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Characterization of Histone Mutants Associated with Mitotic Defects in Saccharomyces cerevisiae

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Characterization of Histone Mutants Associated with Mitotic Defects in *Saccharomyces cerevisiae*

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Abstract

Nucleosomes, the basic unit and the building blocks of chromatin have an essential role in the tight packaging of DNA into higher order chromatin. Work from our lab and others have provided information on the contributions of different histone proteins and specific domains within the nucleosome made to create the centromeric chromatin structure required for normal chromosome segregation during mitosis. The DNA entry/exit site is a particular region of the nucleosome where histone H2A, H3 and H4 form critical interactions that appear to be essential for the association of Sgo1, a tension sensing protein that monitors kinetochore-microtubule attachment. In our study, we first characterized histone H2A mutants with respect to their chromosome segregation phenotypes. Three mutations that show such phenotypes were in the C-terminal region of H2A, which is located in the DNA entry/exit region of the nucleosome, in close proximity to H3 and H4 residues that show severe chromosome segregation defects when mutated. We then created a double mutant strain that incorporated two single mutations, one in H2A and one in H4, to study their combined effect in chromosome segregation and normal cell cycle progression. The H2A N115S residue, positioned in the C-terminal tail of histone H2A, and H4 K44Q, positioned in H4 L1 histone fold domain, both falling in the region of DNA entry/exit point of the nucleosome, were incorporated in our double mutant. We found that the incorporation of the H2A N115S mutation alleviated the growth defect of the H4 K44Q single mutant and fully suppressed its increase-in-ploidy phenotype. We also found that overexpression of Sgo1 suppressed the sensitivity of both single mutants and the double mutant to the microtubule depolymerizing drug benomyl. We conclude that histone-histone interactions within the DNA entry/exit point of the nucleosome are particularly important in chromosome segregation, most likely in establishing centromere-kinetochore attachments during mitosis. In

addition, and consistent with a previously noted role of the DNA entry/exit point, this nucleosomal region creates a unique surface required for the recruitment of Sgo1, and perhaps other proteins, such as components of the CPC, required for normal microtubule attachment at the centromere.

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Dedication

Dedicated to my father

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Introduction

Since the emergence of life in the form of a single cell, its activity, behavior and survival are being imprinted and carried on from generation to generation in its blueprint, the DNA (Deoxyribonucleic acid) molecule. The double stranded DNA forms the basis of existence and development of every organism, dictates its behavior as it grows up to adult form. This vital molecule of life therefore needs to be packaged and stored in a sophisticated manner inside every single cell of every living organism. The most important proteins take parts in the process are histones. The very long DNA molecule that constitutes each chromosome must be condensed and tightly packaged and then needs to be segregated in proper manner to the daughter cells to maintain the integrity of life and for the survival of the organism. The DNA therefore is packaged into chromatin with the array of nucleosomes in a “beads on a string” manner where the beads are nucleosomes and the string is the DNA molecule. The chromosome number varies in different organisms. They vary both in number and length in terms of DNA content. The histone-DNA interaction in this super compaction is very important in maintaining the integrity, faithful division and segregation of the genetic information.

In our current work we focused on two of the four histone proteins and after initial screening of some potential mutants of those histones we picked two of the mutant candidates, each carrying mutation in a different histone and generated a double mutant through crossing up to several generations. We compared the effect of the double mutation on the organism to the single mutations, tried to detect any significant role of the double mutation in terms of the interactions between two altered amino acid residues from two histones with their neighboring residues and

the DNA molecule. Histones are small positively charged basic proteins which interact with the negatively charged sugar-phosphate backbone of the DNA.

In our current study, we chose to work on *Saccharomyces cerevisiae* as the model organism to look at the role of histones. Out of the many advantages that this simplest unicellular eukaryote offers, the important ones are listed below [Goffeau A et al., 1996]:

- *Saccharomyces cerevisiae* is a single cell organism and hence the simplest among eukaryotes.
- Can be easily grown in synthetic media.
- It has a short generation of around 2 hours which allows us for easy genetic analysis.
- Amenable to genetic manipulation i.e. easy to mutate, add or knock out genes due to efficient homologous recombination and
- It is nonpathogenic.

