p on silencing is through H3K79 methylation. In H3K79 mutants, levels of the silencing proteins Sir2p and Sir3p are greatly reduced (Ng et al., 2002). Therefore, methylation of H3K79 by Dot1p helps to recruit silencing proteins and maintain the heterochromatin at the telomeric regions in *S. cerevisiae*.

The acetylation status of lysine residues in the H3 and H4 tail domains is also important for heterochromatin formation. In *S. cerevisiae* there are three regions of silenced heterochromatin: the mating donor loci *HML* and *HMR*, rDNA and the telomeres. The *URA3* gene can be integrated at these loci, and by virtue of its location will not be expressed, due to its

establishment in heterochromatin. A read out of *URA3* expression is the ability to grow on plates containing 5-FOA, which will kill cells expressing the *URA3* gene. Wild type cells with a *URA3* gene integrated at the subtelomeric loci are able to grow fine on 5-FOA. Cells in which H4K16 has been mutated to either arginine or glutamate de-silence the URA3 gene and the cells die in the presence of 5-FOA (Aparicio et al., 1991). Mutations of three or four of the following H3 lysines (9, 14, 18 or 23) will also result in a loss of telomeric silencing. (Thompson et al., 1994).

A gradient of H4K16 acetylation from the telomere to 20kb from the telomere ends has been established as an important regulator of the heterochromatin state of the sub-telomeric regions. Acetylation of K16 depends on the histone acetyltransferase (HAT) Sas2p (Suka et al., 2002; Kimura et al., 2002). A gradient of K16 acetylation is formed by the opposing action of Sas2p and the histone deacetylase (HDAC) Sir2p. H4K16 acetylation status regulates the silenced state of genes within the telomeric regions. H4K16 is hypoacetylated in the region 1kb from the telomere. A sharp gradient of increasing acetylation is seen from 1-5kb from the telomere, and from 5-30kb away the chromatin is hyperacetylated (Kimura et al., 2002). If H4K16 is mutated to arginine the other three acetylatable lysines on the H4 tail become hypoacetylated compared with WT (Suka et al., 2002). Regulation of H4K16 acetylation levels around the telomere is important in the control of Sir3p spreading into the subtelomeric regions. In a *sas2Δ* strain, where there is a loss of H4K16Ac, Sir3p levels are decreased at the telomeres and increased in the subtelomeric regions, which causes repression of genes located in the subtelomeric regions (Suka et al., 2002; Kimura et al., 2002).

Histone Modifications and Transcription

A belief that a correlation between histone acetylation and methylation is essential for proper gene regulation has long existed. Many studies which focus on changes within the promoter regions of inducible genes have established that many modifications work in concert to ensure both efficient expression and repression of genes.

S. cerevisiae provides a very useful model to study histone modifications within the context of gene expression: the inducible *PHO5* gene. Four stably positioned nucleosomes in the promoter region have been precisely mapped under both repressive and inducing conditions (Barbaric et al., 2001). The *PHO5* gene is silent in phosphate rich medium, and is expressed in the absence of phosphate.

A study from the Horz lab followed the acetylation status of the nucleosomes in the promoter of the *PHO5* gene. It had been previously established that Gcn5p, the catalytic subunit of the SAGA histone acetyltransferase complex, and the chromatin remodeling complex SWI/SNF are necessary for full activation of the *PHO5* gene (Barbaric et al., 2001). They showed that during transcriptional activation of *PHO5* there is a transient increase in acetylation of histone H3 K9 and K18, followed by complete eviction of the nucleosomes in the *PHO5* promoter (Reinke et al., 2003). A study of the same promoter showed that although there is no transient increase in H4 tail acetylation, the NuA4 HAT complex is essential for *PHO5* activation, and is recruited directly to the promoter region. In its absence, the *PHO5* gene remains in the repressed state (Nourani et al., 2004).

Acetylation isn't the only modification important for *PHO5* maintenance. Set1p, a histone methyltransferase required for the methylation of H3K4 (H3K4me), is also important for proper *PHO5* gene expression. Although Set1p dependent H3K4me is traditionally required for gene activation, a study showed that deletion of *SET1* led to derepression of the *PHO5* gene in

rich media (Carvin et al., 2004). A non-methylatable H3K4R mutant also showed *PHO5* derepression, leading the proposal that Set1p regulates *PHO5* expression both positively and negatively.

Histone H2B ubiquitylation (H2Bub) has also been implicated in expression of the *PHO5* gene. H2B is ubiquitylated on K123 dependent on Rad6p and Bre1p in *S. cerevisiae* (Robzyk et al., 2000). Reports have shown that this modification is important for transcriptional activation of inducible genes in yeast. Upon induction of *SUC2*, (Henry et al., 2003) *GAL1* (Henry et al., 2003; Kao et al., 2004) or *PHO5*, H2B is transiently ubiquitylated at the promoter region, peaking at around 30-60 minutes, and then decreasing (Kao et al., 2004). This ubiquitylation is necessary for H3K4me to be enriched at these promoters of the induced genes. Rad6p, an ubiquitin conjugating enzyme, is recruited to the promoter (Kao et al., 2004) during transcription initiation and decreases with the same kinetics as the ubiquitin modification. It has been shown that Rad6p associates with the elongating form of RNA PolymeraseII, and this association is necessary for H2Bub, and H3K4me to be enriched at the promoters of active genes (Xiao et al., 2005). H2Bub is excluded from the silent areas of the genome including telomeres (Kao et al., 2004).

Histone Modifications and Chromatin Compaction

It has long been known that histone acetylation plays a role in transcriptional activation and euchromatin formation, but its effect on chromatin structure has remained elusive. A study using acetylated histones demonstrated that arrays reconstituted with highly acetylated octamers are not able to achieve highly compacted structures (Tse et al., 1998). Octamers were extracted from HeLa cells which had been treated with varying amounts of an HDAC inhibitor and had 8,

23 or 46% of all available lysines acetylated. These octamers were used to reconstitute an *in vitro* chromatin array using the highly characterized 5S rDNA gene segment which contains a known nucleosome positioning sequence. The sedimentation coefficient (S) of these arrays was measured in the analytical ultracentrifuge (AUC). The sedimentation coefficient gives an indirect readout of the shape of a species. In low salt buffer (TE) these arrays are known to sediment as a 29S species (Hansen and Turgeon, 1999) With increasing amounts of Mg^{+2}, WT arrays compact and the S value increases to a maximum of 55S. The arrays reconstituted with the hyperacetylated octamers could only achieve a 35S species. In a test of chromatin self-association, which is thought to mimic long range fiber-fiber interactions *in vivo*, the acetylated arrays required more Mg^{+2} to associate than their less acetylated counterparts.

Another study demonstrated that a single acetylated lysine residue can disrupt the formation of higher order chromatin structure (Shogren-Knaak et al. 2006). Using a native chemical ligation strategy, a homogenous nucleosomal array was reconstituted in which every H4 was acetylated at K16 (H4K16Ac). The findings showed that an array containing H4K16Ac was not able to compact to the maximally folded state of 55S, and only reached a maximum compaction of 44S. These results were comparable to an array containing "tailless" H4, which had been characterized as being defective in compaction (Dorigo et al., 2003). The H4K16Ac array also needed more Mg^{+2} to self-associate than a WT array. An *in vivo* characterization of H4K16Ac complimented the *in vitro* data as K16Ac was found preferentially enriched in euchromatin and excluded from the heterochromatic regions in HeLa cells. This study showed that two acetyl groups/ octamer are able to disrupt higher order folding.

Other modifications have been implicated in chromatin compaction. Histone H3 is phosphorylated in a cell cycle regulated manner (Gurley et al., 1978). Phosphorylation of serine

10 of H3 peaks during mitosis and diminishes as cells enter telophase. Based on the timing of this modification, specifically when the chromatin is at its most compact within the cell cycle, it has been hypothesized that this modification may aid in chromatin compaction. Yet, there is a lack of evidence to support this hypothesis. A study from the Mancini lab showed that in Chinese Hamster Ovaries, treatment with a phosphatase inhibitor (okadaic acid), which maintains H3S10phos throughout the cell cycle, does not cause aberrant chromatin compaction outside of mitosis (Van Hooser et al., 1998). They also found that H3S10phos was not necessary to maintain chromosome condensation. Therefore it can be concluded that phosphorylation is not sufficient to cause compaction, nor necessary for maintenance of compaction.

Ubiquitylation of histones has also been a modification proposed to disrupt higher order structure. Histone H2B is ubiquitinated on K123 in yeast, and K120 in mammals and this is expected to be within the histone fold region of the nucleosome (Jason et al., 2002). Because levels of histone ubiquitylation vary within the cell cycle, disappearing as cells enter mitosis (Wu et al., 1981) it makes sense to hypothesize that such a bulky adduct would inhibit chromatin compaction and higher order chromatin structure. Again, there has been no evidence to support this hypothesis. Nucleosomes can be reconstituted with two molecules of ubiquitinated H2A or H2B (Davies et al., 1994). Arrays reconstituted with ubiquitinated histones do not have an altered Mg-dependent folding in vitro (Jason et al., 2001). A role of histone ubiquitylation in chromatin structure, if there is one, has yet to be discovered.

A recent paper from Shelley Berger's lab has proposed that the phosphorylation of histone H4 serine 1 (H4S1phos) aids chromatin compaction *in vivo* (Krishnamoorthy et al., 2006). They noticed that in yeast undergoing meiosis that H4S1phos increases at the end of meiosis during sporulation and persists until the cell cycle resumes. This modification was also

seen during fly and mouse spermatogenesis. In an *S. cerevisiae* strain in which the serine cannot be phosphorylated (H4S1A), these cells are deficient in sporulation, with an efficiency only 30% that of wild type. They also see an increase in the nuclear volume of cells with both the HS1A mutation or are cells that lack the responsible meiosis-specific kinase Sps1p. In an effort to study this hypothesis *in vitro*, we tested the contribution of H4 serine 1 phosphorylation on chromatin folding in the analytical ultracentrifuge. The results of this study are presented in Chapter IV.

DNA Double Strand Break Repair: The Chromatin Connection

The process of DNA double strand break (DSB) repair in the eukaryotic cell must contend with the repressive structure of chromatin. The role of histone modifications in DNA damage repair, the two main pathways of DSB repair, DNA damage checkpoint signaling, and chromatin remodeling at the DSB will be discussed to give an overview of how the cell repairs the deleterious break.

Chromatin Modifications and DNA Damage

One of the most highly characterized modifications in response to DNA damage is the phosphorylation of a serine residue in the C-terminal domain of histone H2AX, traditionally referred to as γ-H2AX. Serine 129 of the *S. cerevisiae* H2A, or serine 139 of H2AX in higher eukaryotes is phosphorylated in response to DNA double strand breaks. The phosphorylation depends on the DNA damage checkpoint kinases ATM, ATR and DNA-PK in higher eukaryotes and Mec1p and Tel1p in yeast (reviewed in Altaf et al., 2007). γ-H2AX is seen within minutes of DSB induction, and by chromatin immunoprecipitation (ChIP) is shown to peak at 3-5kb from

the break and spread up to 50kb on both sides of the break in *S. cerevisiae* (Shroff et al., 2004) and cover megabases on both sides of the break in mammals (Rogakou et al., 1998).

The contribution of H2AX phosphorylation to cell survival in *S. cerevisiae* was studied in the presence of DNA damaging agents. Cells lacking a phosphorylatable serine in the C terminal domain of H2A showed a slight phenotype in the presence of 0.02% MMS, a DNA alkylating agent, or with increasing amounts of phleomycin, a DSB-inducing agent (Downs et al., 2000). These same cells were not sensitive to UV exposure, indicating that the sensitivity may be DSB specific. In fact, these cells were partially deficient in the DSB repair pathway of non-homologous end joining (NHEJ) but were not deficient in homologous recombination (HR). An *S. cerevisiae* strain with the H2AS129A mutation is also sensitive to camptothecin (CPT), which creates DSBs during the S-phase of the cell cycle but does not activate the intra-S phase cell cycle checkpoint. This indicates that the modification is not important for checkpoint response but is for DSB repair (Redon et al., 2003). Although this modification is robustly increased in response to DSBs, the phenotypes in response to DSB-inducing agents are very subtle.

The contribution of γ-H2AX in mammals has also been studied. Mice lacking both copies of histone H2AX were generated to test the importance of H2AX phosphorylation (Celeste et al., 2002). The $H2AX^{-/-}$ mice were radiation sensitive, growth retarded, and immune deficient. The mouse embryonic fibroblasts (MEFs) generated from these mice showed poor proliferation. Metaphase spreads of these MEFs showed an increase in chromosome breaks and dicentric chromosomes as compared with WT cells. Immunostaining of the checkpoint factors NBS1, 53BP1 and BRCA1 showed that their recruitment to the site of damage (i.e. focus formation) was impaired in $H2AX^{-/-}$ MEFs, but RAD51 was recruited normally compared with

WT. These results show that the histone variant H2A.X is important for DNA damage resistance, but does not directly address the contribution of Serine 139 phosphorylation.

The order of recruitment as measured via focus formation in mammalian cells has been well characterized, and γ-H2AX appears to be a key organizer of the DNA damage focus. After phosphorylation of histone H2AX, the scaffold protein MDC1 is recruited to the site of a break (Lukas et al., 2004). A direct interaction between the two tandem BRCT domains and phosphorylated H2AX is thought to recruit and retain MDC1 directly at the site of a DNA DSB. An X-ray structure of the tandem BRCT domains interacting specifically with a phospho-peptide of the C-terminal domain of H2AX helped to advance the hypothesis that MDC1 directly interacts with γ-H2AX at the site of DNA damage (Stucki et al., 2005). Disruption of the MDC1 protein results in a loss of focus formation by the DNA damage proteins NBS1, 53BP1 and BRCA1 (Lou et al., 2006). Conflicting studies have shown that NBS1, BRCA1 and 53BP1 do not need γ-H2AX (Celeste et al., 2003) and that 53BP1 can be recruited to the site of DNA damage through the interaction of its Tudor domains with methylated H3 (Huyen et al., 2004).

Recent studies have pointed to interplay between γ-H2AX and H2A ubiquitylation at the site of DNA damage. The RING and FHA domain containing protein RNF8 is recruited to a DSB dependent upon both γ-H2AX and MDC1 localization, and is necessary for the downstream recruitment of BRCA1, NBS1 and 53BP1 (Mailan et al., 2007; Huen et al., 2007; Kolas et al., 2007). The FHA domain is a phospho-peptide binding motif and the RING domain is a protein-protein interacting motif implicated in ubiquitylation.

RNF8 is found to bind phosphorylated MDC1 via its FHA domain and colocalize at DNA damage foci within a minute of irradiation (Mailand et al, 2007). RNF8 contains a RING domain which implies it is an E3-ubiquitin ligase capable of transferring ubiquitin from an E2

conjugating enzyme to a specific substrate. Indeed, with the depletion of the E2 conjugating protein Ubc13 from HeLa cells, formation of 53BP1 foci was diminished (Kolas et al., 2007). Therefore, the downstream recruitment of many players of the damage response requires protein ubiquitylation to be recruited to sites of DNA damage. It was found that RNF8 is responsible for ubiquitylation of γ-H2AX. This ubiquitylation is responsible for the downstream recruitment of RAP80, a ubiquitin interacting motif (UIM) containing protein, as well as 53BP1 and BRCA1 (Huen et al., 2007; Mailand et al., 2007). In summary, an initial phosphorylation of H2AX leads to recruitment of proteins that are necessary to ubiquitinate γ-H2AX, which in turn further recruits later players in DNA damage response.

Another established role of γ-H2AX is recruitment of the cohesion complex directly to the site of a DNA DSB. The GAL::HO system in *S. cerevisiae,* which will be explained in detail later in the introduction, has been helpful in studying the dynamics of a single break. Cohesin is loaded onto chromatin during S-phase to maintain sister chromatid cohesion until the cells are ready to divide during M phase (Nasmyth, 2002). The different subunits of the cohesion complex are also loaded onto chromatin surrounding a DNA DSB in a manner dependent upon γ-H2AX formation (Strom et al., 2004; Unal et al., 2004). The cohesin subunits are enriched 7-10 fold 8kb from a DSB, and yet are excluded from the region 1-2kb directly surrounding a DSB (Unal et al., 2004). This enrichment is specific for S and G2 phases of the cell cycle but is not found during G1. The spreading of cohesin stretches as far as 50kb from both sides of the break, correlating with that of γ-H2AX. Importantly, cohesin recruitment is necessary for DSB repair via the homologous recombination pathway using the sister chromatid.

Although many studies have shown H2A.X phosphorylation to be important for recruiting and retaining many downstream proteins, such as chromatin remodeling enzymes,

cohesion, and members of the checkpoint cascade, the fact remains that loss of phosphorylation does not show a dramatic phenotype. Phosphorylation covers up to 50 kb on both sides of a DSB in yeast, but cohesion, and the chromatin remodeling complexes are only recruited within 8kb of a DSB. Therefore, there is not a clear explanation as to the role phosphorylation is playing in the DSB response.

Once the DSB has been repaired, the chromatin needs to be restored to its original status, and the DNA damage checkpoint, a signaling cascade which inhibits cell cycle progression in the presence of DNA damage, needs to be turned off. De-phosphorylation of γ-H2AX is an important part of checkpoint recovery. In mammalian cells, the responsible phosphatase is PP2A (Chowdhury et al., 2005). In WT cells that are treated with CPT for 1Hr, γ-H2AX foci are reduced to 20% by 12 hours, but in cells in which PP2A is inhibited the foci persist. PP2A was also found to dephosphorylate nucleosomal γ-H2AX *in vitro*. In irradiated HeLa cells, PP2A is recruited to damage foci dependent on H2AX phosphorylation.

In *S. cerevisiae* the responsible phosphatase is Pph3p, which exists as a complex of three subunits termed the HTP-C (histone H2A phosphatase complex) (Keogh et al., 2006). In a strain in which *pph3* has been deleted, γ-H2AX persists longer than in WT as seen by western blot but levels at the break are comparable to WT as seen by ChIP. Similar results are seen in cells that have been irradiated. Both WT and *pph3Δ* cells have lost their foci with 6 hours, but by western blot γ-H2AX is elevated in the *pph3Δ* mutant. It is therefore hypothesized that H2AX is not dephosphorylated until it is removed from chromatin. It is also interesting to note that persistence of γ-H2AX in the cell affects checkpoint recovery, as *pph3Δ* cells take longer to turn off the checkpoint even after repair has been completed, and this is dependent upon γ-H2AX.

Although γ-H2AX has been the most characterized histone modification studied in response to DNA DSBs, it is certainly not alone. The importance of S122 of H2A in *S. cerevisiae* was reported to be necessary for survival in the presence of many DNA damaging agents including MMS, phleomycin, CPT, HU and UV (Harvey et al., 2005). This residue was found to be phosphorylated in the presence of DNA damage, the most robust of which was in the presence of menadione, an oxidative DNA damaging agent (Moore et al., 2007). Phosphorylation of S14 of histone H2B is also reported in the presence of DNA damaging agents. Antibodies against the modified residue show that it colocalizes with γ-H2AX foci in response to DNA DSBs and that this modification needs γ-H2AX. Similar to γ-H2AX it is also seen during programmed rearrangements in B cells (Fernandez-Capetillo et al., 2004).

Phosphorylation of histone H4 serine 1 in response to DNA DSBs has also been examined (Cheung et al., 2005) We have found that Serine 1 is phosphorylated specifically in response to DNA DSBs within 30 minutes of treatment with a DNA damaging agent. The modification is stable; in the presence of chronic damage the modification does not disappear. Serine 1 of H4 is phosphorylated on both sides of the DNA DSB as seen by ChIP, as far as 10kb from the site of the break, but its timing follows γ-H2AX localization by two hours. The modification does not need activation of the checkpoint kinases Mec1p or Tel1p, which are responsible for both γ-H2AX and activation of the DNA damage checkpoint response. The responsible kinase is CK2 (formerly known as Casein Kinase II) which we also show phosphorylates histone H4 *in vitro.* We further characterize the role of H4S1phos in DNA damage in Chapter II. We show in Chapter III that this modification requires an intact histone deacetylase complex, SIN3/RPD3, to be recruited to the site of a DSB.

Two Pathways of DSB Repair: Homologous Recombination and Non-homologous End-Joining

The cell has two major DSB repair pathways: homologous recombination (HR) and non-homologous end-joining (NHEJ). These two repair pathways have to contend with the restrictive nature of chromatin to make sure the genome is faithfully restored.

Although in the model system *S. cerevisiae* the main repair pathway for DSBs is HR, it has proven useful to discover factors that are responsible for NHEJ. The yeast proteins Yku70p and Yku80p are essential for NHEJ and are conserved throughout all eukaryotes. Cells lacking either Yku70/80p are sensitive to bleomycin and MMS, and are sensitive to IR if the HR repair pathway is compromised (Mages et al., 1996; Feldman et al., 1996; Siede et al., 1996) The Ku proteins bind DNA ends as a heterodimer and are of the first proteins recruited to a DSB. The Mre11/Rad50/Xrs2 complex (MRX) has also been implicated in NHEJ repair in *S. cerevisiae*, but its homolog in higher eukaryotes, the MRN complex, has not been established in NHEJ in mammals. Using an HO nuclease inducible DSB in *S. cerevisiae*, the MRX complex has been shown to be necessary for WT levels of NHEJ in yeast (Moore et el., 1996).

The ligase responsible for NHEJ in yeast is the Dnl4/Lif1 complex, which are the counterparts of the DNA ligase IV/ XRCC4 complex in mammals. Deletion of either gene yields a strong phenotype in a plasmid based repair assay which can screen for mutants that are unable to repair a break via NHEJ (Teo et al., 1997; Wilson et al., 1997). In this assay, a plasmid linearized with a restriction endonuclease is transformed into WT and mutant yeast cells. The cells are plated on a selective media on which only cells which have repaired the plasmid via NHEJ will be able to grow. The transformation efficiency of the cells is a read-out of their

ability to perform NHEJ compared with the WT cells. Another gene has been revealed from such a screen is Nej1p (Valencia et al., 2001; Ooi et al., 2001). Although there is no biochemical function known for Nej1p, it is known that it is encoded by a haploid specific gene.

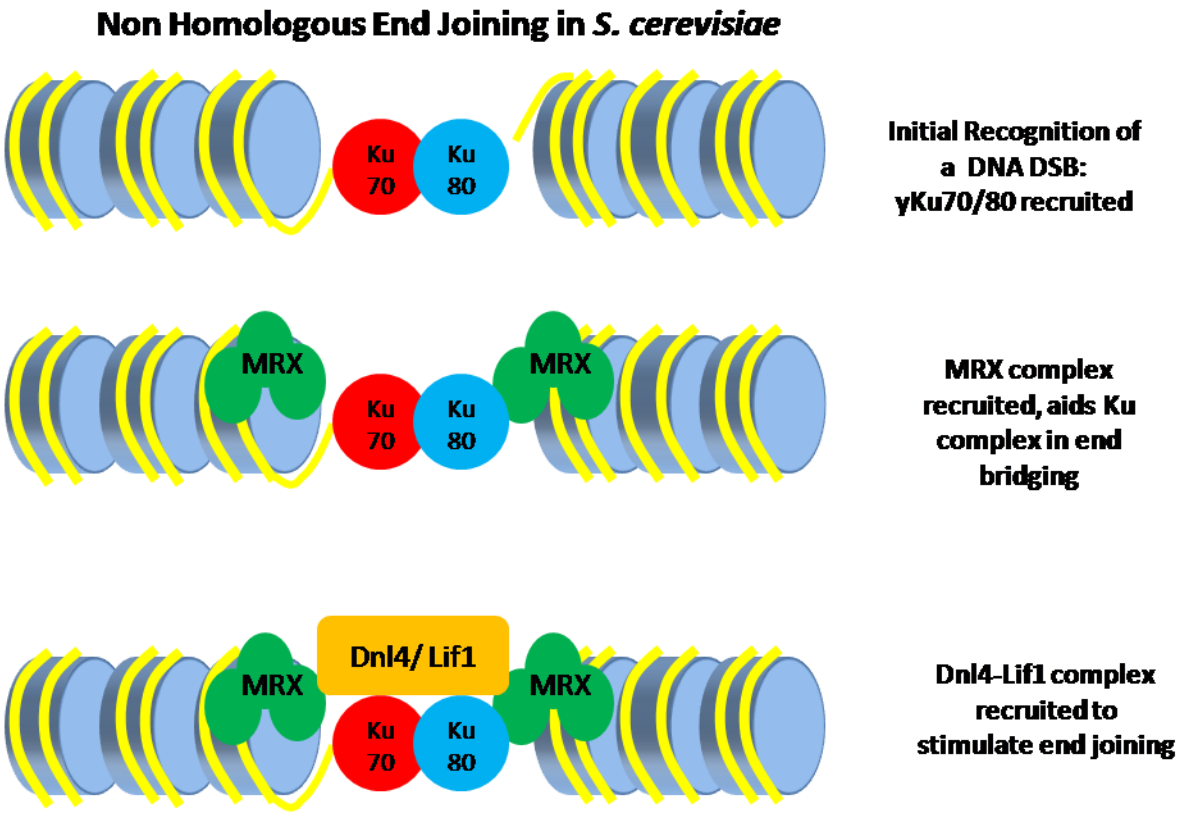

Figure 1. The basic steps of NHEJ in *S. cerevisiae*.

NHEJ is enhanced in haploid cells, because there is no homologous donor chromosome to be used as a template for HR.

S. cerevisiae has also proven to be a very useful model system for studying the steps of homologous recombination *in vivo*. Epistatic analysis of mutants which share the inability to repair a DNA DSB by HR has led to the establishment of the *RAD52* epistasis group, which are proteins that are essential to the HR process *in vivo*. (See Figure 2 for a basic schematic of HR)

The proteins that contribute to each step have also been highly characterized both *in vivo* and *in vitro*. A brief review of HR would not be complete without discussing the roles of the members of the *RAD52* epistasis group and their contribution to recombinational repair.

A major player in this pathway is Rad51p, a recombinase protein which shares homology to the bacterial RecA protein (reviewed in San Filipo et al., 2008). Rad51p is required for formation of a protein filament which searches for homology and engages the broken 3' end of DNA with the homologous template. An early essential step in the HR pathway is the exonucleolytic processing of the break ends, creating long stretches of ssDNA. RPA, which has a strong affinity for ssDNA, is recruited to these single strands on both sides of the break. It is hypothesized that RPA enhances Rad51p binding by removing secondary structure of the ssDNA (Sugiyama et al., 1997). RPA also inhibits Rad51 filament formation by coating the ssDNA and excluding Rad51p from binding. In order for Rad51p to be able to bind ssDNA, mediator proteins are needed to overcome the inhibition by RPA.

Rad52p is a Rad51p interacting protein and is thought to help recruit Rad51p to the site of a DSB (San Filippo et al., 2008). In *S. cerevisiae* strains lacking Rad52p, Rad51p binding to a break is impaired (Wolner et al., 2003; Sugawara et al., 2003). Rad51p also needs Rad55p, Rad57p, and Rad54p for proper recruitment to the break (Gaisor et al., 2001; Wolner et al., 2003). The Peterson and Haber labs also studied the timing of recruitment of members of the *RAD52* epistasis group to a DSB. Rad51p, Rad52p , Rad55p and Rad57p are all recruited at early stages to a DSB, most likely to aid in formation of the pre-synaptic filament and the homology search. Rad54p, a member of the SWI/SNF family of ATPases, is recruited later and is required for extension of the broken end following strand invasion of the homologous donor template.

The phenomenon of mating-type switching in *S. cerevisiae* has been essential in studying the steps involved in DSB repair. The details of mating-type switiching have been fairly well dissected and have provided a model in which to track the fate of a single, controlled DSB. Located within Chromosome III is a region called the *MAT* locus. Contained within this locus is the mating-type information of the haploid cell. The cell can be one of two mating types, a or α, depending on what genes are located at the *MAT* locus. Haploid cells are able to mate with another cell of the opposite mating type to become a single diploid cell. These haploid cells are also able to switch mating type which allows them to mate with the recently budded daughter cell from a previous round of cell division of the opposite mating type (reviewed in Haber 1998).

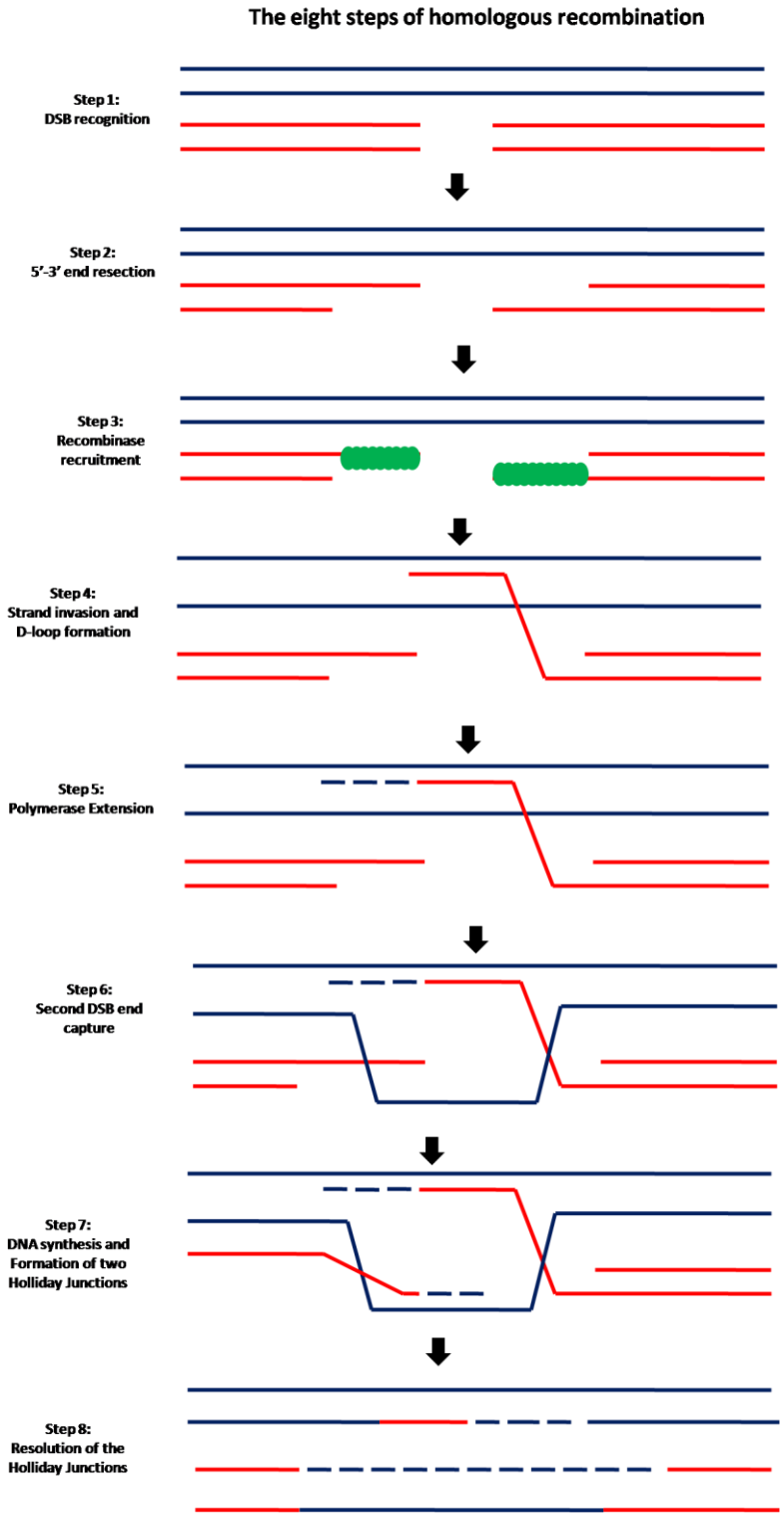

Figure 2. The eight steps of homologous recombination. Adapted from San Fillipo et al. 2008.

On opposite arms of Chromosome III are the silent donor loci, which contain the genes of the two mating types, called *HMLα* and *HMRa*.

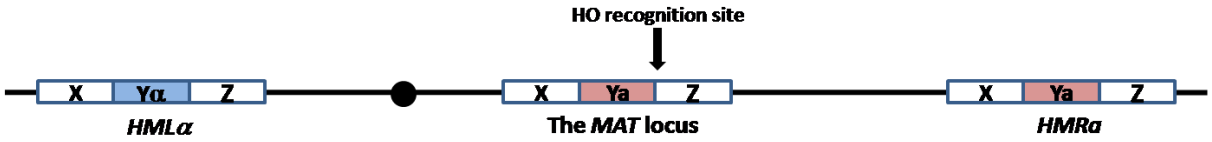

Figure 3. A simple overview of Chromosome III.

Both *HMLα* and *HMRa* are silenced due to packaging in heterochromatin. Although the genes for both mating types are found on Chromosome III, only the genes at the *MAT* locus are expressed. In the example presented in Figure 3, the cell with this chromosome would be an **a** cell, due to the presence of Ya at the *MAT* locus.

The process of mating type switching starts with expression of a site-specific endonuclease called HO. In WT yeast cells the expression of this endonuclease is tightly regulated. It is only expressed during the G1 phase of the cell cycle, and only in a cell that has undergone at least one round of cell division. A newly budded "daughter" cell is not able to switch mating type, but the "mother" cells can (Nasmyth, 1983). The HO endonuclease recognizes a site at the *MAT* locus at the Y/Z interface. Although there are also HO recognition sites at the Y/Z interface in both *HML* and *HMR*, the heterochromatic state inhibits HO from recognizing the restriction site within the donor loci.

After the *MAT* locus has been cleaved, the Z side of the break is involved in the homology search. In this unique system, the homology can be found on both sides of chromosome III, as both *HML* and *HMR* contain the Z region of homology. Since the cell is programmed to switch mating type, a phenomenon called "donor preference" will influence the **a**

cell to find homology at *HMLα* rather than *HMRa* in about 85-90% of switches (reviewed in Haber, 1998).

Another unique aspect of mating type switching is that it is a directional process. As stated previously, only the *MAT* locus is cleaved by HO endonuclease. After the donor locus is found via the homology search, the information is copied and replaced at the MAT locus, but the silent donor locus remains unchanged, in a process called gene conversion. Members of the *RAD52* epistasis group are essential for this process (reviewed in Haber, 1998).

HO cleavage and *MAT* switching have been used to study the dynamics at a DSB, using strains which have the HO endonuclease under regulation of the *GAL1-10* promoter (*GAL::HO*) (Jensen and Herskowitz, 1984). In these strains, HO is expressed upon addition of galactose to the media, creating a controlled site-specific DSB in a population of cells. Furthermore, strains have been used in which both donor loci have been deleted to create a strain in which repair via HR is not possible (Moore and Haber, 1996). Using ChIP in these strains it is possible to follow the recruitment of repair proteins, histone modifications, and chromatin remodeling enzymes to the break site.

The G2/M DNA Damage Checkpoint and DSB Repair

The G2/M DNA damage checkpoint was first described by Weinert and Hartwell in 1988. They recognized that the cell had a way to delay transition from G2 into M phase in the presence of unrepaired DNA DSBs in G2, allowing time to repair the DNA before cell division. The MRN complex is an important player in the initial recognition of DSBs, checkpoint activation, and signal amplification. The MRN (MRX in *S. cerevisiae*) complex is composed of

Mre11, Rad50, and Nbs1 (Xrs2p in *S. cerevisiae*) in mammals. One of its well characterized roles is to bridge the two broken ends of DNA (reviewed in Williams et al., 2007).

Importantly, the MRN complex is also involved in the checkpoint response. MRN is necessary for activation of ATM (Ataxia- Telengiectasia Mutated) *in vivo,* which is one of the key initial checkpoint cascade kinases (Uziel et al., 2003). MRN can also activate ATM *in vitro* (Lee et al., 2004b). MRN and ATM activity are necessary to recruit and activate another checkpoint kinase ATR (ATM- related). MRN and ATM are recruited to DSBs, which in turn activates ATR, which then phosphorylates the Chk1 kinase to proliferate the damage signal (Jazayeri et al., 2006).

MRN has also been implicated in DNA resection which is important for HR repair and amplification of the checkpoint signal (D'Amours and Jackson 2002). An MRN interacting partner, CtIP, is recruited to γ-H2AX foci and is involved in DNA resection and RPA recruitment in U2OS cells (Sartori et al., 2007). MRN and CtIP work in concert to facilitate DSB repair.

The resection of dsDNA to form stretches of ssDNA stimulates recruitment of the ssDNA binding protein RPA. RPA binding to ssDNA is a key signal in the checkpoint cascade. RPA association at the break is necessary for recruitment of ATR and ATRIP (ATR interacting partner) to the break (Zou and Elledge, 2003a.). In *S. cerevisiae*, RPA recruitment to DSBs is important to recruit Ddc2p, the ATRIP homolog.

The RPA coated ssDNA is also a substrate for loading of the DNA damage specific RFC-like clamp loader and the PCNA-like "9-1-1" complex (Zou et al., 2003b). During replication, the RFC complex is necessary to load the PCNA sliding clamp at the primer/ template junction to facilitate DNA replication. A similar scenario occurs at the DNA DSB. The clamp-loader in

this case consists of the members or the RFC complex (RFC2-5) with RFC1 replaced by Rad17 in vertebrates and Rad24p in *S. cerevisiae*. This damage "clamp-loading" complex needs RPA coated ssDNA to load at the break. Subsequently, the DNA damage "sliding clamp" or "9-1-1" complex needs the RFC-like clamp-loader to localize at the break. The "9-1-1" complex consists of Rad9, Hus1 and Mec1 in mammals and Ddc1p, Rad17p and Mec3p in budding yeast. Loading of the RFC and 9-1-1 complexes are necessary for full activation of the ATM/ATR damage checkpoint.

Full activation of the Mec1p/Tel1p or ATM/ATR leads to activation of the effector proteins Rad53p (yeast) and Chk2 (mammals), as well as Chk1. Activation of these effector proteins require the adaptor proteins Rad9p (yeast) or 53BP1 and Brca1 (mammals) which help to increase local concentration of the checkpoint kinases which stimulates activation through autophosphorylation (reviewed in Heideker et al., 2007). Once these effector proteins are activated they can inhibit progression through anaphase by inhibition of cohesion degradation, and sister chromatid separation.

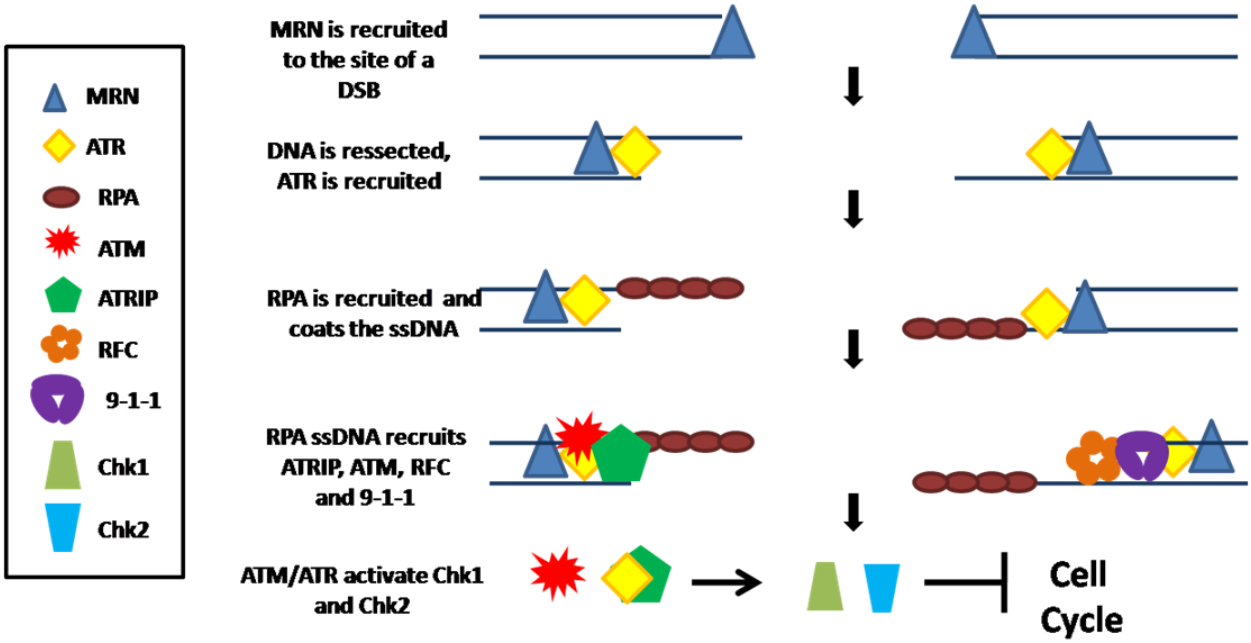

Figure 4. An overview of the mammalian checkpoint signaling pathway.

The Chromatin Modifying Enzymes and DNA DSB Repair

Both chromatin remodeling and histone modifying enzymes have been implicated in many aspects of DNA DSB repair. As discussed previously, phosphorylation of H2AX (H2A in yeast) is essential for recruitment of DNA damage sensing proteins, repair proteins and chromatin modifying enzymes.

Histone acetyltransferase complexes are important for DSB repair. The TIP60 complex has both HAT and ATP-dependent chromatin remodeling activities. Human cells which express a TIP60 protein with a mutation in the catalytic HAT domain are unable to repair DNA DSBs (Ikura et al., 2000). A role for DSB repair of the TIP60 complex in Drosophila has been characterized. The H2AX variant in Drosophila is H2Av, and is phosphorylated in response to DSBs in a manner similar to H2AX. Members of the Workman lab found that both the HAT and ATPase activities of the Drosophila TIP60 complex are necessary to acetylate phosphorylated H2Av, and subsequently exchange the newly acetylated and phosphorylated H2Av for an unmodified version (Kusch et al., 2004). *In vivo*, disruption of TIP60 leads to an increase in the persistence of phosphorylated H2Av. It is possible that TIP60 is being recruited via phosphorylated H2Av and this catalyzes the exchange of the histone variant.

TIP60 has also been shown to be directly recruited to a DSB in human ES cells and to acetylate the chromatin at the break. The HAT cofactor TRRAP is known to recruit HAT complexes to transcription factors, but has also been implicated in recruiting TIP60 to sites of DNA DSBs (Murr et al., 2006). Using the site specific endonuclease I-SceI, it was shown that cells depleted for TRAAP were deficient in DSB repair. The I-SceI break was also used to monitor the recruitment, by ChIP, of acetylated H4 surrounding the DSB. It was found that a transient acetylation of H4 was found 0.5-2.0kb from the break, and this acetylation depends

upon TIP60. In fact, in the absence of this acetylation, RAD51, BRCA1, and 53BP1 focus formation was reduced.

The study of HAT recruitment and histone acetylation around a break has also been extensively studied in *S. cerevisiae*. Strains in which all four acetylatable lysines in the H4 N-terminal tail have been mutated to glutamine are very sensitive to both CPT and MMS. By restoring a single lysine at any position, the sensitivity is rescued (Bird et al., 2002). Mutation of the essential HAT *ESA1* showed similar CPT and MMS sensitivity as the lysine mutants. The *ESA1* mutant, as well as the H4KtoQ strain, were defective for NHEJ.

A study using the GAL::HO inducible break in *S. cerevisiae* followed the status of the 9 acetylatable lysine residues in the H4 (K5, 8, 12 and 16) and H3 (K9, 14, 18, 23 and 27) tails during break repair. Acetylation of all 9 lysines increase 0.6kb from the site of a DNA DSB, and decreases once HO is repressed (Tamburini et al., 2005). Various HATs and HDACs were tested for their sensitivity to HO expression and *gcn5* and *rpd3* strains were sensitive to the DSB produced by HO. In fact an *rpd3Δ* strain was as sensitive to HO expression as loss of *rad52*. The Tyler group also showed the recruitment of the HATs Gcn5p, and Esa1p, as well as the HDACs Rpd3p, Hst1p and Sir2p to the site of a DSB.

Proof that chromatin modifying enzymes directly bind γ-H2AX was shown by a study in which whole cell extracts were fractionated and screened for proteins that specifically bound γ-H2AX peptides and did not bind the unmodified peptide. Subunits from the NuA4 complex, a HAT which contains the catalytic subunit Esa1p, were found to directly bind the phosphorylated peptide. Both Arp4p, a subunit common to a variety of chromatin modifying complexes, as well as Eaf3p, a subunit specific to NuA4, were found in the fraction that binds the phosphorylated peptide (Downs et al., 2004). It was also shown that subunits of the SWR1 and INO80

complexes also bound the phosphorylated H2AX peptide. Using an antibody specific to Esa1p, the catalytic subunit of the NuA4 complex, it has been shown that NuA4 is recruited to the site of a DSB.

The INO80 complex, an ATP-dependent chromatin remodeling complex of the SWI/SNF family, is recruited to the DSB, and has been reported to aid in ssDNA formation, NHEJ, and nucleosome eviction at the break. The catalytic subunit Ino80p, as well as Arp5p and Arp8p are recruited to the site of a break within an hour of break induction, and reach 3-4 fold enrichment between 2-4 hours (Van Attikum et al., 2004; Morrison et al., 2004). The INO80 complex specifically associates with γ-H2AX through its Nhp10p subunit, and its recruitment to the break is diminished in the lack of phosphorylated H2AX. ssDNA formation is impaired in a strain lacking the Arp8p subunit of the INO80 complex (van Attikum et al., 2004).

The INO80 complex has also been shown to disrupt chromatin structure during DSB processing. Nucleosomes are evicted within an hour after DSB induction, as seen by ChIP of both H2B and H4 (Tsukuda et al., 2005). Chromatin structure is also disrupted surrounding a break over time as seen by MNase mapping. This nucleosome loss depends on the INO80 complex.

The INO80 complex is needed for checkpoint adaptation in response to a single DSB. Traditionally, a cell will arrest in the presence of DNA damage, due to the damage checkpoint response. The cell cycle will not continue until the damage is repaired and the checkpoint is turned off. In *S. cerevisiae* the phenomenon of checkpoint adaptation has been characterized. Adaptation occurs when the cell has not repaired a single DSB and yet turns off the checkpoint and resumes the cell cycle (Toczyski et al., 1997). WT cells resume the cell cycle within 16 hours of break induction, but *ino80Δ* cells die in the presence of a single break (Papamichos-

Chronakis et. al., 2006). The *ino80* mutant strains also have a decreased amount of phosphorylated H2AX as seen by western blot and ChIP, and a concomitant increase in Htz1p incorporation at the break.

The SWR1 complex is another ATP-dependent remodeling enzyme that is recruited to the break (Van Attikum at al., 2007). Recruitment increases over 4 hours and spreads approximately 10kb from the break. In the absence of γ-H2AX, recruitment of Swr1p is reduced by 75-80%. Loss of either *ino80* or *swr1* results in decreased recruitment of Yku80p to the break.

Studies in S. *cerevisiae* show that members of the SWI/SNF complex, Snf5p and Snf2p are necessary for bleomycin and HU resistance (Chai et al., 2005). They are both recruited to the break 40 minutes post break induction and continue to increase over 4 hours. Interestingly, both are required for synapsis with the homologous donor loci, and strains lacking either subunit cannot undergo the strand invasion/ extension reaction necessary for completion of HR.

In mammalian cells, SWI/SNF has been shown to be necessary for survival after IR and DSB repair (Park et al., 2006). In cells with a dominant negative Brg1, γ-H2AX foci after IR is impaired and there is reduced levels of γ-H2AX as seen by western blot, although ATM and ATR recruitment is not affected.

Finally, the RSC chromatin remodeling complex has also been characterized for its role in DNA DSB repair. *S. cerevisiae* strains lacking the Rsc1p or Rsc2p subunits are sensitive to MMS, bleomycin and HU (Chai et al., 2005). The catalytic subunit, Sth1p, is recruited to the break within 10 minutes of induction and peaks at 40 minutes. The *rsc* mutants are defective in the post-synapsis step of HR. Many studies have implicated RSC in remodeling nucleosomes at the site of a break (Shim et al., 2007; Liang et al., 2007; Kent et al., 2007). Cells lacking RSC

subunits did not restructure the nucleosomes at the DSB as compared to WT. Also, cells lacking RSC subunits were defective for γ-H2AX induction. Studies of RSC mutants showed reduced recruitment of Mec1p, Tel1p, cohesion, and RPA (Liang et al., 2007), reduced Ku and Mre11p binding at the break (Shim et al., 2007), and decreased ssDNA formation at the site of the break (Liang et al., 2007; Shim et al., 2007; Kent et al., 2007).