***Sachharomyces cerevisiae*: A closer look**

Commonly known as Baker's yeast, *Saccharomyces cerevisiae* is the simplest among all eukaryotic life forms, which can alternate between haploid and diploid states making it ideal for genetic analyses such as gene complementation and performing genetic crosses [Sherman F, 2002]. It has 16 chromosomes in its haploid state and the organism has two mating types "a" and "α". In minimal media, a diploid yeast cell sporulates and gives rise to four haploid cells through

meiosis when favorable environment is given and the characteristics are passed on to these daughter cells in a standard Mendelian fashion. The haploid yeast cell having one copy of each chromosome is suitable for genetic manipulation and analyzing the phenotypes. The two haploid yeast cells of opposite mating types are selected for genetic crosses to create desirable genotypes and thus observing the phenotypes afterwards and analyzing the biomolecular and biochemical changes inside the organism of different pathways. This technique can help us elucidate novel functions and interactions of different biomolecules of different pathways. In our current study, we deploy this very technique to see the outcome of altering a single residue in each of the two different histone proteins (H2A and H4) essentially by incorporating a point mutation in each case. *Saccharomyces cerevisiae* genome has approximately 6000 ORFs (open reading frames) with little intervening sequences and thus offers an easy model to study the effect of altering particular genes. Moreover, it has only two copies of each histone genes comparing to higher eukaryotes, which made our task easier of observing histone gene's functions and interactions that affect chromosome segregation and cell division.

Chromosome segregation and chromatin:

In living organisms, the life's blueprint, the DNA, is stored in the safest place of the cell, the nucleus, from where copies of the blueprints are made in the form of RNA and they are transported outside of the nucleus to cytoplasm for decoding into proteins. The DNA, however, is too large to be accommodated in its extended form in the nucleus and needs tight packaging into chromosomes. And only because of this, the otherwise 2 m long human DNA can be accommodated in the 5-10 μm diameter containing nucleus. In fact, The DNA of eukaryote cell

is never naked. Regardless of its location either in transcriptionally active chromatin or inactive chromatin state, the so called euchromatin or heterochromatin respectively, the DNA is bound with many protein molecules. The complexes between eukaryotic DNA and proteins are called chromatin, which typically contains about twice as much protein as DNA. Among all of these proteins, the histones are the major ones. They are extremely abundant in eukaryotic cell. Taken together, their mass is approximately equal to that of the cell's DNA [Cooper GM, 2000].

The chromosomes need to be distributed properly in the daughter cells during cell division, a process termed chromosome segregation. For proper chromosome segregation, a physical connection between spindle microtubules and chromosomes need to be established during cell division process. This connection is made possible by proteinaceous structures known as kinetochores on centromeric DNA [White CL et al., 2001]. In the process of segregation, many proteins' function must be activated and inactivated in an orderly temporal and spatial manner which make possible the segregation of sister chromatids to opposite poles of a mitotic spindle [Kim J et al., 1999]. The spindle of a dividing cell has two poles and each duplicated chromosome contains two attachment sites, the kinetochores. Attachment of the kinetochores to the opposite poles ensures the distribution of each copy of duplicated chromosomes in each daughter cell. This attachment to the spindle microtubules produces force and tension, ensuring that each replicated chromosome sister chromatid moves to the pole to which its kinetochore is attached [Nicklas RB, 1997].

Chromatin is composed of nucleosomes, which are the universally repeating protein-DNA complexes in eukaryotes and the structure and functions of the nucleosome core particles is conserved among all eukaryotes [White CL et al., 2001].

Nucleosome, histones and histone modifications:

The basic unit and the building blocks of chromatin are the nucleosomes. DNA in chromatin is organized in arrays of nucleosomes: Two copies of each histone protein H2A, H2B, H3 and H4 are assembled into an octamer with 146-147 base pairs (bp) of DNA wrapped around it to form the nucleosome core. The repeating nucleosome core is further and gradually compacted into higher order chromatin structure and hence this nucleosome plays a role as the principal packaging element of DNA within the nucleus and is the primary determinant of DNA accessibility [Luger K et al., 1997]. Around the histone octamer, the 146 bp of DNA are wrapped in 1.65 turns of a flat, left handed superhelix where the DNA sequence binds the octamer with a central base pair at the pseudo-two fold axis of the particle such that DNA is divided into 73- and 72-bp halves, with one base pair falling on the dyad [Luger K et al., 1997]. Many groundbreaking studies that deal with the complex interplay between higher order chromatin and regulation of transcription arise from yeast genetics. This has been made possible for two characteristics of yeasts: First, the *Saccharomycers cerevisiae* has only two genes for each of the core histones and many of the characteristics of chromatin in higher organisms are also seen in yeast such as histone variants, for example, the histone H2A variant H2A.Z (HTZ1) and the centromere specific H3 variant CenpA (Cse4) [White CL et al., 2001; Hartzog and Winston, 1997; Santisteban et al., 2000, Glowczewski et al., 2000]. It also uses targeted ATP dependent chromatin remodeling factors and reversible histone tail modifications such as acetylation, and methylation [White CL et al., 2001].