In summary, many chromatin remodeling complexes are recruited to a break. INO80, RSC and SWI/SNF are involved in regulating γ-H2AX formation. INO80 and RSC have been implicated in ssDNA formation at the site of a break as well as remodeling the nucleosomes in the break region. SWR1 and INO80 are involved in adaptation to the DNA damage checkpoint. All are necessary for proper recruitment of repair and checkpoint proteins. Although chromatin is a repressive structure for DSB repair, the cell has many overlapping remodeling enzymes to ensure efficient repair.

ATP-dependent remodeling enzymes are necessary at each step of the break repair process. Each stage of the damage repair process must occur within chromatin. Nucleosomes need to be mobilized to allow the repair proteins to access the broken DNA ends. They are involved in the DNA damage checkpoint signal (Papamichos-Chronakis et al., 2005). They are recruited to the region of homology to aid in the repair process. Finally, nucleosomes need to be restored to their original density and position, and ATP-dependent remodelers are involved in chromatin restoration.

The literature shows that chromatin surrounding a DSB is extremely dynamic. The contribution of the various modifying enzymes and modifications are being elucidated. In the second and third chapters I will address the histone modifying enzyme, CK2, and the phosphorylation of serine 1 of histone H4 within the dynamics of a DNA DSB.

CHAPTER II

CHARACTERIZATION OF CK2-DEPENDENT HISTONE MODIFICATIONS

Summary

CK2 was identified in a biochemical screen for histone tail kinases. CK2 is an essential enzyme which has two catalytic subunits, one of which is essential for survival. We found that it phosphorylates the H3 and H4 histone tails *in vitro*. Using yeast genetics, we show the *in vivo* characterization of an *in vitro* CK2-dependent histone H3 tail modification, S31 phosphorylation. We performed a phenotypic analysis of the deletion of a single catalytic subunit of CK2, *cka1Δ*. We also show the *in vivo* characterization of a CK2-dependent modification, the phosphorylation of serine 1 of histone H4 in response to DSBs.

The *in vitro* characterization of the H3 tail modification was begun by a former post-doc in the lab, Mike Samuels. He performed a biochemical screen of whole cell lysates from yeast to search for H3 tail kinases. The *in vivo* characterization of H4 Serine 1 phosphorylation was done in collaboration with members of the C. David Allis and Shelley Berger laboratories.

Figure 8 was used for publication in Current Biology, Volume 15, no. 7 (2005).

Introduction

CK2 is a ubiquitous serine/threonine kinase, essential in all eukaryotes. It has many known substrates, and has been implicated in cancer progression (Laramas et al., 2007), cell cycle regulation (Poole et al., 2005), and circadian rhythm maintenance (Smith et al., 2008). The necessity of the CK2 catalytic subunits has been well defined in *S. cerevisiae*. Yeast genetics has allowed the characterization of this essential kinase for each stage of the cell cycle. At least one of the two catalytic subunits (Cka1p and Cka2p) are necessary to enter S phase and complete mitosis (Hanna et al., 1995). The regulatory subunits (Ckb1p and Ckb2p) have been implicated in CK2 substrate specificity but they are not essential for cell survival or CK2 activity.

CK2 has also been implicated in different aspects of DNA DSB repair. A recent publication from the Lukas lab shows that CK2-dependent phosphorylation of MDC1 is necessary for NBS1 retention at a DSB (Melander et al., 2008). They do not, however, see any subunit of CK2 localizing at the break (personal communication). The β subunits of CK2 were found in a screen for mutants defective for adaptation to the G2/M checkpoint (Toczyski et al., 1997). A more recent study has followed up on the role of CK2 in checkpoint adaptation. CK2 phosphorylates the phosphatase, Ptc2p, which is necessary for a Ptc2p-Rad53p interaction (Guillemain et al., 2007). A strain with a mutant *ptc2* allele cannot adapt to the checkpoint and Rad53p remains active. Recently it has been reported that inhibition of CK2 diminishes γ-H2AX phosphorylation in MEFs (Ayoub et al., 2008). We have published that CK2 is responsible for phosphorylation of H4 serine 1 in *S. cerevisiae* (Cheung et al., 2005).

In this study we show that CK2 is a histone H3 and H4 kinase. We found CK2 in a screen for H3 tail kinases using biochemical fractionation of whole cell lysates from *S. cerevisiae*. We were interested in determining the relevant serine(s) or threonine(s)

phosphorylated by CK2 *in vitro*. We were also interested to see if this CK2-dependent modification is relevant *in vivo*, and redundant with H4S1phos. This study shows an *in vivo* characterization of H3S31phos, and a phenotypic analysis of a double mutant strain (H3S31A/H4S1A) to see if H3S31phos is indeed a redundant modification.

In this biochemical screen for histone tail kinases we also determined that CK2 is an H4 tail kinase. We found that CK2 is responsible for phosphorylation of serine 1 of histone H4 *in vivo* and *in vitro* (Cheung et al., 2005). *In vivo* the modification occurs in response to DNA DSBs. Phosphorylation is enriched around the break within two hours of break induction. Substitution of serine 1 with alanine in *S. cerevisiae* does not yield a phenotype. In this study we further characterized this modification *in vivo*. Histones, which are highly basic proteins, do not have the canonical consensus sequence for CK2. Because CK2 is a constitutively active kinase, we were interested in determining how CK2 recognizes H4 as a substrate. We tested whether phosphorylation needs the regulatory subunits of CK2, various members of the DNA damage checkpoint cascade, and histone chaperones for substrate recognition.

CK2 has two catalytic subunits, Cka1p and Cka2p in *S. cerevisiae*. At least one is essential for cell cycle progression. Because Cka1p is also necessary for full levels of histone H4 phosphorylation we studied the effect of deletion of *cka1* on DNA DSB resistance and repair.

Results

CK2 phosphorylates Serine 31 of histone H3 *in vitro*

In a screen for histone tail kinases we found that CK2 is an H3 kinase *in vitro*. In brief, GST-H3 tails were used as a substrate for radioactive *in vitro* kinase assays of chromatographic fractions of yeast whole cell lysate in the presence of [γ-^{32}P] ATP. Two "peaks" from a Mono Q column contained H3 kinases. Following further purification, two different kinases were identified. The kinase responsible for the first peak of activity from the column was determined via mass spec to be Ksp1p. The second chromatographic peak contained the catalytic subunits of CK2 (Figure 5).

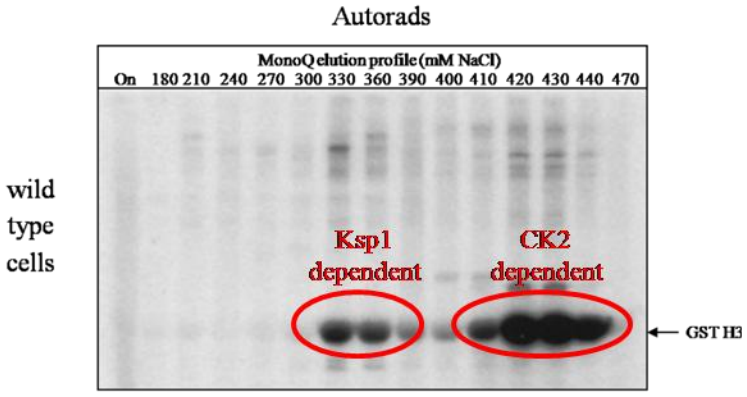

Figure 5. Two new histone tail kinases: Ksp1p and CK2. Fractionation of *S. cerevisiae* whole cell lysates in the presence of γ-[^{32}P]ATP and GST-H3 substrate yields two distinct kinase pools. The peak which elutes between 410-440mM NaCl is CK2-dependent as determined by mass spectrometry. (Courtesy of Mike Samuels)

The specificity of CK2 on GST fusions of the four histone tails showed that CK2 phosphorylates both the H3 and H4 tails. The only serine/ threonine within the H4 tail is serine 1, but the specificity of the H3 CK2 phospho-acceptor site needed to be determined. To do so, three different GST-H3 tail fusions were used as substrates in an *in vitro* kinase assay: one with amino acids 1-45, another with amino acids 1-25, and a third with amino acids 23-45. These

GST fusion proteins were subjected to *in vitro* kinase assays using TAP-purified CK2 from *S. cerevisiae* in the presence of radiolabeled [γ-^{32}P] ATP. Our results show that only the full length GST fusion is phosphorylated, while the other two were not recognized as substrates (Figure 6a).

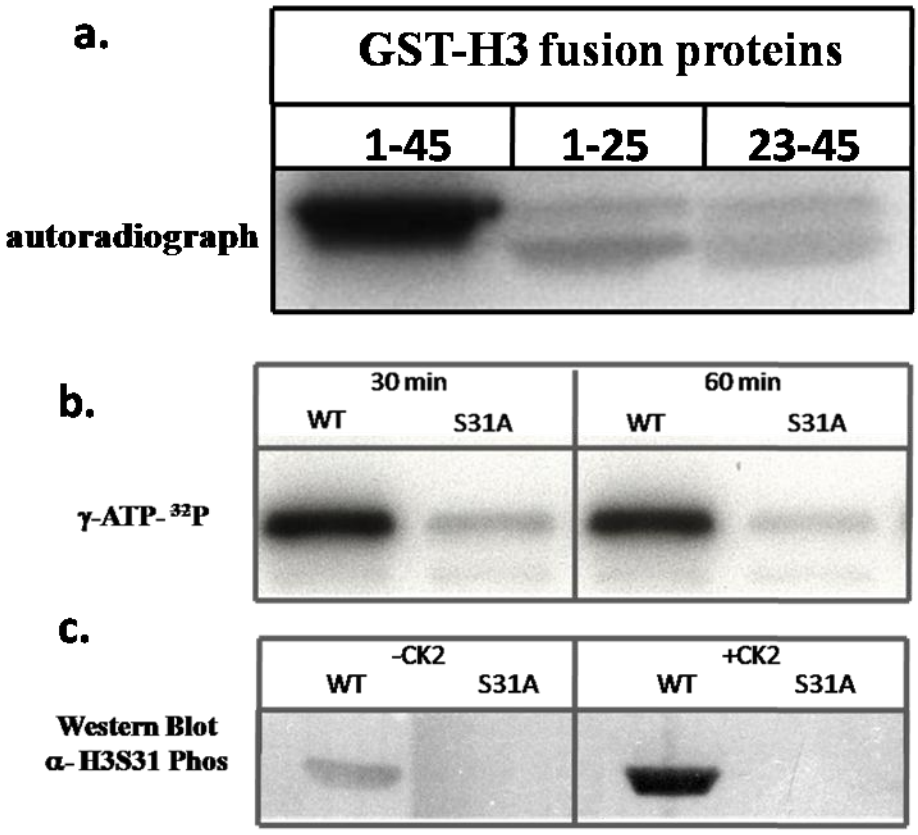

Figure 6. CK2 phosphorylates serine 31 of histone H3. a. An *in vitro* kinase assay using different GST-H3 tails shows that CK2 phosphorylates the full-length H3 tail but not the truncated versions. **b.** Results of an *in vitro* kinase assay show that substitution of serine 31 with alanine eliminates the CK2-dependent phosphorylation. **c.** Western blot with an antibody specific for H3S31phos confirms that CK2 phosphorylates S31 *in vitro*.

The first 45 amino acids of H3 contain 9 serine or threonine residues. In order to map the relevant residue(s) we focused on the following amino acids: S22, S28, S31 and T32. These residues are located close to the area of overlap between the two truncated versions of the GST

fusions. Each individual serine or threonine was mutated to an alanine. Then each mutated GST fusion protein was subjected to an *in vitro* kinase assay to see which mutated residue would result in loss or diminished phosphorylation by CK2. Mutation of serine 31 to alanine resulted in a loss of CK2-dependent phosphorylation (Figure 6b). To confirm that H3S31 is phosphorylated by CK2, western blots using an antibody specific for H3S31-phsophorylation was performed on an *in vitro* kinase assay using WT and H3S31A GST-fusions as substrates. In the presence of CK2, S31 was phosphorylated, while the S31A mutation was not recognized by the antibody (Figure 6c).

After determining the substrate of CK2 *in vitro* we wanted to test if this modification was generated by CK2 *in vivo*. H3S31phosphorylation has been previously characterized by the Allis lab (Hake et al., 2005). They showed that H3S31 phosphorylation is enriched during mitosis. Using the antibody to H3S31phos we probed cells with and without treatment with MMS, to see if S31phos is induced in response to DSBs similar to H4S1phos. H3S31 phosphorylation appears to be a constitutive modification in an asynchronous population (Figure 7a). H3S31 phosphorylation also does not appear to need CK2 as levels are similar in a strain in which a single subunit is deleted, and the second contains a temperature sensitive mutation (Figure 7b).

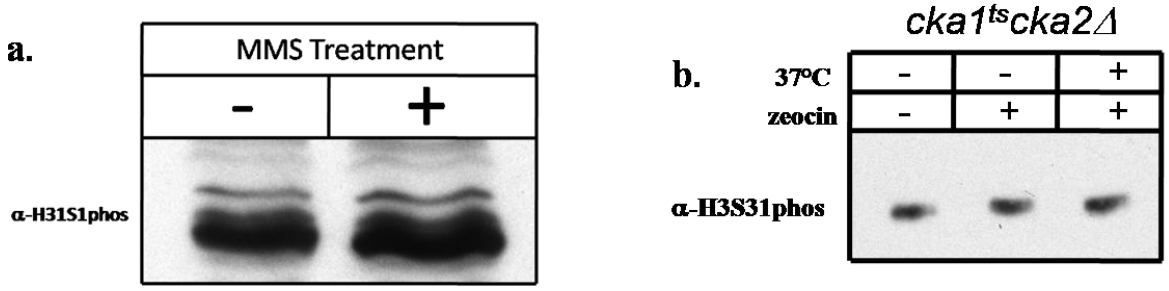

Figure 7. H3S31phos is neither damage inducible nor CK2-dependent. a. Western blot of treated and untreated asynchronous WT cells do not show a change in levels of H3S31

phosphorylation. **b.** Western blot using H3S31phos specific antibody shows that at the restrictive temperature, which disrupts CK2 activity, H3S31 is still phosphorylated.

CK2 phosphorylates H4S1 *in vitro* and *in vivo*

Work done in collaboration with Shelley Berger's and C. David Allis' laboratories also showed that H4S1 is phosphorylated by CK2 *in vivo* and *in vitro*. The discovery of CK2 as a histone kinase is an interesting phenomenon. The minimal consensus sequence of CK2 (Ser-Xaa-Xaa- Acidic, where the acidic residue may be Glu, Asp, pSer or pTyr) (Litchfield, 2003) consists of at least one, and traditionally more than one acidic residues, and the basic histones are an unusual substrate for this kinase.

H4S1 phosphorylation is induced in response to DNA DSBs. The modification is distributed around a DSB, as seen by ChIP at the *MAT* locus in the GAL::HO donorless strain, peaking two hours after break induction. A strain in which the sole copy of histone H4 has serine 1 mutated to alanine does not show any sensitivity to DSB inducing agents.

H4S1phos requires CK2 *in vivo*. In a strain in which *cka1* is deleted and *cka2* contains a temperature sensitive mutant, H4S1 phosphorylation is abolished at the restrictive temperature. Deletion of even the single subunit *cka1* greatly reduces H4S1phos levels. CK2 also phosphorylates H4S1 *in vitro*. Using TAP purified CK2 and recombinant yeast histone H4, we showed by western blot, with an antibody specific for H4S1phos, that CK2 could generate the epitope in an *in vitro* kinase assay (Figure 8). It was also shown that recombinant CK2 could phosphorylate chicken H4 when the histone octamer was used as the substrate. CK2 can not recognize a nucleosomal array as a substrate in an *in vitro* kinase assay.

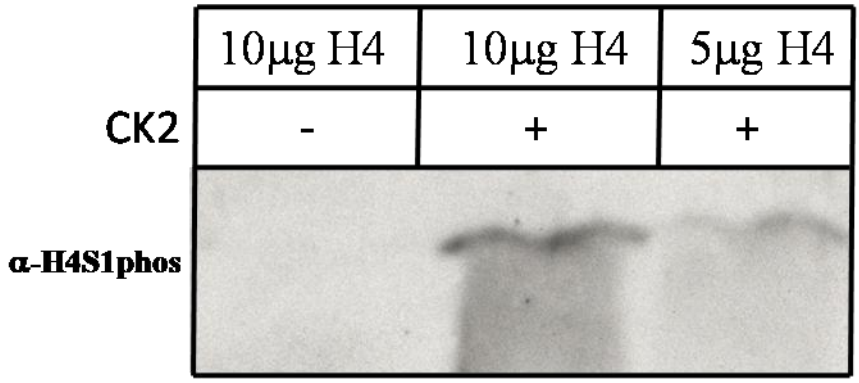

Figure 8. CK2 phosphorylates H4S1 of recombinant yeast H4. Western blot of an *in vitro* kinase assay using an antibody to H4S1phos shows that CK2 phosphorylates serine 1 of recombinant yeast histone H4.

The DNA damage checkpoint is dispensable for H4S1phos

The *in vivo* data showing that CK2 is responsible for the damage inducible modification of H4 begs the question as to how CK2, a constitutively active enzyme, recognizes H4 as a substrate, only within the context of DSBs. Phosphorylation of H2AX at the DSB requires the checkpoint kinases Mec1p and Tel1p in yeast. An intact DNA damage checkpoint is required for full induction of γ-H2AX. This is not the case for H4 serine 1 phosphorylation. Both *mec1* and *tel1* can be deleted without a loss of H4S1phos (Cheung et al., 2005). The same is true for downstream members of the checkpoint cascade. H4S1 is induced in both *rad9* and *rad17* deletion strains (Figure 9). Work from Jacques Cote's lab also showed that H4S1 phosphorylation does not need Dun1p, Chk1p, or Rad53p (Utley et al., 2005).

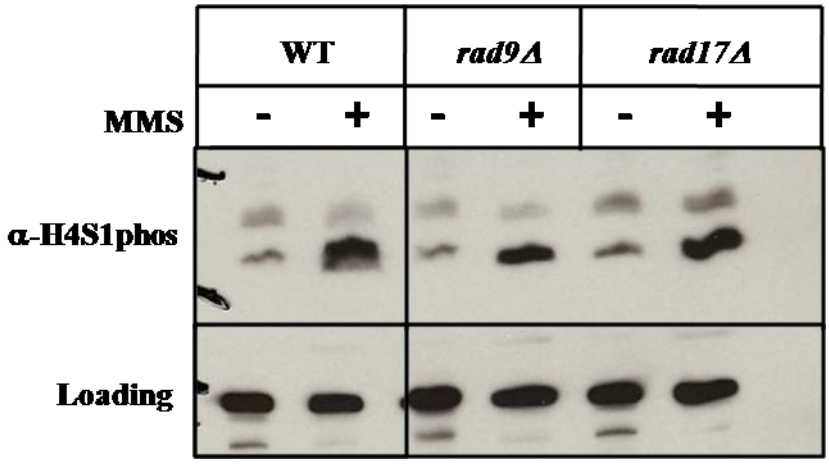

Figure 9. *RAD9* and *RAD17* are not necessary for H4S1phos. Western blot of whole cell extracts from strains lacking either Rad9p or Rad17p before and after treatment with MMS shows that disruption of the DNA damage checkpoint does not diminish H4S1phos.

The regulatory (β) subunits of CK2 are dispensable for H4S1phos *in vivo*

The CK2 holoenzyme complex consists of two catalytic (α) subunits and two regulatory (β) subunits. The catalytic subunits are constitutively active. The regulatory subunits have roles in both inhibiting CK2 activity towards select substrates while enhancing phosphorylation of others (reviewed in Bibby and Litchfield, 2005). In *S. cerevisiae* the regulatory subunits are Ckb1p and Ckb2p. Neither is necessary for cell survival. We were interested to see if these regulatory subunits would aid recognition of H4S1 in the presence of DNA DSBs. In a strain in which both *ckb1* and *ckb2* have been deleted, H4S1 is still phosphorylated in the presence of MMS, signifying that the regulatory subunits are dispensable for substrate recognition (Figure 10).

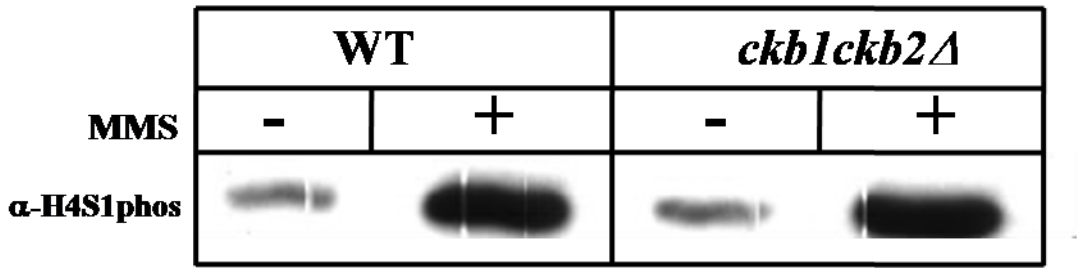

Figure 10. The regulatory β subunits of CK2 are dispensable for H4S1phos. Western blot of whole cell extracts from WT and *ckb1ckb2Δ* strains before and after MMS treatment show that in a *ckb1ckb2Δ* strain H4S1phos is still induced in response to DNA DSBs.

H4S1phos is not a modification of newly synthesized histones

The first report of H4S1 phosphorylation was in a paper by the Allfrey group in 1975 (Ruiz-Carrillo et al., 1975). They reported that H4S1phos is a modification of newly synthesized histones in avian erythrocytes. H4 is phosphorylated in the cytoplasm, shuttled into the nucleus, and is dephosphorylated after incorporation into chromatin. We wanted to elucidate if H4S1phos was a modification of newly synthesized histones in *S. cerevisiae* as well.

In cells that have been arrested in G1 using α-factor and released into the cell cycle, no cell cycle dependent increase of H4S1phos is seen (Cheung et al., 2003). Cells arrested in different stages of the cell cycle do not show an increase in H4S1phos unless treated with zeocin, a DSB inducing agent, except for those arrested in S phase using 200mM HU (Figure 11a).

Treatment with 200mM HU has been speculated to induce DNA DSBs (Lundin et al., 2002), so this HU-dependent increase of H4S1phos may not be due to an accumulation of newly-synthesized histones during an S-phase arrest but to DSBs. To address this we looked at the contribution of histone chaperones on the accumulation of phosphorylation. Histone chaperones bind histones in the cytoplasm after synthesis and are responsible to shuttle them into the nucleus and allow for proper incorporation into chromatin.

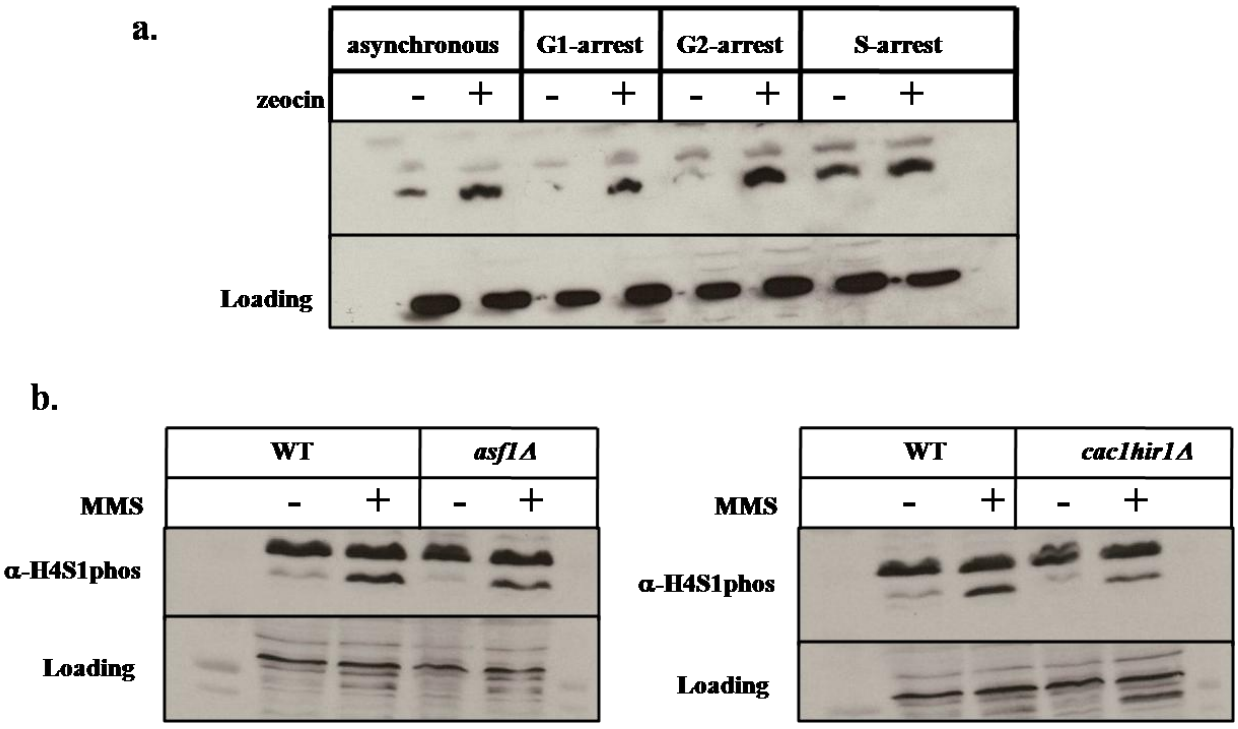

Figure 11. H4S1phos is not a modification of newly synthesized histones. **a.** Cells arrested in G1 with α-factor, or G2 with nocodozole do not show an increase in H4S1phos unless treated with zeocin, as seen by western blot of whole cell lysates. Cells arrested in S-phase with HU show an increase in H4S1phos, most likely due to DSBs created by high HU treatment. **b.** Western blot of cells lacking Cac1p and Hir1p or Asf1p show that H4S1phos does not need these histone chaperone complex subunits.

In brief, *S. cerevisiae* has three main histone chaperone complexes. CAF-1, which is composed of Cac1p, Cac2p, and Cac3p, interacts with the PCNA during replication to ensure proper coordination between DNA replication and nucleosome formation as well as epigenetic inheritance (Zhang et al., 2000). The HIR complex is a chaperone complex which consists of Hir1p, Hir2p, Hir3p and Hpc2p. The HIR complex is responsible for histone replacement outside of S phase (reviewed in Loyola and Almouzni, 2004). RCAF is a complex of the histone chaperone Asf1p and histones H3 and H4 (Tyler et al., 1999). Asf1p interacts with both CAF-1 and the HIR complex to coordinate proper histone deposition throughout the cell cycle.

Asf1p was also shown to interact with Rad53p during an unperturbed cell cycle, but is released during DNA damage checkpoint activation (Emili et al., 2001). It is through this interaction that the histone chaperones have also been implicated as part of the checkpoint response.

Strains in which one of the three histone chaperone complexes have been inactivated are still viable. Therefore we were able to see if the histone chaperone complexes contributed to H4S1phos. In a *cac1hir1Δ* strain, H4S1phos was not compromised when treated with zeocin (Figure 11b). There was no increase in H4S1phos without treatment in this strain. Therefore, we conclude that unlike in higher eukaryotes, H4S1phos is not a modification of newly synthesized histones and the chaperones are dispensable for phosphorylation.

The H4S1A containing strain does not have a defect in checkpoint downregulation

It had been previously reported that the β subunits of CK2 are necessary for adaptation to the DNA damage checkpoint. We wanted to test the hypothesis that it could be affecting the downregulation of the checkpoint through phosphorylation of H4S1. To test this hypothesis, we monitored the phosphorylation status of Rad53p after induction of DSBs using an *in situ* kinase assay (Pellicioli et al., 1999). Cells were treated with a low dose of zeocin for an hour at 30°C, washed, and returned to rich media. Phosphorylation of Rad53p was monitored for 18 hours. If cells fail to turn off the checkpoint, Rad53p remains phosphorylated indefinitely until the cell dies. As seen in Figure 12, both WT and H4S1A are able to turn off the checkpoint within 18 hours of zeocin treatment.

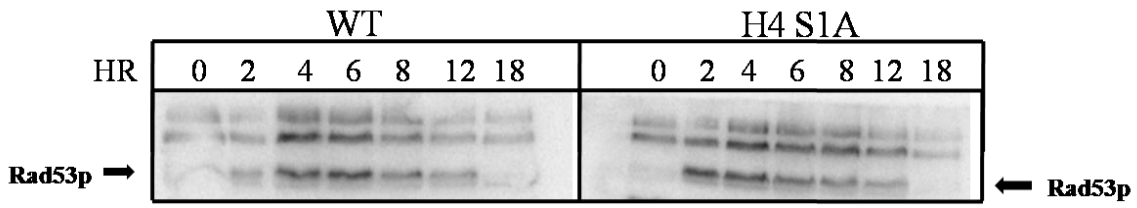

Figure 12. The H4S1A mutant can turn off the DNA damage checkpoint. An *in situ* kinase assay of Rad53p kinase activity shows that both WT and an H4S1A strain turn off the DNA damage checkpoint by 18 hours post treatment.

The double H3S31A/H4S1A mutant does not have a DNA damage phenotype

Because H3S31phos and H4S1phos are CK2-dependent modifications *in vitro*, we were interested in determining whether these were redundant modifications *in vivo*. Alone, mutation of H4S1A does not yield a phenotype in response to DSBs. We wanted to create a strain for phenotypic analysis in which both serines have been substituted with alanine.

There are two chromosomal loci for each of the four replicative histones in *S. cerevisiae*. We used a strain in which both copies of H3 and H4 have been deleted and replaced by plasmid-borne copies of H3 and H4. On this plasmid both the H4S1A and H3S31A mutations can be made and "shuffled" back into the strain containing the WT copy. Using auxotrophic selection markers, a colony can be selected in which the plasmid containing the WT histones have been lost and the sole source of H3 and H4 for the cell is the mutant versions.

Testing the double H3/H4 mutant on various DNA damaging and stress agents did not yield a phenotype. Cells were grown on zeocin, HU, MMS, hydrogen peroxide, and at high temperature (37°). No growth defects were seen for any treatment (Figure 13).

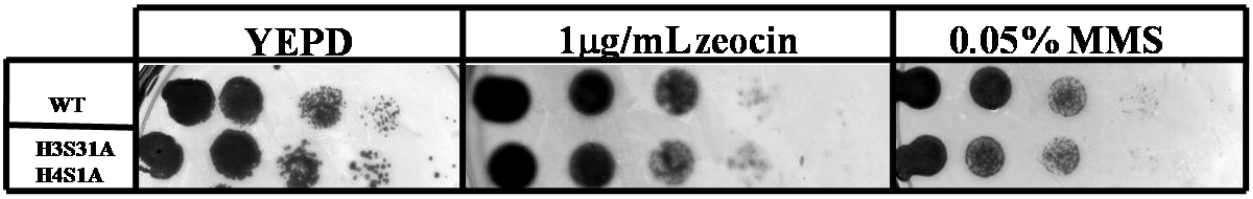

Figure 13. The double histone mutant does not show a DNA damage phenotype. Ten- fold serial dilutions of WT and the double mutant H3S31A/H4S1A strain in the presence of various DNA damaging agents show that the mutant strain grows as well as WT.

Deletion of a single catalytic subunit of CK2 has a mild DNA damage phenotype

The bulk of the H4S1phos can be depleted when a single CK2 subunit, Cka1p, is deleted. To test if a redundant CK2-dependent modification may have been overlooked, we deleted *cka1* to test for a DNA damage phenotype. The only observable phenotype was a mild sensitivity to zeocin, which specifically induces DNA DSBs. There was no sensitivity to HU or MMS.

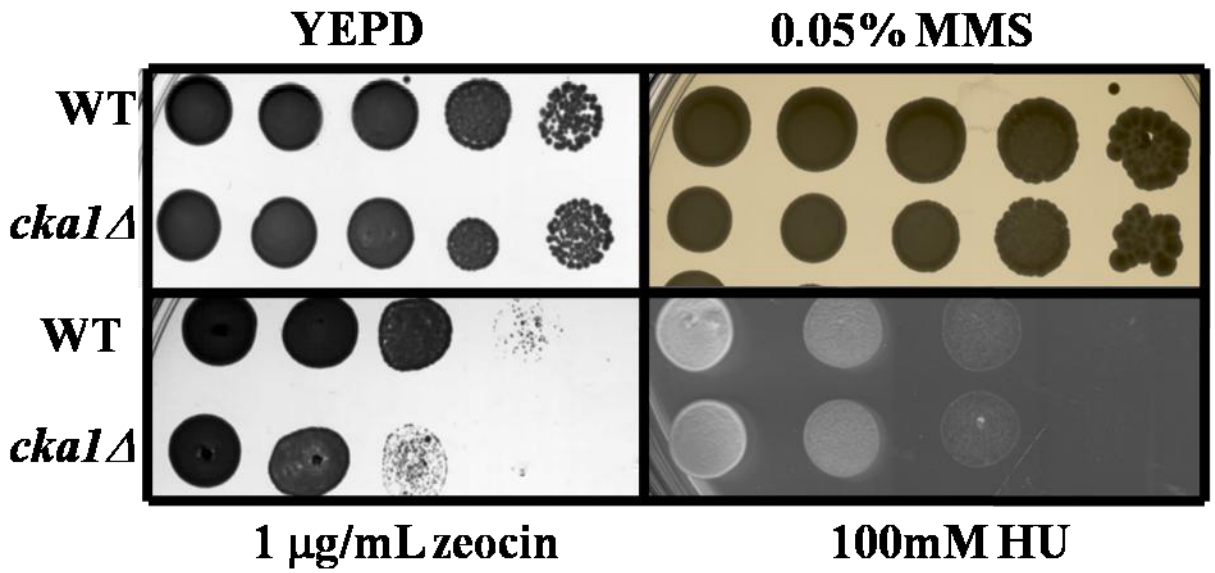

Figure 14. A *cka1Δ* strain has a subtle DNA damage phenotype. Ten-fold serial dilutions of WT and *cka1Δ* strains in the presence of various DNA damaging agents shows that the *cka1Δ* strain can grow similarly to the WT strain, except in the presence of zeocin.

We also looked at the kinetics of DSB repair using a GAL::HO containing strain in which the donor loci are still intact. When the Z region of the DSB invades the homologous donor, DNA synthesis, which is primed from the invading 3' strand, occurs in the process of gene conversion. This strand invasion/ extension (SIE) step can be captured by PCR using a primer which hybridizes at the donor region and one just outside of the *MAT* locus (Figure 15a). The kinetics of switching can be monitored by adding galactose to induce the break and monitoring the formation of the SIE product by PCR. The single subunit deletion of CK2 did not have a delay in the SIE reaction compared with WT CK2 (Figure 15b).

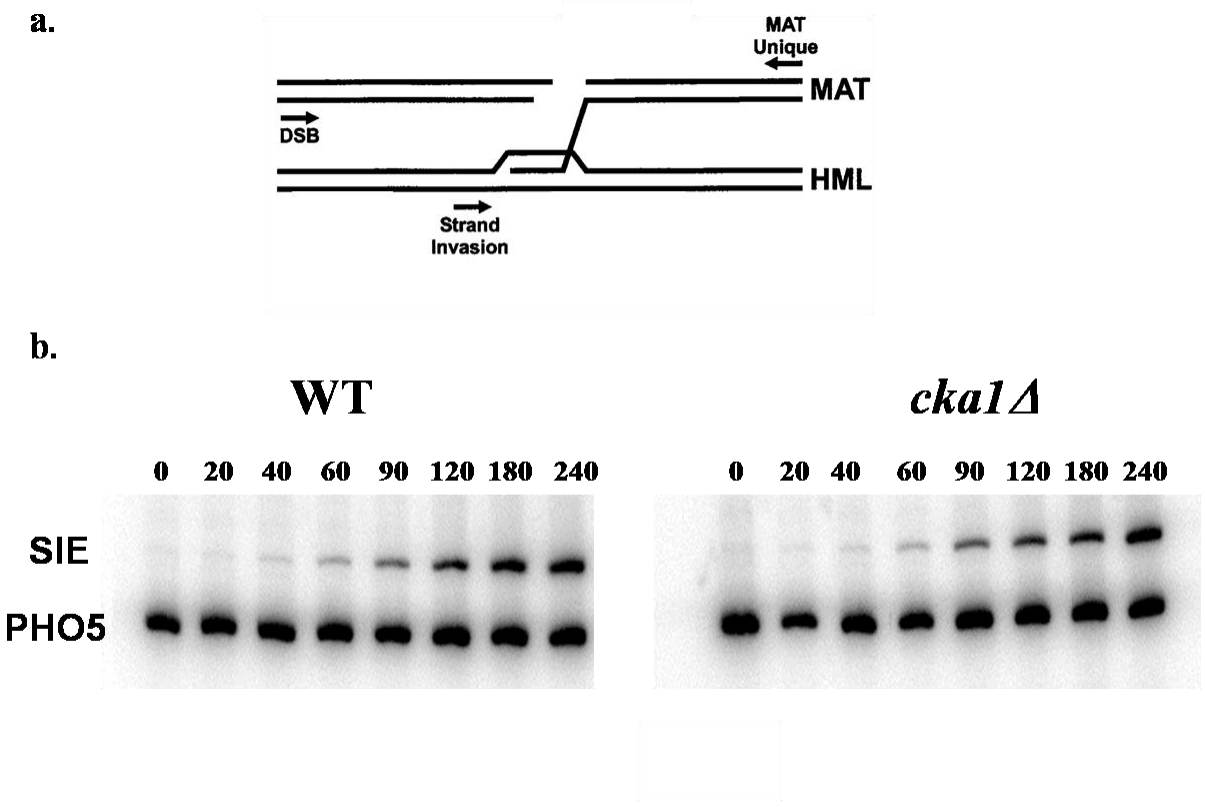

Figure 15. Deletion of a single CK2 subunit does not lead to a delay in SIE. a. Schemata of primers used to capture strand-invasion kinetics adapted from Papamichos-Chronakis et al., 2006. **b.** The results of the strand-invasion/ extension assay show that in both WT and *cka1Δ* strains SIE occurs at around 40 minutes and peaks at 240 minutes.

We also looked to see if there is a defect in NHEJ in the absence of Cka1p. Using the GAL::HO strain in which the donors are deleted, the only way for the cell to survive in the presence of a DSB at the *MAT* locus is for the two ends of the break to repair via the NHEJ pathway. To test the efficiency of NHEJ, cells were grown in galactose for one hour to induce a break and were then spotted on glucose containing plates, shutting off HO expression. Only cells which are proficient for NHEJ will be able to grow on glucose containing plates. *cka1Δ* mutants did not show a defect in this assay (Figure 16a).

We also looked to see if the *cka1Δ* cells were more likely to repair the break via an error-prone NHEJ pathway. The GAL::HO donorless strains are spotted on plates containing galactose as the sole carbon source. Therefore, HO will be expressed constitutively. The only way the cells can survive on these plates is to repair the break via mutagenic end joining, causing the cell to lose the HO recognition site. We found that there was no phenotype for mutagenic

end joining (Figure 16b).

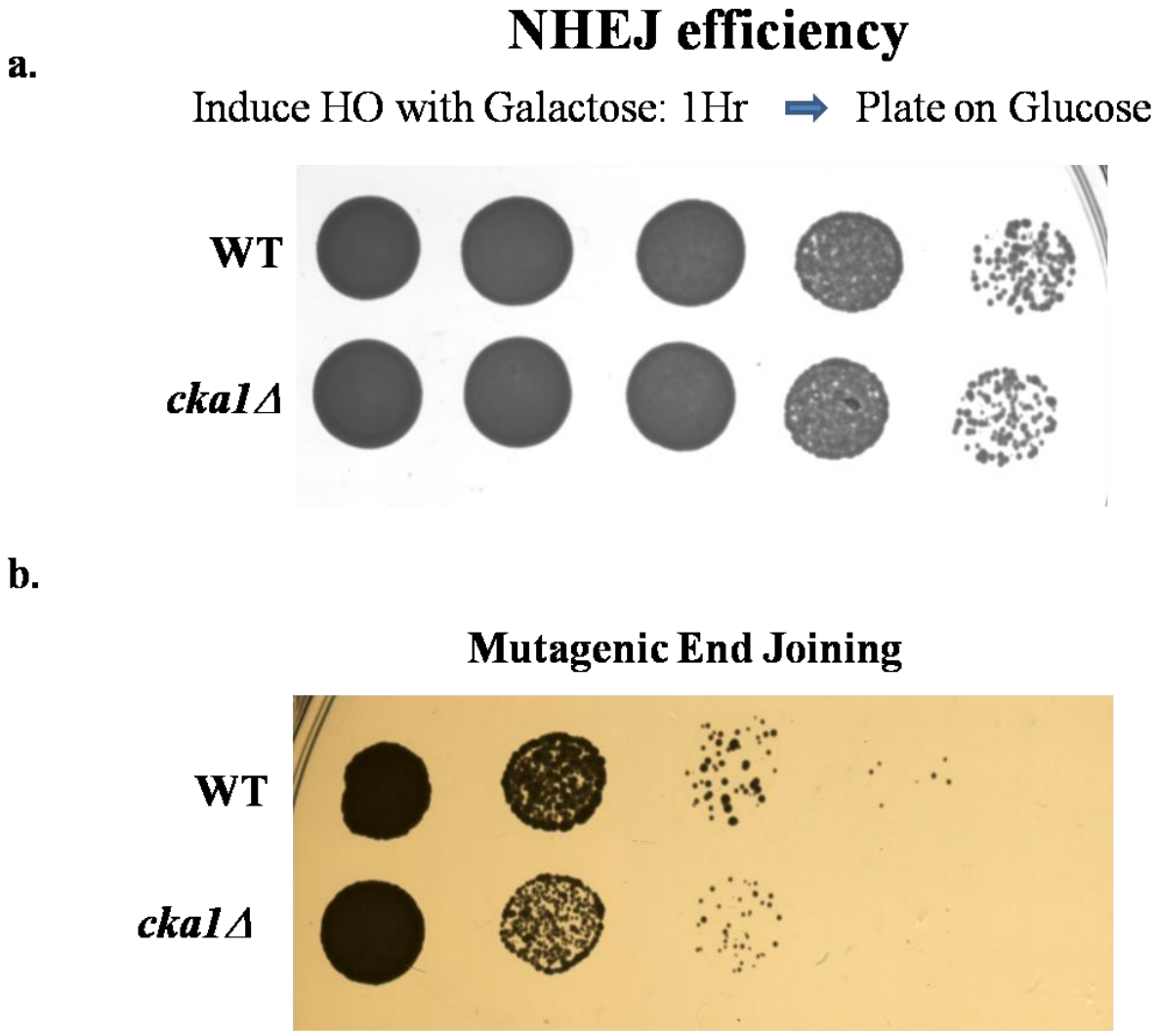

Figure 16. *cka1*Δ cells do not have a defect in NHEJ. a. Ten-fold serial dilutions of WT and *cka1Δ* strains on glucose containing plates after DSB induction show that *cka1Δ* cells are able to repair a single DSB with similar efficiency as WT. **b.** *cka1Δ* cells do not have a phenotype for mutagenic end joining.

Discussion

CK2 phosphorylates both the H3 and H4 N-terminal tails. We have determined the phospho-acceptor site of both tails *in vitro*. CK2 phosphorylates S31 of histone H3, and S1 of histone H4.

The replicative histone H3 in *S. cerevisiae* resembles the H3.3 variant of higher eukaryotes. H3.3 in mammals differs from H3.1 (the main replicative H3 variant) by five amino acids, one of which is Serine 31 (alanine in H3.1). In mammalian cells, H3.3 and H3.1 are deposited by different histone chaperones (Tagami et al., 2004), and H3.3 is synthesized throughout the cell cycle and deposited into chromatin around regions of active transcription (Ahmad and Henikoff, 2002). In HeLa cells, H3.3 Serine 31 is phosphorylated in mitosis and is localized to the outside of the centromeres (Hake et al., 2005).

In *S. cerevisiae,* H3S31 is phosphorylated in asynchronous cells as seen by western blot. The H3 in *S. cerevisiae* differs from the replication-independent H3.3 variant of mammals in that it is expressed only during the S phase of the cell cycle. In asynchronous cells H3S31phos is present even without treatment with a DNA damaging agent. We also see that this modification is not CK2-dependent *in vivo*. The kinase responsible for this modification in HeLa cells was not reported.

It is surprising that loss of a single CK2 catalytic subunit does not have a more dramatic phenotype in the presence of DNA damaging agents. We found that deletion of *cka1* showed a very slight phenotype in the presence of zeocin but not with MMS or HU. Deletion of a single subunit also did not show any defect on SIE or NHEJ. Furthermore, the double H3/H4 serine mutant does not have a DNA damage phenotype.

The specificity of CK2-dependent phosphorylation of H4 provides an interesting question. *In vitro*, CK2 phosphorylates recombinant yeast H4, a yeast GST-H4 tail, and recombinant yeast octamer. It does not, however, recognize a chromatin array reconstituted with Xenopus histone octamer. This evidence would lead to the hypothesis that CK2 phosphorylates H4 before it is incorporated into chromatin which agrees with the report from the Allfrey lab that

H4S1phos is a modification of newly synthesized histones. Surprisingly, cells arrested in G1 with α-factor and released to proceed through the cell cycle synchronously do not show a cell cycle dependent increase in phosphorylation. Cells arrested in S phase with 0.2M HU show an increase, but it is possible that the HU treatment itself caused the increase in phosphorylation, possibly by causing DSBs at stalled replication forks.

Another hypothesis as to how CK2 could recognize H4 after synthesis is the association of new histones with histone chaperones. There is very little, if any, free histone protein within a cell. It is either incorporated into chromatin or is sequestered from binding DNA non-specifically by one of various histone chaperones. Possibly CK2 recognizes the histone chaperone/ H4 complex as a substrate. Our data show that this is not the case. In strains in which three major histone chaperone complexes have been deleted, H4S1phos is still induced in response to DSBs.

Materials and Methods

Strains and plasmids. CY1067 was a gift from the Allis lab (Cheung et al., 2005) JHY86 [*MATα ura3-52 leu2-3,112 trp1-289 his3Δ1 Δ(hht1 hhf1) Δ(hht2 hhf2)* pJH18 (*CEN ARS TRP1 HHT2 HHF2*)] This strain has both copies of H3 and H4 deleted and rescued with WT copies of each on a plasmid. To test the phenotype of the double H3S31A/H4S1A this strain was used and shuffled with pTK54 (*hhf2 S1A-hht2 S31A CEN-ARS1 LEU*). CY915, the donorless GAL::HO strain, was a gift from J. Haber, and described in Moore and Haber 1996 (JKM179 *Δho Δhml::ADE1 MATα Δhmr::ADE1 ade1-110 leu2,3-112 lys5 trp1::hisG ura3-52 ade3::GAL10:HO)*. The "switching" strain, CY924, is isogenic to JKM179 except that it is *MAT***a with *HML* and *HMR***. *cka1* was deleted in CY924 using the *E. coli* kan^r gene as described in Longtine et al., 1998. The strain containing the double CK2 catalytic subunit deletions (*ckb1ckb2*Δ) was a gift from C. Glover and was described in Bidwai et al., 1995 (*MATa ade2-101, his3-Δ200, leu2-801, ura3-52, trp1-Δ63, ckb1Δ::HIS3, ckb2Δ::LEU2*). The *rad9*Δ and *rad17*Δ strains were purchased from Open Biosystems (BY4741 *MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*). The mutant histone chaperone strains were a gift from P. Kaufman (PKY971 *MATa, leu2-3; ura3-1; his3-11,15; trp1-1; ade2-1; can1-100; (*either *caclΔ::LEU2; hirlΔ::HIS3;* or *asf1Δ::TRP1*). The plasmids used for expression of GST-H3 and GST-H4 N-terminal tails (a gift from M. Grunstein) are derivatives of pGEX2T, and were described in Hecht et al., 1995.

CK2 purification. *CKA1* was C-terminally tagged, in frame, at the endogenous locus with the "tandem affinity purification" TAP tag. Six liters of the *TAP-CKA1* strain are grown in YEPD to an OD_{600} of approximately 1.0. Cells are harvested and lysed using a mortar and pestle, keeping cells frozen with liquid nitrogen. Pulverized yeast extracts were resuspended in E Buffer at 4°C

(20mM HEPES pH 7.4, 350mM NaCl, 10% glycerol (v/v), 0.1% Tween (v/v) and protease inhibitors). Lysates were clarified by a high speed spin, 40,000 RPM at 4°C for 1 hour. Cleared lysates were incubated with IgG agarose beads for 2 hours at 4°C with shaking. The bound protein was incubated with TEV protease overnight to cleave off the IgG resin. The eluted protein was incubated with Calmodulin Resin in E Buffer containing 2mM $CaCl_2$. Purified CK2 was eluted from the calmodulin resin with E Buffer containing 10mM EGTA. The complex was concentrated and aliquoted for storage at -80°C.