The nucleosomes in yeast are closely spaced with a repeat length of 162 ± 6 bp, resulting in a shorter linker length of 15-20 bp than the 175 to 240 bp repeat length in metazoans [Horz and

Zachau, 1980; White CL et al., 2001]. The core histones share a structurally conserved motif called “histone fold” about 70 amino acids long. The extensions of histone fold are responsible for defining the surface of the nucleosome, mediating protein-protein interactions within the nucleosome and contribute to DNA binding. Other than this extension, there are parts of histones that extend from the confines of DNA superhelix, and are termed as histone tails [Luger and Collins, 2001]. These flexible N-terminals emanate from the nucleosome and important for nucleosome-nucleosome interactions and have a role together with linker histone H1 and other nonhistone proteins to form the higher order chromatin [White CL et al., 2001]. The histone fold is made up of three alpha helices connected by two short loops in a α1- loop1- α2 - loop2- α3 manner creating a shallow slot [Luger and Collins, 2001]. The principal DNA-binding sites in the nucleosomes are the eight L1L2 loop and four alpha helix structures in the histone folds [Luger K et al., 1997]. Nucleosome assembly is promoted by histone chaperones [Loyola and Almouzni, 2004] that stimulate the deposition of histones onto DNA. Some of these chaperones have been reported to work in tandem with ATP dependent remodeling factors. The nucleosome array resulting from histone deposition onto DNA gives rise to 30 nm diameter chromatin fibers by further compaction in association with linker as well as non-histone proteins. In this fashion, chromatin is eventually organized into functional domains, such as constitutive heterochromatin, which is located at defined chromosome regions and stably maintained by specific epigenetic marks such as H3K9 methylation and HP1 binding. However, this stable association is challenged at each cell cycle during DNA replication [Polo and Almouzni, 2006] when cell needs to proliferate and distribute its genetic machinery into the daughter cells in correct proportion. In events such as transcription, most DNA-binding factors need DNA to be disassociated from histones to allow access. Because of the fact that histone octamer is bound to

DNA at multiple sites, histone disassociation would occur in semi-cooperative stages beginning at the entry and exit points of DNA, then proceeding into H2A-H2B dimer and eventually into H3-H4 tetrameric regions. For example, SWI/SNF (SWItch/Sucrose Non-Fermentable) nucleosome remodeling complexes, found predominantly at the H4 L1 loop and nearby H3 L2 loop, are required to allow transcription in the presence of nucleosomes by making chromatin accessible to transcription factors [Luger K et al., 1997].

Histone proteins are the basic building blocks of chromatin and therefore this is no surprise that the four core histones, H2A, H2B, H3 and H4 are among the most evolutionarily conserved proteins known [Van Holde, 1988]. Not only sequence conservation is enough, but also maintaining proper histone levels are essential for transcription, chromosome segregation and other related cellular processes. *S. cerevisiae* possesses two genes encoding each of the four major core histones, plus single genes encoding H2A.Z (*HTZ1*), centromeric H3 (*CSE4*), and H1 (*HHO1*). The major core histone genes are organized into four loci in the genome each containing two histone genes divergently transcribed from a central promoter. The cell requires at least one *HHT-HHF* locus for survival, indicating that H3 and H4 are essential [Smith and Andresson, 1983; reviewed by Eriksson PR et al., 2012]. The histones H2A and H2B are encoded by two gene pairs, named *HTA1-HTB1* located in chromosome IV and *HTA2-HTB2* located in chromosome II. When *HTA2-HTB2* is deleted, *HTA1-HTB1* dosage compensates at the transcriptional level. The other two histones, histone H3 and H4 are encoded by two gene pairs *HHT1-HHF1* located in chromosome II and *HHT2-HHF2* located in chromosome XIV [Libuda and Winston, 2006 and yeastgenome.org] (Figure1). Luger K et al., in 1997 and some other studies demonstrated that the C-terminal histone fold domains of core histones possess similar conformations and take part in histone-histone and histone-DNA interactions which is a critical