Expression of GST-H3. The pGEX2T plasmid containing the GST-H3 tail was transformed into BL21(DE3) *E. coli*. A single colony of the plasmid-containing *E. coli* was used to inoculate a 50 mL LB-AMP culture and grown overnight at 37°C. The entire 50mL culture was used to inoculate 500mL LB-AMP for 1 hour and expression was induced by adding 0.2mM IPTG for 5 hours. Cells were harvested and resuspended in NETN Buffer (20mM Tris-HCL pH8.0, 100mM NaCl, 1mM EDTA, 0.5% NP-40 (v/v)) and sonicated at 4°C. The lysate was cleared with a 13,000 RPM spin at 4°C. Glutathione-sepharose beads were added to the cleared lysate and rocked overnight at 4°C. Beads were pelleted by centrifugation, washed with NETN, and protein was eluted with 10mM glutathione. Glycerol was added to 20% and aliquots were stored at -80°C.

GST-H3 mutagenesis. Various GST-H3 mutants (either S22, S28, S31 or T32 to A) were created using the QuickChange Mutagenesis (Stratagene) protocol. In brief, complementary primers are designed which contain the site-specific mutation. PCR using the complementary primers is performed using a high-fidelity polymerase. The completed reaction is incubated with

DpnI to digest the methylated parent template plasmid. Then the reaction is used to transform ultracompeent *E. coli*. Single colonies are selected. Plasmids are purified and sequenced to confirm correct mutagenesis.

***In vitro* kinase assays.** Kinase assays were performed at 30°C for 30 minutes. CK2 purified via Tandem Affinity Purification was added to the 25μL reaction containing kinase buffer (25mM HEPES pH7.4, 10mM $MgCl_2$, 1μmM ATP, 1mM DTT, and 10μCu [γ-^{32}P] ATP (New England Nuclear)) and 5μg GST-H3 substrate. Samples were boiled in 2x SDS sample loading buffer and run on 18% SDS PAGE. The gels were dried and exposed using a phosphoimaging screen. The kinase reactions with recombinant H4 were done similarly using 5 or 10 μg recombinant protein.

Western Blots. For the western blots of H3S31phos: kinase reactions of GST-H3 were performed as described without 10μCu [γ-^{32}P] ATP. After SDS-PAGE, the gel was transferred to PVDF membrane. The membrane was blocked using TBST-5% milk. The membrane was washed and incubated overnight in 1:5000 dilution of H3S31phos antibody (Upstate). The membrane was again washed, incubated in 1:5000 anti-rabbit IgG for 1 hour and exposed using chemiluminescence. For the H4S1phos or H3S31phos western blots of treated cells: Cells at an OD_{600} of approximately 0.5 were treated with 0.1% MMS for two hours. An equal amount of cells, before and after treatment, were harvested, and resuspended in 100μL of water and 100μL of 0.2N NaOH and incubated at room temperature for 5 minutes. The samples were spun at high speed in a table top centrifuge for five minutes. The supernatant is aspirated and the pellet

resuspended in 1xSDS sample buffer and boiled for three minutes. The sample is spun at top speed in a table top centrifuge. The supernatant is saved and the pellet is discarded. Ten microliters per sample were loaded onto 18% SDS-PAGE. The western proceeds as described, using a 1:5000 dilution of the appropriate primary antibody. For loading controls with the H4S1phos antibody a non-specific band was used to show equal loading.

Spotting assays. Cells were grown overnight in YEPD to stationary phase and equal amounts of cells were collected. Ten fold dilutions were made in sterile water. Five microliters of each was spotted onto YEPD plates supplemented with 0.05% MMS, 1μg/mL zeocin, or 100mM HU. Cells were grown for two days at 30°C.

Cell cycle arrest: All cells were grown to an OD_{600} of approximately 0.5 in YEPD. For cells arrested in G1, α-factor was added to a final concentration of 100μM for two hours. Cells arrested in S phase were treated with 0.2M HU for two hours. Cells arrested in G2 were treated with 2.5μg/mL nocodozole for two hours.

Strand Invasion Extension Assay. Cells were grown overnight in YEP 2% raffinose to an OD_{600} of approximately 0.5. Galactose was added to a final concentration of 2%. The same amount of cells was collected at each time point, and genomic DNA was extracted and used for PCR. PHO5 was used as a loading control.

NHEJ assays. To test for mutagenic end joining, cells were grown overnight in YEP-2% raffinose and spotted onto YEP-2% galactose plates. To test for NHEJ, cells were grown

overnight in YEP-2% raffinose, galactose was added for 1 hour to induce a DSB, and cells were spotted onto YEPD plates.

***In situ* kinase assay.** 100mL culture was grown overnight to an OD_{600} of approximately 0.5. 5μg/mL zeocin was added to the culture for 1hr. Cells were spun down and washed with YEPD at 30°C. Cells were spun down and resuspended in 100mL YEPD and incubated at 30°C. Samples were taken at specific time points over 18 hours. Protein was extracted with trichloroacetic acid and resuspended in 1xSDS sample buffer. Samples were run on 10% SDS-PAGE and transferred to PVDF. Membranes were denatured for 1hr at room temperature in Denaturing Buffer (7M guanidine, 50mM DTT, 2mM EDTA, 50mM Tris pH 8.0) followed by two five minute washes in TBS. Membranes were renatured at 4°C for 12-18 hours with at least 4 buffer changes in Renaturing Buffer (2mM DTT, 2mM EDTA, 0.04% Tween, 10mM Tris pH 7.5, 140mM NaCl, 1% bovine serum albumin). The membrane was washed in 30mM Tris pH 7.5 for one hour at room temperature. The membrane was equilibrated in Kinase Buffer (1mM DTT, 0.1mM EGTA, 20mM $MgCl_2$, 40mM HEPES pH 8.0, 100μM sodium orthovanadate) for 30 minutes at room temperature. The membrane was then incubated in Kinase Buffer supplemented with 10μCi/mL [P^{32}]γ-ATP for one hour at room temperature. The membrane was subjected to high stringency washes to reduce background, air dried, and exposed overnight on film.

CHAPTER III

PHOSPHORYLATION OF SERINE 1 OF H4 AT THE SITE OF A DNA DSB REQUIRES THE SIN3/RPD3 HISTONE DEACETYLASE COMPLEX

Summary

After further *in vivo* characterization of the phenotype of H4S1phos, we were interested in determining how H4S1phos was recruited to the site of a break. We focused on the timing of the modification and the interaction of CK2 with a histone modifying complex recruited to the break with similar timing. We use ChIP, yeast genetics, and *in vitro* biochemistry to narrow down two possible models of H4S1phos localization and to determine the necessity of the SIN3/RPD3 complex for the localization of H4S1phos to a break.

Introduction

After further characterization of H4S1phos *in vivo* we were still left with a major unanswered question: How does H4S1phos get to the site of a DNA DSB? We came up with two possible models:

1. Based on the previous report that H4S1phos is a cytoplasmic modification (Ruiz-Carillo et al., 1975), we hypothesized H4 is phosphorylated in the cytoplasm and incorporated into chromatin at the site of a break.

2. In a more straightforward model, we hypothesized that CK2, or a CK2-containing complex, is recruited to the site of the break and directly phosphorylates H4 within a chromatin context.

To address these two models we needed to consider the characteristics of the responsible kinase. CK2 is located both in the nucleus and the cytoplasm. It has been reported to interact with many different chromatin-related proteins. Through genome-wide affinity-capture studies, CK2 was shown to interact with histones H3 and H2A (Krogan et al., 2006). CK2 was also found to interact with members of FACT, a complex reported to stimulate RNAPII transcription through a chromatin template (Krogan et al., 2002). Another genome-wide affinity-capture screen reports that *CKA1* interacts with the HDAC complex SIN3/RPD3 (Gavin et al., 2002). We needed to tease out how CK2 recognizes H4 at the DSB when it is known to be constitutively active and to interact with so many different proteins.

Also, the timing of the increase in phosphorylation at the break must be taken into consideration. Both H2AX phosphorylation and H4 acetylation are reported to increase within minutes surrounding a DSB (Downs, et al., 2004). Members of the NuA4 complex are shown to

be recruited to the break at the initial stages of DSB recognition. However, in a report of acetylation status at the site of a break, acetylation decreases concomitantly with recruitment of various HDAC complexes, which also correlate with the timing of H4S1phos localization (Tamburini et al., 2005).

In this chapter I will focus on a model of H4S1phos localization to a DSB. We believe that CK2 is recruited to a break due to its interaction with the SIN3/RPD3 complex. We believe that CK2 directly phosphorylates H4 within chromatin. We propose a model in which acetylation and phosphorylation at the site of the break are opposing modifications which allow for restoration of chromatin after break repair.

Results

H4S1phos is not a cytoplasmic modification

If CK2 is recruited to the site of a break, the catalytic subunits should localize to the break concomitant with the timing of increased phosphorylation. Histone modifying enzymes have been shown to be recruited to the site of a break, including NuA4 (Downs et al., 2004). To test this straightforward model we myc-tagged each of the two catalytic subunits of CK2 (*CKA1* and *CKA2*) separately in the GAL::HO donorless strain. We took time points to check for recruitment of CK2 using ChIP for up to 4 hours following galactose induction. Neither subunit was enriched above background. We therefore needed to consider the possibility that H4S1phos is a cytoplasmic modification.

One way to test whether H4 is phosphorylated outside of chromatin is to perform a chromatin fractionation assay and look to see if H4S1phos is enriched in the cytoplasmic fraction. In this assay, cells are lysed and the cytoplasm is separated from the chromatin with a high speed spin. The chromatin and cytoplasmic fractions are run on SDS-PAGE and western blot is used to detect where the modification is enriched.

In this assay, cells were separated into two groups, untreated and treated with 0.1% MMS. In the untreated cells there was no increase in H4S1phos in the whole cell extract (WCE), the cytoplasmic fraction (sup), or the chromatin fraction (pellet). The cytoplasmic fraction was immunoprecipitated with H4S1phos antibody to enrich for the modification. In cells which have been treated for two hours with MMS, H4S1phos is enriched in both the WCE and the chromatin

pellet, but there is no trace in the cytoplasmic fraction (Figure 17).

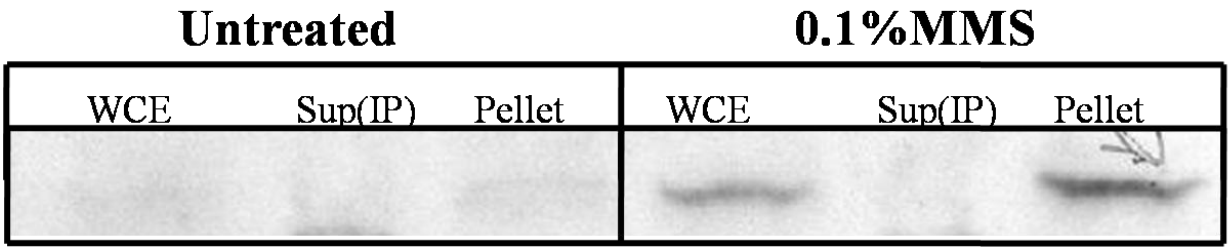

Figure 17: H4S1phos does not appear to be a cytoplasmic modification. Western blot of a chromatin fractionation assay shows that H4S1phos is found in WCE and chromatin after MMS treatment, but is not found in the cytoplasmic fraction (Sup (IP)).

These results were a preliminary reason to believe that this was mainly a nuclear modification, and that CK2 phosphorylates H4 within the chromatin, though negative results in this assay are not uncommon. Free histones are detrimental to a cell and there are many pathways to regulate their levels, so it is difficult to see histones in the cytoplasmic fraction.

H4S1phos does not need DNA resection to be recruited to a break

One of the first steps of HR is the processing of the DSB, creating long stretches of ssDNA so that the 3' end can engage in the homology search. This processing does not occur in the G1 phase of the cell cycle, when most cells repair via NHEJ (Ira et al., 2004). It has also been reported that nucleosomes are lost during DNA resection (Tsukuda et al., 2005). Therefore, during repair, new nucleosomes would be delivered to the site of the break to restore the chromatin to its original nucleosome density. We wanted to see if H4S1phos localization needs chromatin remodeling during DNA resection at the break. To address this, we performed ChIP on asynchronous cells, and cells arrested in G1 with α-factor. The cells arrested in G1 will not undergo resection. We found, two hours post-break induction, that H4S1phos was recruited to

the site of a DSB with the same intensity in the G1 arrested cells as in the asynchronous population (Figure 18). Therefore we conclude that resection is not necessary for H4S1phos localization.

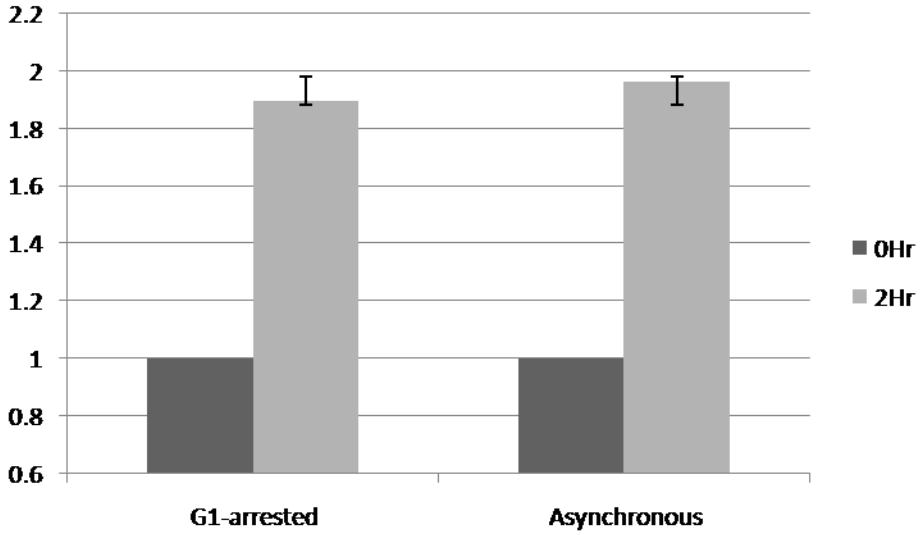

Figure 18. H4S1phos localization to a break does not need DNA resection. ChIP for H4S1phos at a region 2.5kb from the break shows that it is recruited with the same intensity in both G1-arrested and asynchronous populations.

H4S1phos localization to a break requires RPD3p

Considering the results from the chromatin fractionation assay, as well as the fact that resection is unnecessary, we focused on H4S1phos as a nuclear modification. We therefore questioned how CK2 was being recruited to a break. CK2 interacts with many chromatin-related proteins, but two pieces of evidence led us to focus on its interaction with the SIN3/RPD3 complex. Both *SIN3* and *RPD3* have been implicated in the DNA DSB repair pathway (Jazayeri et al., 2004, Tamburini and Tyler, 2005). Deletion of either subunit leads to sensitivity to DSBs. Also, a report from the Cote lab showed that phosphorylation by CK2 is inhibitory to NuA4 HAT activity (Utley et al., 2005). We also know the timing of H4S1phos localization coincides

with chromatin deacetylation and the recruitment of Rpd3p to the site of the break (Tamburini and Tyler, 2005). Both catalytic subunits of CK2, Cka1p and Cka2p, physically interact with Sin3p (Gavin et al 2006), and Cka1p interacts with Rpd3p (Gavin et al., 2006)

To test for the necessity of the SIN3/RPD3 complex for H4S1phos localization, we deleted each subunit individually in the donorless GAL::HO strain. We then looked at the localization kinetics of H4S1phos at the break. In the *sin3Δ* strain, localization is not compromised compared with that in the WT 2.5kb from the break (Figure19).

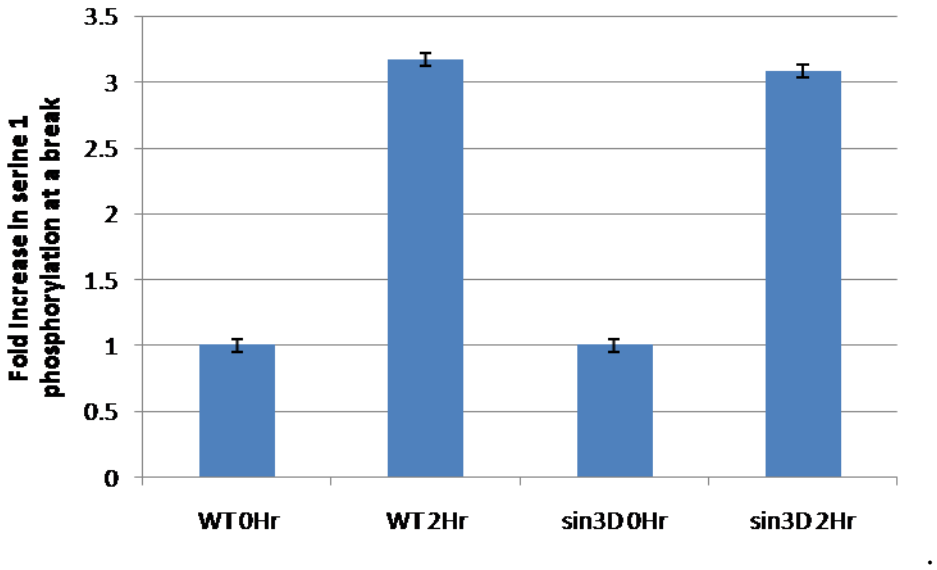

Figure 19. *SIN3* is unnecessary for full H4S1phos localization to a break. ChIP for H4S1phos at a region 2.5kb from the break shows that deletion of SIN3 does not disrupt H4S1phos localization.

Deletion of the catalytic subunit of the HDAC complex, *RPD3*, shows a dramatic decrease in phosphorylation surrounding the break (Figure 20).

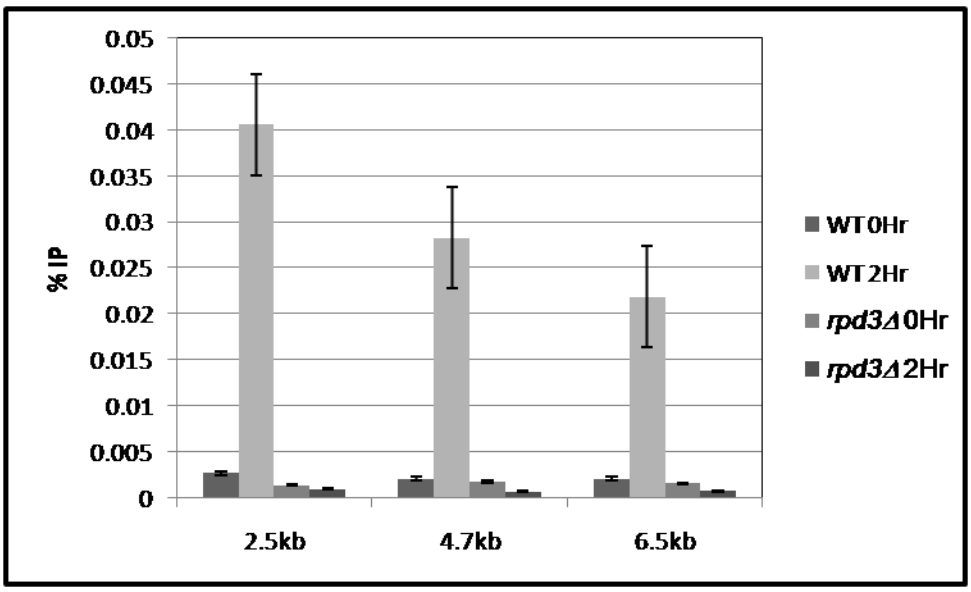

Figure 20. *RPD3* is necessary for localization of H4S1phos to the DSB. ChIP for H4S1phos at various regions surrounding the DSB shows that localization is lost in an *RPD3* delete strain.

From these results we conclude that Rpd3p but not Sin3p is necessary for H4S1phos localization to the DSB.

Mock acetylation inhibits CK2 activity on GST-H4 tails

We concluded from the ChIP data that H4S1phos needs the Rpd3p to be recruited to the site of a break. We were not sure, however, whether the subunit itself was necessary to physically bring CK2 to the break, or if its HDAC activity is necessary to deacetylate the chromatin surrounding the break before CK2 can recognize the H4 substrate. To test if the HDAC activity might be necessary for substrate recognition, we used various GST-H4 tails in which different combinations of the 4 acetylatable lysines have been mutated to gluamine, to mimic acetylated lysine. We found that the various lysine to glutamine mutations inhibit CK2 activity (Figure 21a).

To see which specific lysines contributed to this inhibition, we made individual muations of the four acetylatable lysines to glutamate. Of the four lysines, mutation of 5, 12 and 16 showed inhibition of CK2, while K8Q was not inhibitory to CK2 activity (Figure 21b).

To test if it is specifically the mock acetylation that blocks substrate recognition, or simply that CK2 needs a lysine in these positions to phosphorylae serine 1, we also mutated lysines 5 and 12 to arginine. We found that the lysine to arginine substitutions did not block CK2 substrate recognition (Figure 21c) We therefore conclude that acetylation of the tail blocks CK2 activity.

Based on the results from the *in vitro* kinase assay, we wanted to see if acetylation blocks H4S1phos *in vivo*. Specifically, we wanted to determine if the HDAC activity of Rpd3p is necessary for H4S1phos localization to the break. To test this, we used a strain in which *RPD3* was deleted, and carried a plasmid containing a "catalytic-dead" mutant (*rpd3-H150,151A*) (Kadosh and Struhl, 1998). This mutant has been characterized *in vitro* and *in vivo*. We found that only the complete deletion of *RPD3* affected H4S1phos localization. In the strain with the *rpd3-H150,151A* allele, H4S1phos is recruited to the break with levels seen in WT (Figure 22).

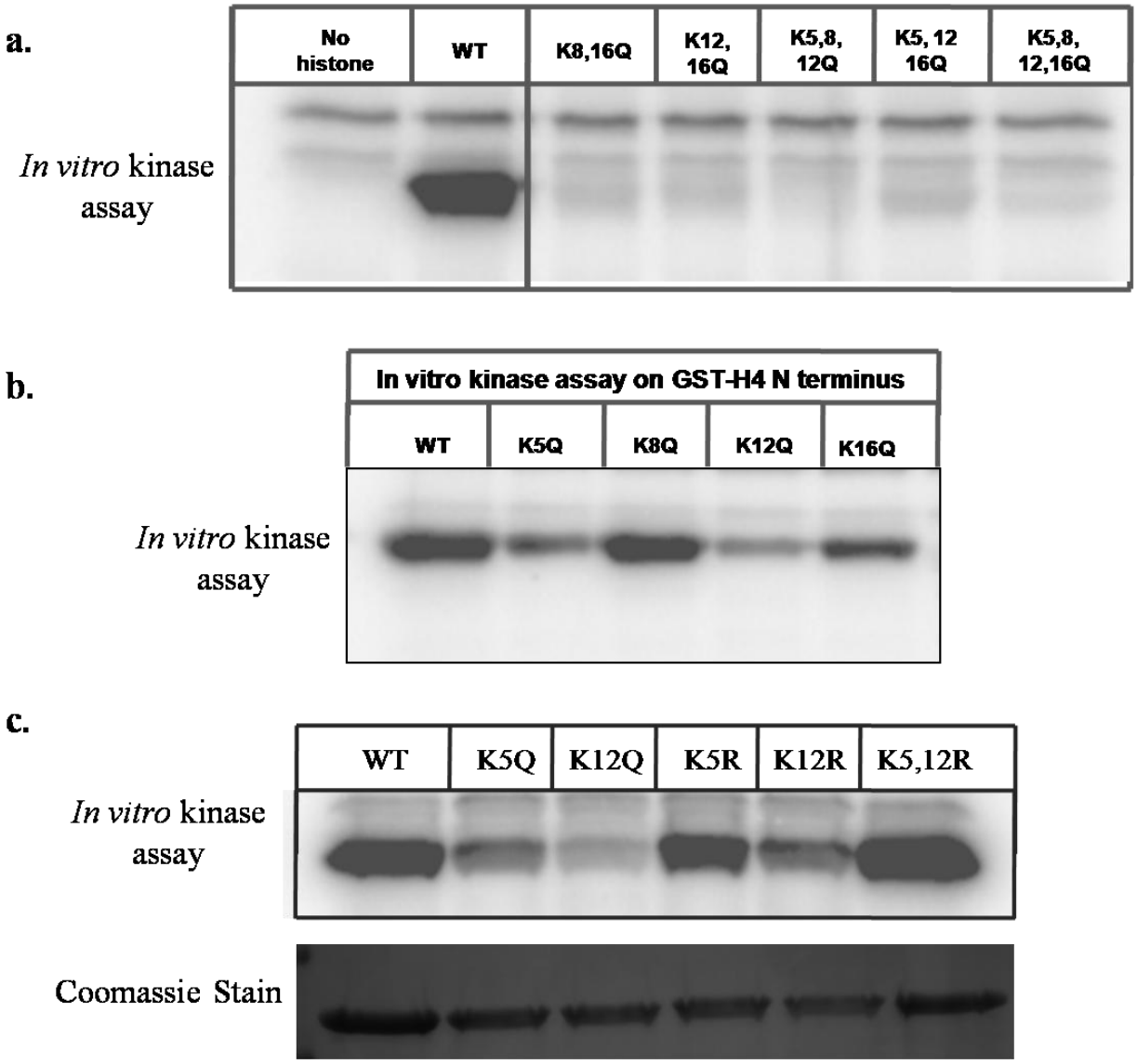

Figure 21. Lysine mock- acetylation of the H4 tail inhibits CK2 activity. a. Substitution of various lysines with glutamine, which mimics lysine acetylation, inhibits CK2 activity in an *in vitro* kinase assay. **b.** Mock acetylation of lysine 5, 12 and 16 inhibit CK2 activity. **c.** Substitution of lysines with arginine does not inhibit CK2 activity. Activity is specifically inhibited by lysine to glutamine substitutions.

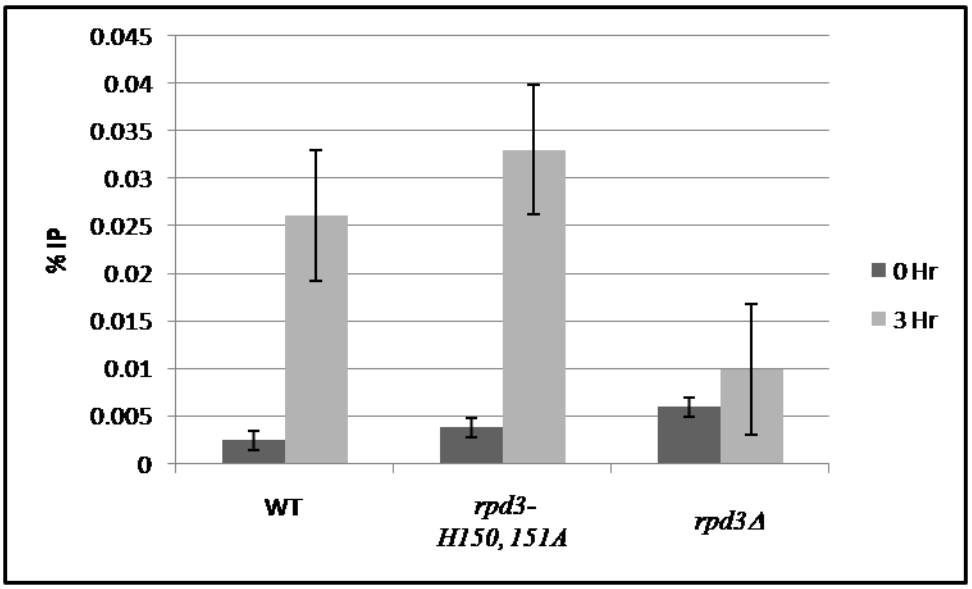

Figure 22. The HDAC activity of Rpd3p is dispensible for H4S1phos localization to a DSB. ChIP of H4S1phos in a strain which contains an allele of *RPD3* which lacks HDAC activity (*rpd3-H150, 151A*) shows the modification is still recruited to the break.

Discussion

The ubiquitious and constitutive nature of CK2 has led to a plethora of questions regarding how it is able to recognize H4 as a substrate in response to DNA DSBs. We have previously ruled out simple explanations for this modification. It does not require the regulatory subunits of CK2. It is not a modification of newly synthesized histones. It does not require the histone chaperones, or the DNA damage checkpoint.

It does, however, require Rpd3p for localization to the site of the break. In strains in which it has been deleted, H4S1phos localization to the break is compromised. We find in a strain in which the HDAC activity of Rpd3p is compromised, that H4S1phos is still localized at the break. It would be easy to conclude that CK2 is directed to the site of the break by a physical interaction with the HDAC complex, and that deacetylation of the chromatin is not necessary for phosphorylation.

That would, however, contradict the *in vitro* kinase assay where the lysine to glutamine mutants inhibit CK2. We find that glutamine, which mimics acetylation, inhibits CK2 activity. To reconcile the opposing data one must take into consideration that there are various HDACs recruited to the break (Tamburini and Tyler, 2005). It is possible that these are redundant, and the chromatin surrounding the break is deacetylated even in the absence of the HDAC activity of Rpd3p. ChIP of H4 acetylation in the *rpd3Δ* and *rpd3-H150,151A* strains would need to be done to determine the H4 acetylation level.

These findings are also interesting in light of the finding by the Cote lab, that H4S1phos inhibits the HAT activity of the NuA4 complex. The "Access, Repair, Restore" model proposes that immediately following a break, the chromatin is phosphorylated (H2AX) and acetylated,

which allows access to repair proteins and remodeling enzymes for DNA repair. After repair the chromatin must be restored as well. During break repair, acetylation blocks H4S1phos. During chromatin restoration, chromatin is deacetylated. This allows for H4 phosphorylation, which then blocks further rounds of acetylation by NuA4. The opposing nature of acetylation and phosphorylation allows the chromatin to remain accessible until repair has been completed and then be restored to its original modification status (Figure 23).

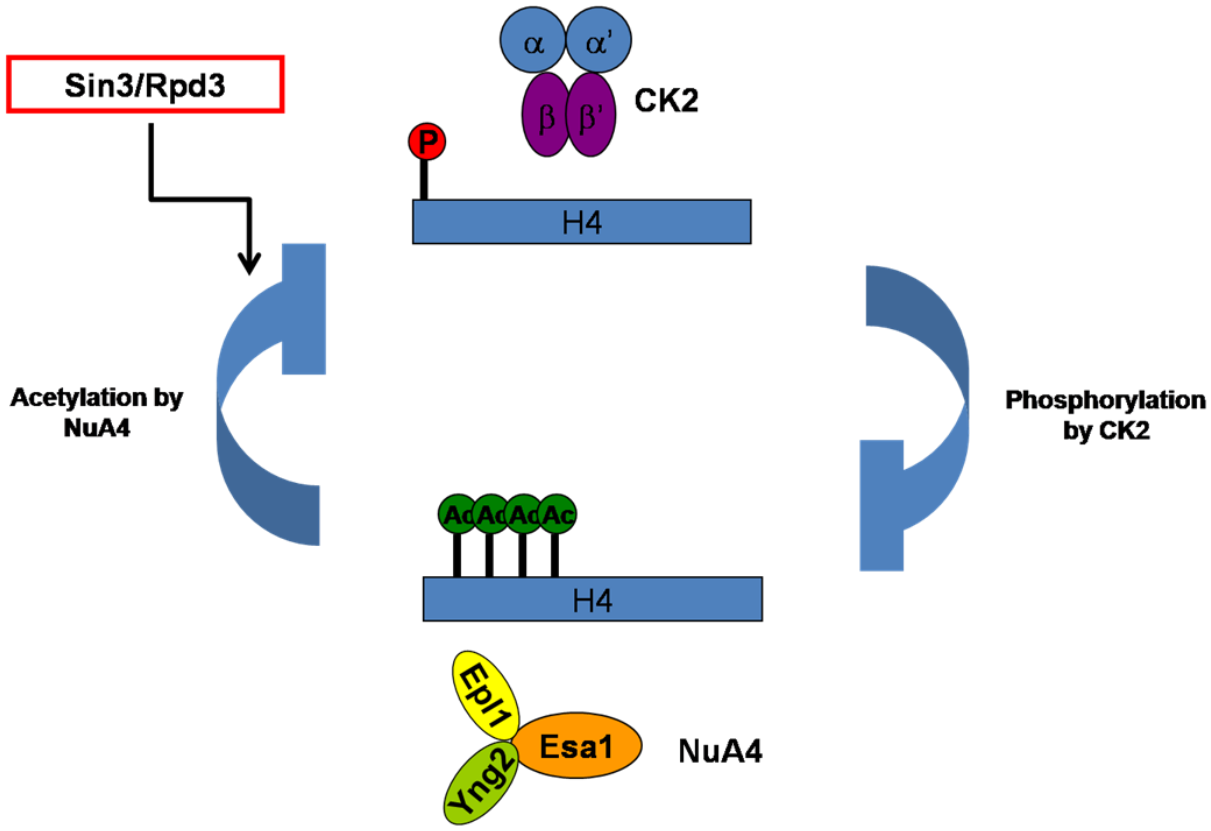

Figure 23: Model of opposing modifications. Phosphorylation by CK2 inhibits NuA4-dependent acetylation of the H4 tail. Acetylation of the H4 tail inhibits CK2 activity. An HDAC complex, such as SIN3/RPD3 must deacetylate the chromatin before CK2 is able to phosphorylate H4.

Materials and Methods

Strains and plasmids. The strain for the ChIPs of H4S1phos was JKM179 (CY915), the GAL::HO donorless strain. This strain was used to tag both *CKA1* and *CKA2* with the myc epitope tag (Longtine et al., 1998) *SIN3* and *RPD3* were deleted using the kan^r gene as described in Chapter II. The pGEX2T plasmid, containing GST-H4, was a gift from M. Grunstein. The lysine to gluatmine or arginine mutants were made using the Stratagene Quickchange Mutagenesis kit. The YEplac112 WT and mutant *rpd3-H150,151A* plasmids were a gift from K. Struhl and were described in Kadosh and Struhl, 1998.

***In vitro* kinase assays.** As previously described in chapter II with the exception that GST-H4 was used as the substrate for the reactions.

ChIP for H4S1phos. To test the localization of H4S1phos, cells were grown overnight in YEPR. Galacose was added to induce a break at an OD_{600} of approximately 0.5. Samples were taken at specific time points, 50mL of culture was harvested and crosslinked with 1% formaldehyde for 30 minutes. Crosslinking was quenched by the addition of 125mM glycine to the sample and incubated for five minutes at room temperature. Cells were spun at 4°C and washed twice in TBS. Pellets were frozen at -80°C. Pellets were resuspended in FA-Lysis buffer and lysed by vortexing with glass beads. The chromatin was sheared by sonication. $3x10^7$ cells were used per IP. Ten percent of each sample was taken as input control. One microliter of antibody (anti-H4S1phos or anti-γ-H2AX as a control) was added to each IP sample. IP sampled were nutated overnight at 4°C. Chromatin bound by antibody was precipitated with Protein A

Sepharose, washed with high stringency buffers, and eluted by overnight incubation with Proteinase K at 37°C. Crosslinks were reversed by incubation at 65°C for five hours. DNA was purified by phenol::chloroform extraction and ethanol precipitation and resuspended in twenty microliters of TE. One microliter if IP DNA, and 1 microliter of a 1:10 dilution of input DNA was used for semi-quantitative radioactive PCR using primers designed at various regions around the DSB. For the experiments using the plasmid-borne copies of WT *RPD3* or *rpd3-H150,151A*, cells were grown in selective SD media to maintain the plasmid. For the cells arrested in G1, 100μM α-factor was added to YEPR for five hours before addition of galactose.

Chromatin fractionation. Cultures were grown overnight in YEPD at 30°C to an OD_{600} of approximately 0.5. Cultures were split in half: one was left untreated, while one was treated for two hours with 0.1% MMS at 30°C. $5x10^8$ cells were harvested, washed with 5mLs of water, and resuspended in 3mL Pre-Spheroplast Buffer (100mM PIPES, pH 9.4, 10mM DTT) and incubated at 30°C for 30 minutes. Cells were harvested and resuspended in 2mL Spheroplast Buffer (50mM KPO_4, pH 7.5, 0.6M Sorbitol, 10mM DTT) and 20mg of 20T zymolyase was added to each sample. Samples were incubated at 30°C for approximately 15 minutes and monitored with the light microscope. Cells were spun and washed with Lysis Buffer (20mM PIPES pH 6.8, 0.4M Sorbitol, 150mM KOAc, 3mM MgOAc, 0.5mM PMSF) at 4°C. Cells were spun and resuspended in 500μL Lysis Buffer and TritonX was added to 1%. Half of the sample was collected for the WCE fraction and 2xSDS sample buffer was added. The other half was spun at 4°C at 12000rpm for 15 minutes. The supernatant was saved for IP, and the pellet was resuspended in 1xSDS sample buffer. To the supernatant 1μL antibody was added and nutated at 4°C overnight. Phosphorylated histone H4 was pulled down with Protein A Sepharose beads,

and protein was eluted by boiling in 1xSDS sample buffer. Samples were run on 18% SDS-PAGE, transferred to PVDF, blocked in 5% milk (TBST) and incubated overnight at 4°C in 1:5000 H4S1phos antibody. Blots were washed and incubated in 1:5000 anti-rabbit antibody, washed and visualized using chemiluminescence.

CHAPTER IV

THE INVOLVEMENT OF H4 SERINE 1 PHOSPHORYLATION IN CHROMATIN COMPACTION

Summary

A report from the Berger lab showed compelling evidence that H4S1phos is necessary for chromatin compaction *in vivo* (Krishnamoorthy et al., 2006). This modification is specific to meiosis and is not dependent on CK2. We were interested in studying this phenomenon *in vitro*. Specifically, we wanted to look at the contribution of this phosphorylation on the folding of a chromatin array. Using recombinant Xenopus histone H4 with serine 1 substituted with glutamic acid to mimic phosphorylation, we found a folding phenotype *in vitro*. At 1mM Mg^{+2} we found that a fraction of the H4S1E array would self-associate. Further analysis of this mutant revealed that the N-terminal methionine was not cleaved off during expression of the histone. A mutant with a glutamic acid inserted at the second position, keeping the N-terminal serine intact, allowed for removal of the methionine, but did not show a folding phenotype *in vitro*.

The data presented in this chapter was part of an on-going collaboration with the Berger lab. Much help and support for the data analysis was given by Borries Demeler at the University of Texas Health Science Center at San Antonio.

Introduction

A recent paper from the Berger lab presented a few lines of evidence that H4S1phos is involved in chromatin compaction *in vivo* (Krishnamoorthy et al., 2006). They showed that H4S1phos increases during meiosis in yeast and remains until the cells begin proliferation. During yeast meiosis, the chromatin is very compact, and the nuclear volume decreases. The modification is not dependent on the kinase CK2, but instead on the meiosis specific Sps1p, whose expression increases during meiosis, concomitant with the onset of H4S1phos.

ChIP with the H4S1phos antibody during meiosis shows that the modification is genome-wide. In a strain which lacks serine 1 phosphorylation, sporulation efficiency falls to 30% that of WT. Also, the nuclear volume is increased, leading to the hypothesis that lack of H4S1phos leads to lack of chromatin compaction. MNase digestion of the WT and *sps1Δ* chromatin during the sporulation program shows a resistant MNase band in the WT cells which is lacking in the *sps1Δ* mutants. The MNase resistant band seen in the WT could be due to inaccessibility of the enzyme, due to enhanced compaction which does not exist in the *sps1Δ* strain.

One way to tease out if chromatin compaction is a direct or indirect effect of H4S1phos is to study its contribution to chromatin folding in the analytical ultracentrifuge. The AUC is able to measure the sedimentation coefficient (S) of a species in solution. The sedimentation coefficient is a measure of a species' shape and molecular weight. A change in shape will change the rate of sedimentation towards the bottom of the cell in the centrifuge.

Creating arrays *in vitro* from purified components is a well-defined process. Recombinant histones are expressed in bacteria and purified via chromatography. Purified histones are combined at equimolar ratios to make the histone octamer. Octamers are added to

the DNA template at specific molar ratios and dialyzed from high to low salt to create a defined array with twelve octamers/ template. This array is then analyzed in the AUC to measure the change in S with different salt conditions.

The DNA template used for these studies (601-177-12) has been characterized in Dorigo et al., 2003. It consists of 12 tandem arrays of 177bps of the 601 nucleosome positioning sequence. The "solubility boundary" of an array made with this template has been reported. In TE buffer, the chromatin is in an extended beads-on-a-string conformation, or the 10nm fiber. With the addition of up to 2mM Mg^{+2}, the chromatin begins to compact, taking on the shape of the 30nm fiber. Adding between 2mM-80 Mg^{+2} will cause the array to self-associate and precipitate out of solution. Adding over 80mM Mg^{+2} allows the array to resolubilize.

Another *in vitro* assay to study chromatin folding is the *in vitro* self-association assay. As stated previously, increasing the Mg^{+2} concentrations close to 2mM causes the arrays to self-associate and precipitate out of solution. A modification which enhances chromatin folding may be expected to need less Mg^{+2} to self-associate than WT. It is known that acetylation of H4K16 shows a phenotype in this assay. H4K16ac inhibits chromatin folding as measured by the AUC. It also requires more Mg^{+2} in the self-association assay before it precipitates out of solution.

We studied the effect of substituting the first serine of histone H4 with glutamic acid, which mimics phosphorylation. This array showed a compaction phenotype in the AUC, but did not have a phenotype in the self-association assay. Unfortunately, we later discovered that the N-terminal methionine was not cleaved off during expression in *E. coli*. To circumvent this problem we inserted a glutamic acid reside at the second position in the protein, leaving the N-terminal serine intact (the "SE" mutant). As a control, we created a "WT" version in which a

serine was inserted at the second position, the “SS” version. We found that the “SE” mutant did not have an *in vitro* folding phenotype compared with the “SS” control.

Results

Studying Chromatin Folding *In Vitro*

It is well established that chromatin undergoes various levels of compaction to fit within the confines of a nucleus. The most basic level of chromatin organization is an array of nucleosomes separated from each other by linker DNA. This is the canonical "beads-on-a-string" chromatin fiber, seen as the 10nm fiber by EM. This is, however, an *in vitro* artifact, as the salt conditions to achieve this structure are not physiologically relevant. With increasing amounts of monovalent and divalent cations, the beads-on-a-string array will compact into a moderately folded and maximally folded chromatin array (Hansen 2002).

It is possible to study the compaction of a chromatin array *in vitro* using the analytical ultracentrifuge, which measures the sedimentation velocity of a sample. Sedimentation velocity measurement is a hydrodynamic technique which will yield the sedimentation coefficient (S), a measure of the mass and shape of a multi-component system within solution. The AUC is equipped with an optical detection system which can measure the concentration of a sample within a centrifuge cell in real time (Cole and Hansen, 1999). At the beginning of the experiment, the concentration of the sample is uniform throughout the cell. As a centrifugal force is applied, the molecules closest to the meniscus begin to move towards the bottom of the cell. This depletes the area closest to the meniscus of sample and forms a moving "boundary," which is a region of increased concentration of sample. The concentration of the sample near the bottom of the cell does not change until the encountered by the boundary.

The rate at which this boundary moves towards the bottom of the cell is used to calculate the sedimentation coefficient of a sample. The sedimentation coefficient is defined by the Svedberg equation, where S depends on velocity and mass of the molecule, strength of the

centrifugal field, frictional coefficient of the molecule, and viscosity of the solute (Cole and Hansen, 1999). The frictional coefficient (f) is a measure of a molecule's shape and size in solution. A sphere has a frictional coefficient of 1. If this sphere takes on a more elongated form the frictional coefficient increases.

What can confound the measure of the S of a sample is the fact that the molecules in the cell are not only subject to transport by the centrifugal field applied, but by diffusion away from the moving boundary with time. This diffusion broadens these moving boundaries and obscures the different components of the sample. The contribution of diffusion must be removed from the boundary measurements before a valid S value can be reported. The data from the analytical ultracentrifuge is a measurement of the boundary movement over time. The centrifuge will scan the absorbance of the cell throughout the experiment, and each scan is recorded.

The data from the AUC is then analyzed to determine the distribution of sedimentation coefficients within the sample. The data is a series of absorbance scans taken over time as the sample proceeds to sediment towards the bottom of the cell (Figure 24).

This set of scans is taken from a sample with one component, a chromatin array. As stated above, as a molecule travels towards the bottom of the centrifuge cell it is subject to transport by both sedimentation and diffusion. Over time it is possible that the contribution from diffusion may distort the boundary in such a way that it spreads, masking the potential of a heterogeneous species within the cell. K. Van Holde and W. Weischet developed a method of analyzing this data that removes the contribution of diffusion from the sedimenting species (van Holde and Weischet, 1978). They realized that because diffusion proceeds with a velocity proportional to the square root of time, and sedimentation proceeds proportional to the first

power of time, the contribution of each can be separated. Therefore, a "diffusion-corrected" sedimentation coefficient can be calculated using the "van Holde- Weischet" method.

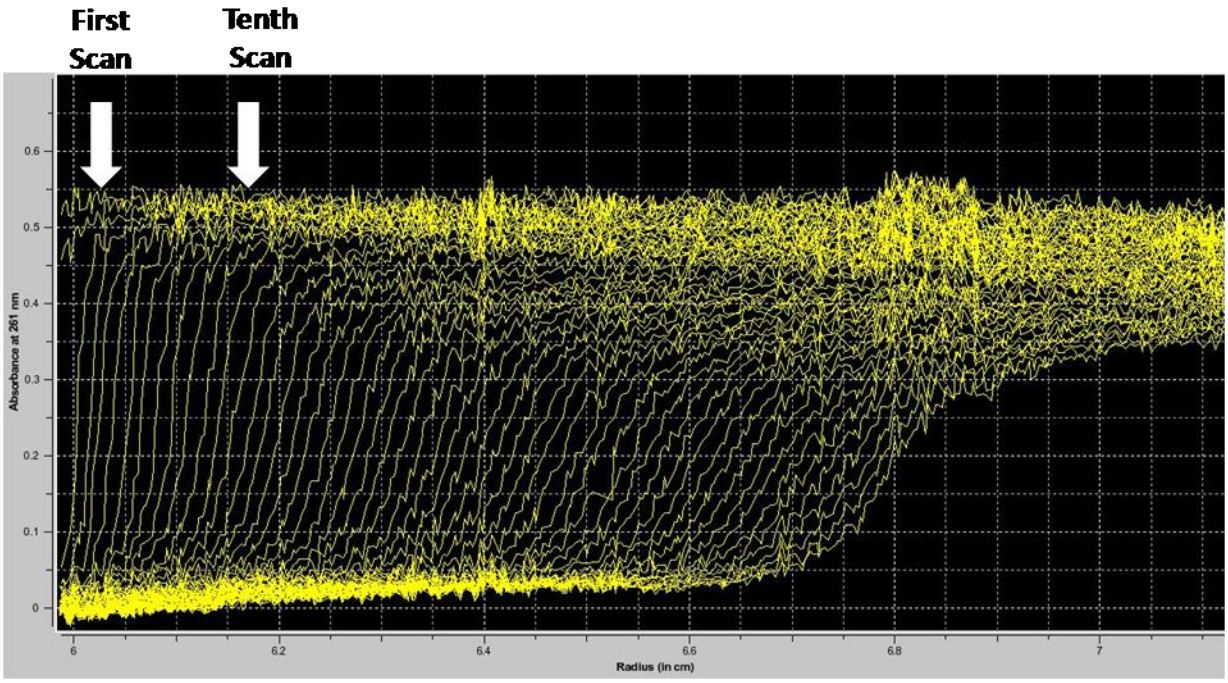

Figure 24. Raw AUC data before analysis. With time, the concentration of sample is moving towards the bottom of the cell, as demonstrated by the moving boundaries.

The first step of this method is to divide each of the scans into evenly spaced boundary fractions from the baseline absorbance to the absorbance plateau. Traditionally 50 fractions are used for each analysis but for simplicity only 4 fractions are shown in Figure 25.

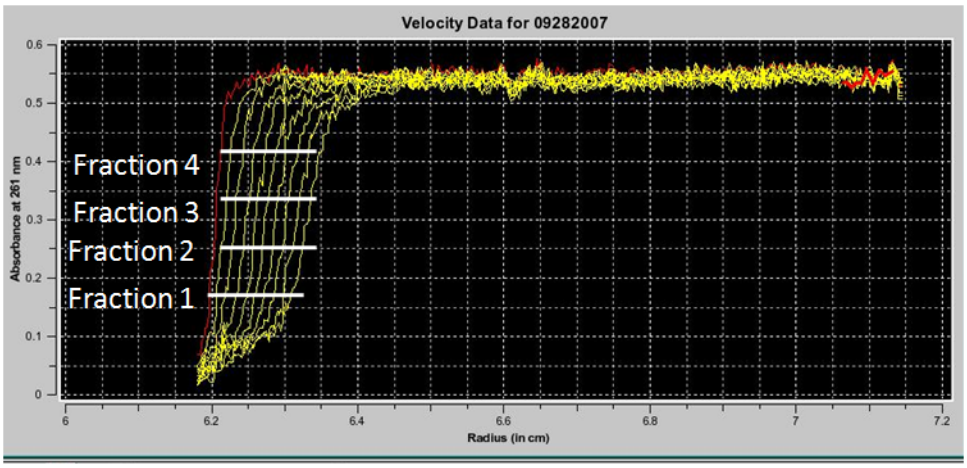

Figure 25. Ten scans from the AUC. Each scan is divided into four fractions for simplicity. Fifty fractions are traditionally used for a van Holde-Weischet analysis.

The following is an example where the first ten scans from an AUC run are being used to determine the sedimentation coefficient. For each scan, an apparent sedimentation coefficient can be determined at each fraction using the Svedberg equation, which takes into account the radial position of the sample at each given time. The apparent sedimentation coefficient can then be plotted with respect to time for each fraction for each scan. Each fraction can then be extrapolated out to infinite time to correct for diffusion, to form the van Holde-Weischet fan plot (Figure 26). The point where each line (the best fit for each boundary fraction) crosses the Y-axis is the diffusion-corrected sedimentation coefficient.

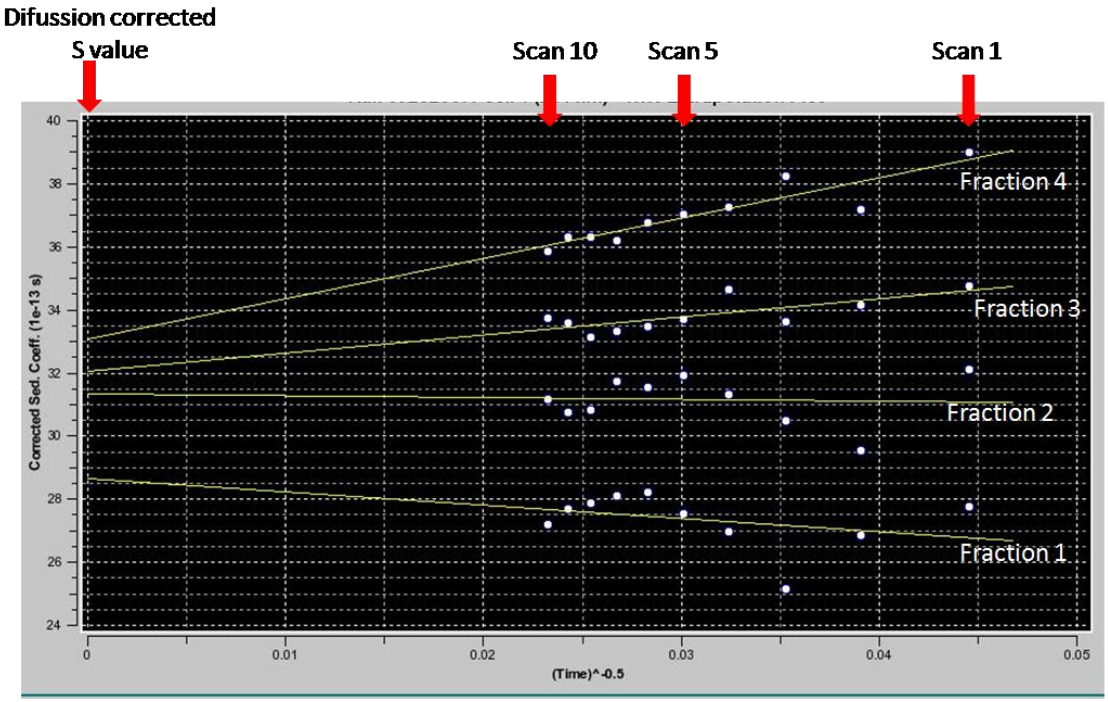

Figure 26. A van Holde-Weischet fan plot. The apparent S from each fraction of each scan is plotted and then extrapolated out to infinite time. The diffusion corrected S value for each fraction can be determined where the extrapolation crosses the Y-axis

From this van Holde-Weischet "fan plot," a distribution chart of the sedimentation coefficients can be plotted as a percentage of the boundary fraction that sedimented at each S value (Figure 27).

As mentioned previously, this method is able to distinguish between heterogeneity within a sample population. The amount of each species within the sample can be approximated based on the % of the boundary fraction it occupies. The distribution plot in Figure 28 shows two distinct species.

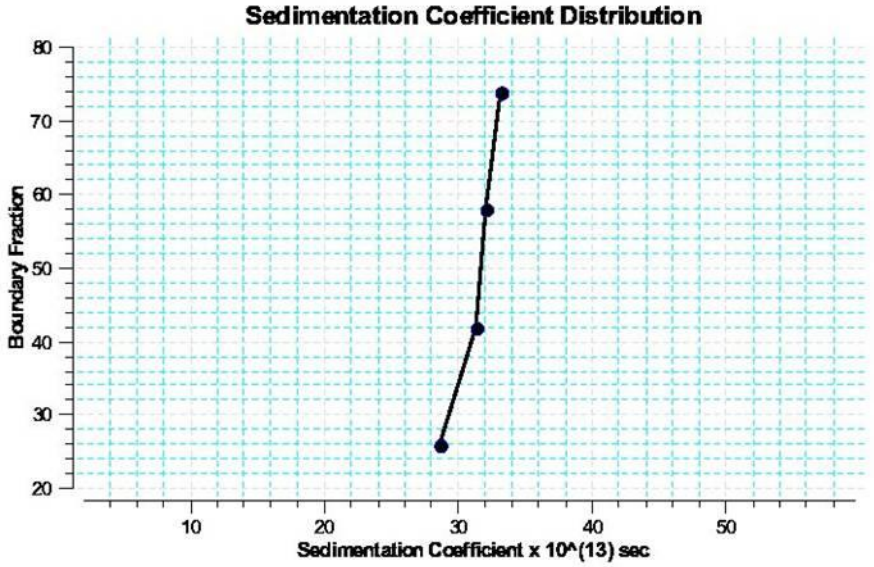

Figure 27. A sedimentation coefficient distribution plot. A distribution plot is created by plotting the diffusion- corrected S value for each boundary fraction. In this distribution plot the species sediments between 29S and 34S.

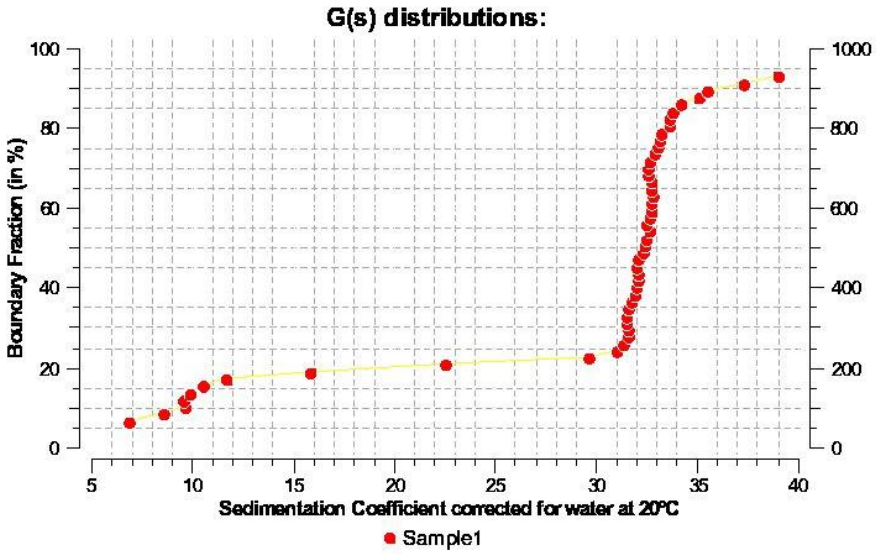

Figure 28. A distribution plot with two distinct species. In this plot a small portion of the sample, approximately 15%, sediments at 7-10S. The rest of the population sediments at 32-40S.

Homogenous chromatin arrays can be reconstituted *in vitro* from purified histone octamer and a DNA template. An octamer can be reconstituted *in vitro* by mixing the four histone proteins in equi-molar ratios under denaturing conditions and dialyzing into 2M NaCl. The octamer is then purified via gel filtration chromatography (Luger at el., 1999a,b). The DNA template is a tandem array of 12 copies of the 177 base pair (bp) "601" nucleosome positioning sequence (602-177-12) (Lowary and Widom, 1998; Dorigo et al., 2003). The DNA template and octamer are added together in 2M NaCl in a ratio such that there is one octamer per each 177 bp positioning sequence. During the step-wise dialysis to 2.5mM NaCl, the DNA wraps around the octamer to form a homogenous array. The folding properties of the array can then be studied using different salt conditions using the AUC.

The folding properties of chromatin in the presence of mono- and divalent cations are well characterized. Chromatin arrays will reach maximal compaction (53-55S) in the presence of Mg^{+2} but not Na^{+1}. With increasing amounts of Mg^{+2}, up to approximately 2mM, the arrays will sediment with higher S values. Increasing the Mg^{+2} concentration past 2mM causes the array to "self-associate," which sediments at S values greater than 200S, up to 10,000S. Self-association is reversible. Lowering the Mg^{+2} concentration to 1mM will yield arrays that sediment at 55S. Self-association is thought to mimic long range fiber-fiber interactions *in vivo* (Schwarz et al., 1996).

The H4S1E mutant shows enhanced folding properties in 1mM Mg^{+2}

If the Berger lab's hypothesis is correct, and H4S1phos aids in chromatin compaction, we expect to see the H4S1E mutant reach the compact 30nm fiber with less Mg^{+2} than in the WT. Testing folding phenotypes in the AUC requires a fully saturated array, meaning the DNA

template contains one octamer per nucleosome positioning sequence. An under saturated array is unable to reach maximal compaction whereas an oversaturated array will have histone octamer non-specifically bound in between nucleosomal positioning sequences and the array will not be homogenous.

To achieve the correct saturation level, the ratio of octamer to DNA template added to the reconstitution (recon) is crucial. This is also known as the "R" value of the recon. The DNA template has 12 tandem repeats of the "601" positioning sequence. This sequence has a high affinity for the octamer, and only one translational position. To avoid oversaturation, a DNA "sink" is added to the recon to bind excess octamer, preventing non-specific binding to the chromatin array. The sink DNA is a nucleosome positioning sequence with a lower affinity for the octamer than the 601. We used the MMTV nucleosome positioning sequence reported in Flaus et al., 2004.

To reconstitute an array, DNA template, histone octamer, and sink DNA are combined in the presence of 2M NaCl and dialyzed over time to 2.5mM NaCl. To obtain a purified array absent of sink DNA and the mononucleosomes that formed from the sink DNA, the self-association property of the arrays is exploited. The arrays are incubated in 4mM Mg^{+2} to induce self-association and spun at high speed in a table top centrifuge. The arrays will precipitate out of solution while the sink DNA and mononucleosomes will remain in solution. The pelleted array is resuspended in TE and is ready for analysis.

To test the saturation levels, a restriction digest of the reconstituted array is performed. Between each nucleosome positioning sequence within the DNA template is a ScaI site. Complete digestion of the naked DNA template yields a single band of 177bps. If an octamer

occupies the positioning sequence, the band will shift. A properly saturated array will have approximately 5% unoccupied fragment. If the array is oversaturated, an octamer will occupy the ScaI site and a larger shifted band will appear (Figure 29).

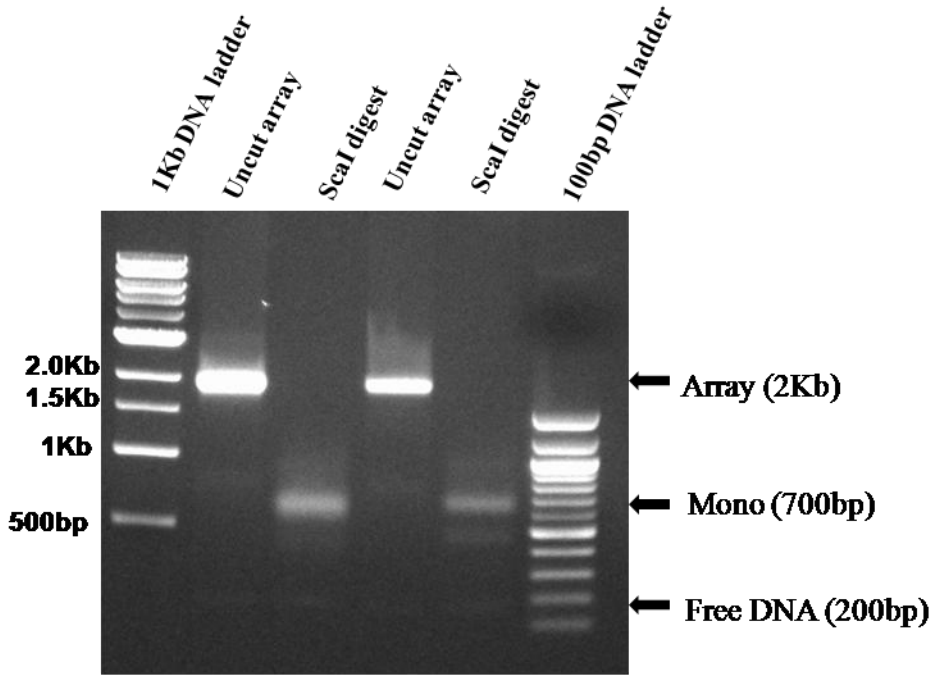

Figure 29. ScaI digest of a saturated array. Very little free DNA is seen in this ScaI digest, showing that most of the nucleosome positioning sequences are occupied.

To test the folding of the array in the AUC, a run is performed in TE to further confirm that the array is saturated. A saturated array will have a sedimentation coefficient of approximately 33S. Over or under saturation changes the S accordingly (Figure 30).

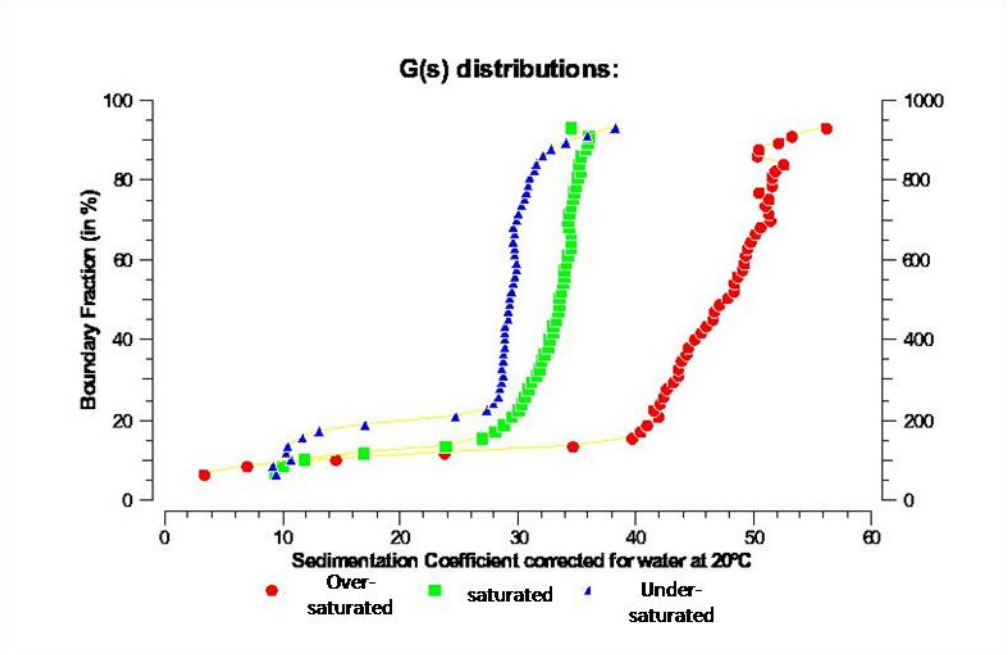

Figure 30. Example of distribution plot of arrays in TE. The blue triangles represent an undersaturated array which sediments lower than 30S. The green squares represent a saturated array and sediments between 30-35S. The red circles represent an oversaturated array which sediments between 40-60S.

We first characterized a WT array to make sure it folds as previously described. We used TE buffer and 1mM Mg^{+2} throughout experimentation because maximum compaction of the 601 array was reported to occur at 1mM Mg^{+2} (Dorigo et al., 2004). We found that the WT array behaved as described (Figure 31).

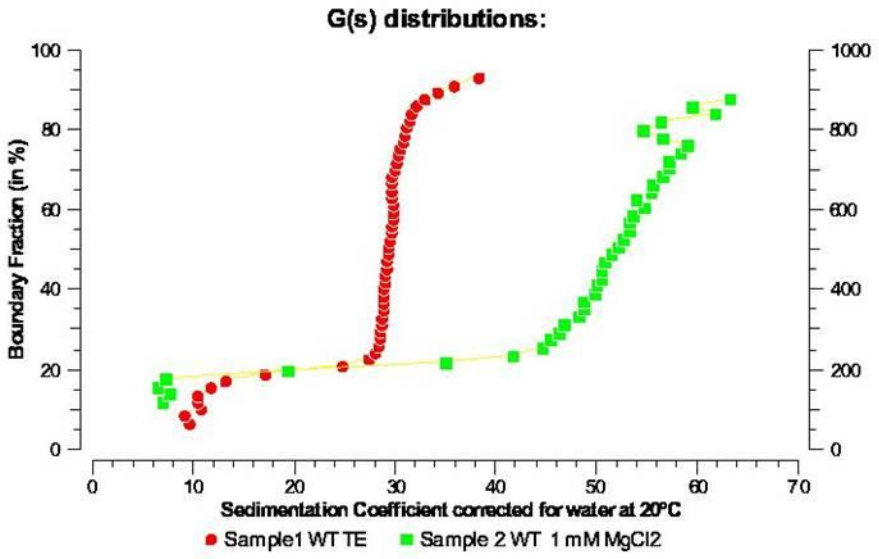

Figure 31. The WT array achieves the 53S species in 1mM $MgCl_2$. In TE the array sediments at 30S (red circles). This same array in the presence of 1M $MgCl_2$ sediments between 50-60S, showing maximal compaction (green squares).

We next sought to test the sedimentation properties of the H4S1E mutant. With 1mM Mg^{+2}, half of the sample reached maximum compaction while half sedimented between 100-250S, a phenotype of self-associated chromatin (Figure 32).

The ability of a chromatin array to self-associate with increasing amounts of Mg^{+2} is thought to mimic long range fiber-fiber interactions *in vivo*. Self-association is a reversible process. Saturation levels of the chromatin array do not affect its ability to self-associate. To test the self-association characteristic of the array differing concentrations of Mg^{+2} are used, from 0-4mM. The arrays are incubated in Mg^{+2} for 15 minutes and subjected to a high speed spin in a table top centrifuge. The amount of DNA remaining in the supernatant in measured. Although this array was able to sediment with less Mg^{+2} than WT, we found that the H4S1E and WT arrays needed similar amounts of Mg^{+2} for self-association (Figure 33).

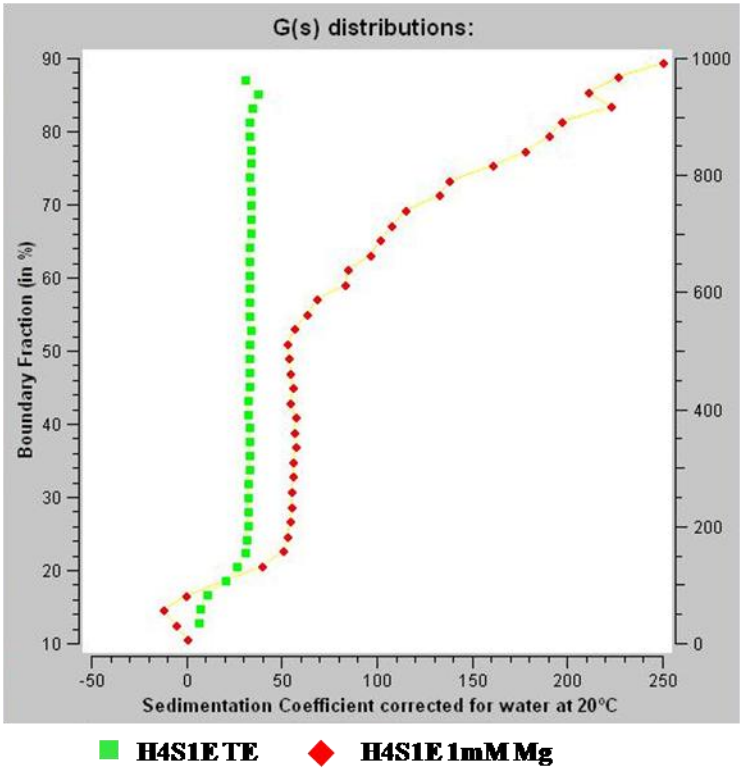

Figure 32. At 1 mM Mg^{+2} the H4S1E array begins to self-associate. In the presence of 1mM $MgCl_2$ the saturated array sediments between 53-250S (red diamonds), a property of self-associated chromatin.

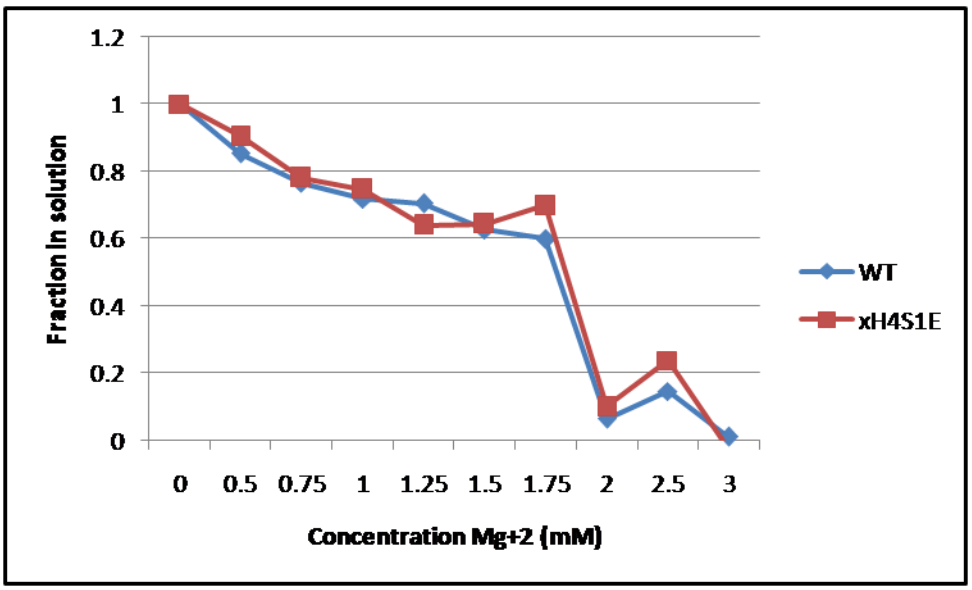

Figure 33. The self-association pattern of H4S1E and WT are the same. In the presence of increasing amounts of $MgCl_2$, both the WT and mutant array self-associate between 1.75-2mM $MgCl_2$.

It has been published that there are specific amino acid residues necessary for proper cleavage of the N-terminal initiator methionine in *E.coli* recombinant protein expression (Flinta et al., 1986). There are also specific amino acids which protect the initiator methionine from being cleaved off. Seven amino acids (glycine, alanine, serine, threonine, proline, valine, and cysteine) will direct methionine cleavage (Walker and Bradshaw, 1999), while others protect the methionine. Therefore it is important to check a recombinant protein by mass spec when mutating the penultimate residue.

The results of the mass spec analysis revealed the molecular weight (MW) of the H4S1E histone to be 11409Da. The expected MW was 11278Da. We concluded that the initiator methionine was not cleaved off of this mutant H4. Therefore, we could not conclude that the change in folding was due necessarily to the S1E mutation, the retention of the methionine, or a combination of both.

The "SE" nucleosomal array does not enhance chromatin compaction

To address the issue of the retention of the initiator methionine, we designed constructs which would retain the serine in the first position. A glutamic acid was introduced at the second position, and the rest of the amino acids were maintained ("SE"). As a control, a serine was inserted at the second position ("SS"). In these constructs a serine would be maintained adjacent to the initiator methionine and cleavage would not be affected.

Unfortunately, this new SE construct did not mimic the phenotype we found previously with the H4S1E construct. We found that the SS and SE arrays sedimented with the same sedimentation coefficient at 1mM Mg^{+2} (Figure 34). We therefore conclude that H4S1phos does not, by itself, contribute to chromatin folding *in vitro*. This could indicate that the folding phenotype *in vivo* is due to downstream effects such as the recruitment of non-chromatin proteins.

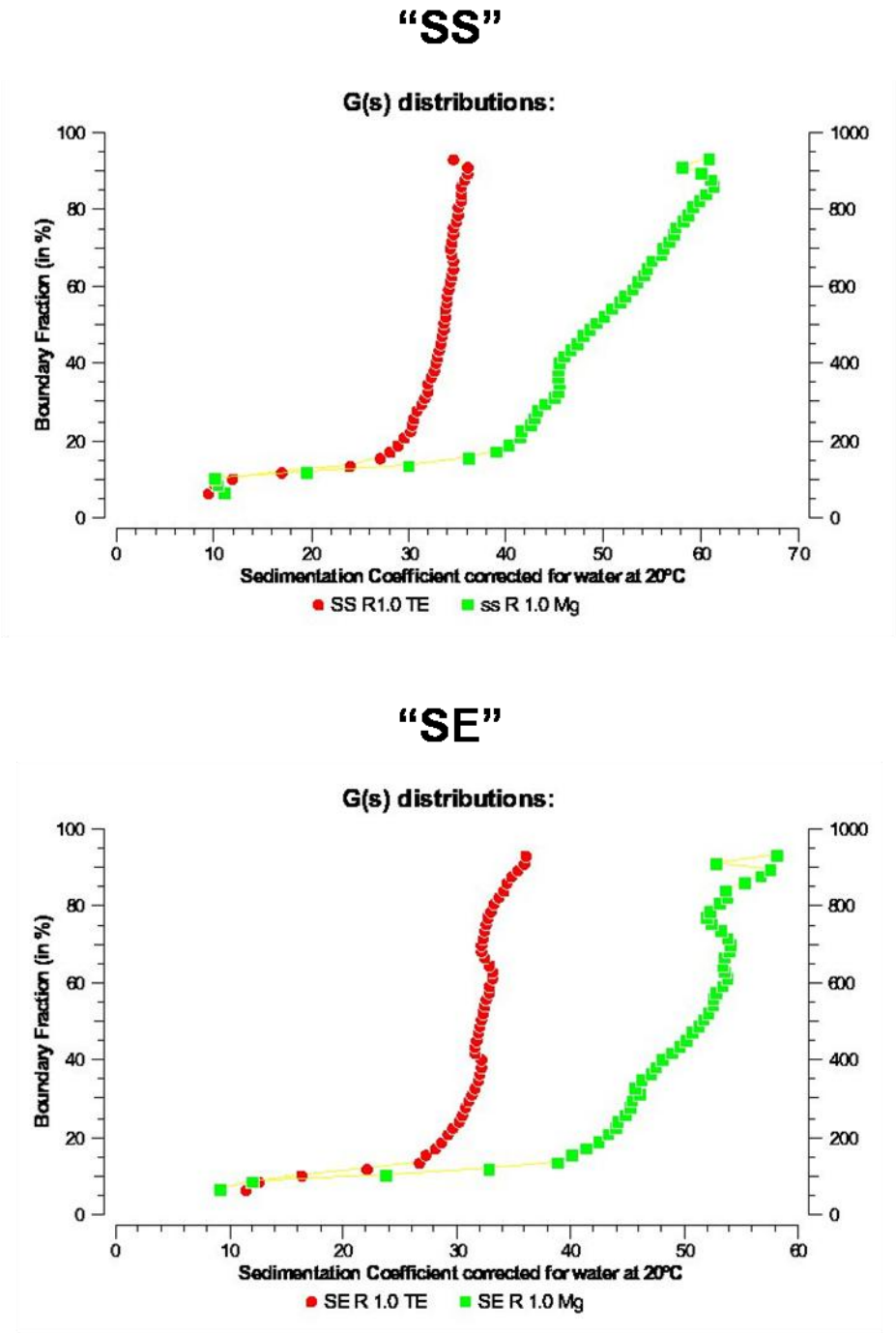

Figure 34. The SS and SE mutant show similar folding properties in the AUC. In the presence of 1mM $MgCl_2$ both the SS and SE arrays sediment at 53S.

Discussion

In a study of the contribution of each of the histone N-terminal tails to chromatin folding, the H4 N-terminal tail was found to be unique in its necessity for compaction (Dorigo et al. 2003). Testing compaction of arrays in which each tail was individually deleted, only the H4 N-terminal deletion could not reach WT levels of compaction, sedimenting at a maximum of 44S. Analysis of the important residues within the H4 tail determined that residues 14-19 contribute to chromatin compaction.

The crystal structure of the nucleosome has shown that residues within the histone H4 N-terminal tail interact with an "acidic patch" found within the H2A/H2B dimer (Luger et al., 1997a+b). The H4 tail interacts with the acidic patch if a neighboring nucleosome. A report from the Richmond lab has also shown evidence for the "two-start model" of the chromatin fiber, in which the chromatin fiber has two stacks of nucleosomes and a nucleosome at position "1" within a chromatin fiber interacts with a nucleosome at position "3" to achieve the 30nm fiber (Dorigo et al., 2004). These lines of evidence explain the necessity of a fully saturated array to achieve maximal compaction of the chromatin array.

The analytical ultracentrifuge is a straightforward way to measure the folding properties of a defined chromatin array. The Berger lab hypothesized that H4S1phos enhances chromatin compaction *in vivo* based on their data studying its phenotype in meiosis. We were able to use the AUC and arrays reconstituted with glutamic acid to mimic constitutive acetylation to test whether the folding phenotype is a direct or secondary effect of phosphorylation. We show that the *in vivo* chromatin compaction defect is most likely due to a secondary effect because the serine 1 substitution with glutamic acid does not directly affect the compaction of a chromatin array in the AUC. It is true that glutamic acid is not a perfect phospho-mimetic. An ideal

measure of the contribution of H4S1 phosphorylation would be to use native chemical ligation to create an array with each H4 phosphorylated on serine 1 to study in the AUC.

Materials and Methods

Plasmids. The Xenopus expression plasmids used were previously described in Luger et al., 1999a+b. The H4S1E plasmid was made using the Stratagene Quickchange Mutagenesis kit. The "SE" and "SS" plasmids were a gift from the Berger lab. The plasmid containing the 601-177-12 template was a gift from T. Richmond. The sink plasmid was a gift from T. Owen-Huges.

Histone Expression and Purification. Histone expression was done as previously described in Luger et al., 1999. Five individual colonies of transformed BL21(DE3) pLysS were used to start 5- 1mL cultures in 2xYT broth at 37°C. After three hours, all five cultures are used to inoculate a 50mL culture of 2xYT at 37°C. After three hours this is used to inoculate 6x500mL 2xYT to an OD_{600} of 0.005 at 37°C. Once the OD_{600} reaches 0.4, IPTG is added to 0.2mM and cells are harvested after 4 hours. The pellet from one liter of cell culture is resuspended in 30mL Histone Wash Buffer (50mM Tris pH 8.0, 100mM NaCl, 1mM EDTA, 1mM DTT, 1mM benzamidine) and stored at -80°C. Cells are thawed and lysed by sonication. Histones are isolated as inclusion bodies and stored at -80°C. Histones are resolubilized in Unfolding Buffer (7M guanidine, 20mM Tris pH 7.5, 10mM DTT) and dialyzed overnight into Urea Dialysis Buffer (7M urea, 10mM Tris pH 8.0, 1mM EDTA, 1mM DTT). Histones are purified using tandem Q Sepharose and SP Sepharose columns. Fractions containing purified histones are dialyzed into water containing 1mM DTT, lyophilized, and stored at -80°C.

Octamer reconstitution and purification. Lyophilized histones are resuspended in Unfolding Buffer at room temperature. Equimolar amounts of histone were combined in Unfolding Buffer to a concentration of 1mg/mL. Histones are dialyzed against three changes of Refolding Buffer (2M NaCl, 10mM Tris pH 7.5, 1mM EDTA) at 4°C overnight. Octamers were purified from tetramers and monomers using a Superdex-200 column. Fractions which contained octamers are combined and concentrated using Vivaspin Centrifugal Concentrators and stored on ice at 4°C.

601-177-12 template purification. The 2.1kb DNA template is flanked by EcoRV sites so it can be cut from the plasmid backbone. Purified plasmid was digested with EcoRV for 3 hours and phenol::chloroform extracted, ethanol precipitated, and resuspended in TE. The template was purified from the backbone by precipitation using 6% PEG in 0.5M NaCl. The pelleted template was dialyzed against TE to remove the residual PEG.

Sink DNA purification. The MMTV sink template was PCR amplified in bulk. To purify it away from the primers and unincorporated nucleotides we purified the fragment on an agarose gel and extracted the DNA using the Qiagen Gel Extraction kit.

Array reconstitution. Varying ratios of octamer to DNA template were mixed together in dialysis buffer containing 2M NaCl in a total volume of 1mL. A constant amount of sink DNA was added each time (ratio of octamer: sink DNA was 1:0.3) Step-wise salt dialysis was performed, switching buffers every three hours. The final salt concentration was 2.5mM NaCl.

The array was precipitated by incubation in 4mM Mg^{+2} for 20 minutes at room temperature followed by a 20 minute high speed spin in the table top centrifuge. The pellet was resuspended in TE and stored on ice at 4°C. A fraction of the array (5μg) was digested by ScaI for 30 minutes at 37°C and run on an agarose gel to check the saturation level.

AUC runs. The array was brought to a volume of 400μL and an OD_{260} of 0.5 for the AUC runs. For the TE runs the arrays was simply diluted into TE. For the 1mM Mg^{+2} runs the array was diluted to 200μL 10mM Tris pH 8.0, and 200μL of 2mM $MgCl_2$ was added to make a final concentration of 1mM Mg^{+2}. The samples were run at 20,000rpm at 4°C and at least 50 scans were taken for each sample.

Self-association assay. For each Mg^{+2} concentration a final volume of 100μL with an OD_{260} of 0.4 was used. The array was aliquoted into eppendorf tubes at a volume of 50μL. To each sample, 50μL of varying concentrations of Mg^{+2} was added, and the samples were incubated for 20 minutes at room temperature, followed by a 20 minute high speed spin in the table top centrifuge. The OD_{260} of the supernatant was measured using a nanospec. The amount of array remaining in the supernatant was divided by the amount remaining in the 0mM Mg^{+2} fraction and plotted as fraction remaining in the supernatant vs. Mg^{+2} concentration.

CHAPTER V

Perspectives

Histone modifications have been shown to contribute to a plethora of DNA templated events. Their signature in transcription, replication, heterochromatin formation, and DNA damage have led to the hypothesis of a "histone code " (Strahl and Allis, 2000). It is now known that histone modifications cannot be read as a straightforward "code" leading to specific downstream events, but instead they work in combination to coordinate cellular processes.

CK2 is a ubiquitous, essential, serine/threonine kinase that is conserved from yeast to man. It has been extensively studied *in vivo* as well as in biochemical assays. The casein kinase family is amongst the first kinases discovered (Morales and Carpenter, 2004). CK2 purified from mammalian cells appears to be constitutively active. The preferred consensus sequence for CK2 has been loosely defined as acidic amino acid residues surrounding the serine or threonine phospho-acceptor.

CK2 has been implicated in apoptosis, transcription and the cellular stress response (Morales and Carpenter, 2004). It was only within the past decade that it has emerged as a player in the DNA damage response. CK2 is essential for efficient single strand break repair (SSBR) in human cells. It was reported that CK2 phosphorylates XRCC1, a scaffold protein important in SSBR (Loizou et al., 2004). Inhibition of CK2 decreases XRCC1 interactions with polynucleotide kinase (PNK) and eliminates repair of the break. CK2 is also known to phosphorylate XRCC4, a member of the ligase complex necessary to complete NHEJ. Phosphorylation by CK2 promotes an interaction between XRCC4 and PNK, and loss of this interaction increases radiosensitivity in these cells (Koch et al., 2004).

CK2 is also emerging as an important player in repair of DSBs. In mammalian cells it has been reported that CK2 is necessary for retention of the MRN complex at DNA damage foci in living cells (Melander et al., 2008; Spycher et al., 2008). CK2 phosphorylates the MDC1 scaffold protein which directly binds γ-H2AX. Without this phosphorylation, the FHA domain of NBS1 cannot interact with MDC1, and the MRN complex cannot be retained at the DSB.

Work in our lab has shown that CK2 phosphorylates the H3 and H4 histone N-terminal tails, and the globular domain of both H2A and H2B *in vitro*. In an *in vitro* kinase assay, CK2 recognizes serine 31 within the histone H3 N-terminal tail. However, characterization of H3S31 phosphorylation *in vivo* did not reveal it to be a CK2-dependent modification.

H4S1phos and DNA DSBs

Work from our lab, in collaboration the Berger and Allis labs, showed that H4S1 is the substrate of CK2 *in vivo* and *in vitro*. The modification is enriched after assault with DNA DSB-inducing agents. It was first published as a modification of newly synthesized histones in avian erythrocytes (Ruiz-Carillo et al., 1979), but this does not appear to be the case in *S. cerevisiae*. The modification is enriched at sites surrounding a DSB within two hours of break induction.

Further characterization of H4S1phos reveals that it is specific for DNA DSBs but does not appear to need a functioning DNA damage checkpoint. γ-H2AX, which is also enriched at a DSB, depends on the damage checkpoint kinases Mec1p and Tel1p. However, H4S1phos is enriched in a strain which lacks both. In fact, the responsible kinase, CK2, is constitutively active. The two regulatory "β" subunits of CK2 have been shown to enhance its kinase activity for certain substrates, but they too are dispensable for H4S1 recognition.

Using a strain in which serine 1 of H4 has been substituted with alanine, we show that loss of H4S1phos does not yield a phenotype upon treatment with DNA damage inducing agents. Because H3S31phos is a CK2- dependent modification *in vitro*, we tested if this was a redundant modification to H4S1phos *in vivo*. Using a strain in which the H3 and H4 serines have been substituted with alanine, we found that this too did not yield a phenotype in response to DNA DSBs.

So far, none of the assays conducted using the H4S1A strain have yielded a discernable phenotype. It is possible that an as yet unknown modification acts in concert with H4S1phos. It would also be interesting to see if it is responsible for recruiting other proteins to the break, similar to γ-H2AX. Whole cell extract can be incubated with a peptide consisting of the H4 tail amino acids phosphorylated at serine 1, or an unphosphorylated control. Using mass spectrometry, it is possible to identify proteins which bind specifically to phosphorylated H4. After identifying interacting proteins, it is possible to use ChIP to see if they localize with H4S1phos at the break and if lack of phosphorylation abolishes recruitment.

It would also be interesting to discover the phosphatase responsible for downregulation of H4S1phos. The phosphatase responsible for reduction of γ-H2AX was discovered in *S. cerevisiae* as Pph3p, part of a three subunit complex (Keogh et al., 2006). They found that H2AX was only dephosphorylated after removal of the histone from the chromatin. Because there are only 9 nuclear phosphatases in *S. cerevisiae*, it would be possible to test the contribution of each individually to removal of H4S1phos. Western blots can be performed in each of the phosphatase delete strains to see which is responsible for reduction of H4S1phos after DNA damage.

CK2 has been implicated in the DNA damage response in *S. cerevisiae*. It was shown that a strain in which either of the regulatory subunits (*CKB1* or *CKB2*) of CK2 have been deleted cannot adapt to a single, unrepairable DSB (Toczyski et al., 1997). During the process of adaptation, a cell retains the DSB and yet the DNA damage signal that causes cell cycle arrest is eliminated in the presence of damage, allowing the cell to continue through mitosis. It is thought that cells that are defective for adaptation are defective for turning off the DNA damage checkpoint. One way to test how long the checkpoint is active is to check the levels of Rad53p phosphorylation. We found in a strain containing H4S1A that loss of H4S1phos does not inhibit the ability of the cell to turn off the checkpoint, as Rad53p phosphorylation is decreased to pre-damage levels by 18 hours in both WT and H4S1A cells.

In an effort to determine how H4S1phos is enriched at the break we looked at the recruitment of the catalytic subunits to a DSB. We were not able to see any enrichment above background. We tested the hypothesis that H4S1 was phosphorylated in the cytoplasm and recruited to the site of the break. In the chromatin fractionation assay we did not see an enrichment of H4S1phos in the cytoplasm, even after arresting the cells with HU to enrich the cytoplasmic pool of histones, immunoprecipitation of the cytoplasmic fraction to enrich for the modification, or concentrating the cytoplasmic fraction. Also, the Allis lab showed that after release from α-factor into the cell cycle, here is no S-phase specific increase in H4S1phos that would show that this is a modification of newly synthesized histones. We also tested whether the histone chaperones were necessary to enrich for H4Sphos. We tested different combinations of chaperone delete strains and fond that members of the CAF-1, RCAF, and HIR complexes are dispensable for enrichment of phosphorylation.

We therefore focused on this being a nuclear modification, and wanted to learn how CK2 was recruited to the site of the break. Although CK2 itself has not been localized to the break in higher eukaryotes, it is known to be necessary for localization and retention of NBS1 to the break (Melander et al., 2008). CK2 is also known to associate with many chromatin-related proteins including transcription factors and the histone proteins.

We chose to focus on the interaction between CK2 and the SIN3/RPD3 complex. Deletion of either *sin3* or *rpd3* yields a phenotype in response to DNA DSBs. In a model of chromatin restoration at the site of a break, "Access, Repair, Restore," from the Cote lab, it is postulated that H4S1phos inhibits NuA4 HAT action. Acetylation, an early modification enriched at the break, amongst other histone modifications and chromatin remodeling enzymes, allows access to the DNA for the repair process. H4S1phos is a later modification, which prevents further rounds of acetylation by NuA4, helping to restore chromatin to its original status.

We found that acetylation inhibits CK2 activity *in vitro*. Using GST H4 tails, in which the lysine residues were mutated to glutamine to mimic acetylation, we found that acetylation inhibited CK2 activity. We also found that the SIN3/RPD3 complex is necessary for enrichment of H4S1phos at the site of a break. Testing a strain in which the catalytic subunit of *RPD3* has been rendered inactive, we found that the HDAC activity of *RPD3* is unnecessary for H4S1phos enrichment. It is possible, however, that the chromatin around the break is deacetylated in the absence of the SIN3/RPD3 complex. Other HDACs are recruited to the break which could be redundant with the SIN3/RPD3 complex. It would be interesting to test the acetylation status in the different HDAC mutants to see if the levels decrease over time as they do in WT.

We find that phosphorylation at the break requires *RPD3* and not *SIN3*. Although most work thus far on the HDAC activity of *RPD3* focuses on the necessity of *SIN3* for its role in transcriptional regulation, recent evidence shows that not all *RPD3* in the cell is in a complex with *SIN3* (J. Worman, unpublished results, personal communication). Little work has been done on the role of *RPD3* in the DNA DSB response. It is possible that in the presence of DSBs, the *RPD3* catalytic subunit forms a DNA –damage specific complex, which lacks *SIN3*, but includes CK2. This complex can be directed to the site of a break. Purification of a TAP-*RPD3* complex from cells which have been treated with zeocin, followed by mass spectrometry, would be a simple way to test this hypothesis.

With this new data we can add to the "Access, Repair, Restore" model. Acetylation which immediately follows the DSB inhibits CK2 action at the break. Acetylation allows for access of the repair machinery. Following break repair, chromatin must be restored to its original state. Recruitment of various HDAC complexes allows for deacetylation of the chromatin and phosphorylation of H4S1. This phosphorylation prevents further rounds of acetylation and allows the chromatin to be restored.

H4S1phos and chromatin compaction

H4S1phos also appears to have a role outside of the context of a DNA DSB. A report from the Berger lab reported that this is a modification enriched during meiosis throughout eukaryotes. They hypothesized that it aids chromatin compaction based on phenotypes found in yeast. In a strain lacking H4S1phos, sporulation efficiency is decreased and nuclear volume is increased during meiosis. The modification is enriched throughout the genome and remains until the cell resumes the cell cycle. MNase digestion of chromatin undergoing the sporulation

program yields a resistant band in the WT cells but not in the cells which lack H4S1phos, possibly due to lack of enzyme accessibility in the WT due to chromatin compaction.

The AUC is an ideal instrument to study the contribution of a single modification to chromatin folding. Chromatin arrays can be reconstituted from purified components. Array folding in the AUC in the presence of a divalent cation has been well characterized, but the study of the contribution of single histone modifications to chromatin folding has just begun.

The effect of H4K16ac has been shown to influence chromatin folding as seen in the AUC. *In vivo*, K16ac is enriched in active euchromatin. In the AUC, arrays reconstituted with H4 acetylated at K16 do not reach maximum compaction levels. Acetylation of K16 shows a folding defect similar to an array reconstituted with a tailless H4.

The study of HS1phos on chromatin compaction was not as fruitful. In an original reconstitution where serine 1 was substituted with glutamic acid, we did see a folding phenotype. We found that with 1mM Mg^{+2}, half of the sample self-associated. We later discovered that these arrays were reconstituted using a histone H4 in which the initiator methionine was not cleaved off during recombinant expression in *E. coli*.

To aid the loss of the initiator methionine we studied an array in which the initial serine was maintained and a glutamic acid was inserted at the second position. Mass spectrometry revealed that the initiator methionine was cleaved off of this species. Using this H4 in the chromatin reconstitution showed that H4S1phos, by itself, does not contribute to chromatin folding.

This does not mean, however, that it does not contribute to compaction *in vivo*. It could recruit downstream proteins which aid compaction. It could also have a redundant modification

which helps enhance compaction. The phenotype of the H4S1A mutant needs to be further dissected to determine how serine 1 phosphorylation enhances compaction during sporulation.

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APPENDIX

Yeast Strains Used for Thesis Research

CY915	*Δho Δhml::ADE1 MATa Δhmr::ADE1 ade1-110 leu2,3-112 lys5 trp1::hisG ura3-52 ade3::GAL10:HO*
CY924	isogenic to CY915 but *MATa*, with *HML* and *HMR*
CY1019	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*
CY1033	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 rad9Δ::kan^r*
CY1034	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 rad17Δ::kan^r*
CY1350	CY915 *cka2Δ::kan^r*
CY1351	CY915 *rpd3Δ::kan^r*
CY1352	CY915 *sin3Δ::kan^r*
CY1159	*MATa ade2-101, his3-Δ200, leu2-801, ura3-52, trp1-Δ63*
CY1160	*MATa ade2-101, his3-Δ200, leu2-801, ura3-52, trp1-Δ63, ckb1Δ::HIS3, ckb2Δ::LEU2*
CY1067	*MATα ura3-52 leu2-3,112 trp1-289 his3Δ1 Δ(hht1 hhf1) Δ(hht2 hhf2) pJH18 (CEN ARS TRP1 HHT2 HHF2)*
CY1068	*MATα ura3-52 leu2-3,112 trp1-289 his3Δ1 Δ(hht1 hhf1) Δ(hht2 hhf2) pTK (CEN ARS LEU HHT2:hhf2 S1A)]*
CY1194	*MATa, leu2-3; ura3-1; his3-11,15; trp1-1; ade2-1; can1-100*
CY1199	*MATa, leu2-3; ura3-1; his3-11,15; trp1-1; ade2-1; can1-100; caclΔ::LEU2; asf1Δ::TRP1*
CY1375	CY915 *cka2-myc::kan^r*
CY1418	CY1351 with CP1126 (Yeplac112, *RPD3*, 2μ, TRP1, Amp, Ori)
CY1419	CY1351 with CP1127 (Yeplac112, *rpd3-H150,151A*, 2μ, TRP1, Amp, Ori)