role in nucleosome assembly. However, N-terminal tails are not essential for this purpose and are thought to make more flexible contacts with DNA and nucleosomes possibly by possessing sites for ATP dependent chromatin remodeling factors such as Swi/Snf [Luger K et al., 1997; Lee KM et al., 1999, Cheung P et al., 2000]. However, sequence-dependent positioning of nucleosome can be specifically altered by regulated changes in histone tail-DNA interactions in chromatin [Yang Z et al., 2007]. Another way the flexible N-terminal tails participate in altering the chromatin structure is that these tails can be subjected to a wide and diverse array of post-translational modifications (PTMs) such as acetylation, phosphorylation, methylation, ubiquitylation, etc. that can modulate the contacts between histone and DNA. Also as these modifications are reversible, they can act as chromatin based “on/off” switches and thus can regulate a wide array of DNA related processes [Cheung P et al., 2000; Cosgrove and Wolberger, 2005]. Because of the fact that distinct histone post-translational modifications correlate with specific transcriptional states, a histone-code hypothesis has been proposed [Fischle W et al., 2000; Strahl and Allis, 2000; Turner BM, 2000] which suggests that specific patterns of modifications are read like a molecular bar code which recruit the cellular machinery to form a distinct chromatin state. Although there is a great amount of evidence in support of the histone code recruiting nonhistone proteins to the chromatin fiber, the molecular details by which these proteins shape chromatin dynamics remain to be elucidated. Dorigo B et al., (2003) showed that deletion of all 4 histone tails, with the exception of residues 14-19 at the base of the H4 tail, did not affect the nucleosome folding, ruling out the need of most of the tails for 30 nm fiber formation and establishing the role of the part of H4 histone in chromatin compaction through internucleosomal interaction [Cosgrove and Wolberger, 2005; Luger K et al., 1997]. The importance of the structured histone globular domain, the histone fold, was indicated by genetic

screens in yeast with the identification of numerous globular-domain amino-acid residues important for gene expression [Kruger W et al., 1995; Park JH et al., 2002]. Later on, it was proposed that histone post-translational modifications could be separated into two functional categories: class I and class II histone-code modifications [Cosgrove MS et al., 2004] where class I modifications regulate the recruitment of effector domains, such as the bromo and chromo domains targeting nonhistone proteins to DNA; whereas class II modifications regulate histone-DNA and histone-histone interactions, mainly by direct chemical interference [Cosgrove and Wolberger, 2005]. The properties of histones, and therefore nucleosomes, can be altered by these post-translational modifications, such as addition or removal of acetyl, methyl, phosphoryl, ubiquityl and sumo groups to regulate diverse processes of gene activity, gene silencing by heterochromatin, replication, apoptosis, and DNA damage responses. In histone H3 from most species, the main acetylation sites include lysines 9, 14, 18 and 23 [Van Holde, 1988; Strahl and Allis, 2000].

However, most single histone proteins are not modified at every site, for example, although H4 can potentially be acetylated at four N-terminal lysine residues in *S. cerevisiae* (K5, K8, K12 and K16), 12% of total H4 isolated from yeast cells is not acetylated at these N-terminal lysines, 36% of total H4 is acetylated at one lysine, 28% at two, 13% at three and 12% at four lysine residues [Reviewed by Millar and Grunstein, 2006]. It has been shown for histone H4 that potentially active euchromatin can be modified at all the H4 acetylable lysines (K5, K8, K12, and K16). On the other hand H4 in heterochromatin is hypoacetylated [Clarke DJ et al., 1993]. The HATs (Histone Acetyl Transferases) participate in gene activation and the HDACs (Histone Deacetylases) are implicated with gene repression. In other words, HATs are associated with the active state of chromatin or euchromatin and HDACs are associated with the inactive state of

chromatin or heterochromatin. Indeed, transcription factors activate or repress transcription by associating with HATs or deacetylases as appropriate [Grunstein M, 1997]. Histone PTMs, although considered to mainly occur at the N terminal tails of histones, in fact also exist in the histone fold domains, and the lysine residue is a major target for histone PTM. Since lysine to glutamine (KQ) substitution is known to mimic the acetylated states of particular histone lysine residues in vivo, a recent study has prepared mutant nucleosomes containing K to Q substitutions and found that the C-terminal region of H2A was significantly disordered in the nucleosome containing H4 K44Q [Iwasaki W et al., 2011]. Another recent study using a library of histone point mutations in *S. cerevisiae* divided 24 histone residues into three spatially separated nucleosomal regions designated TBS-I, -II, and -III (TBZ/benomyl-sensitive regions I–III) based on the mutant's response to microtubule depolymerizing drugs. It suggested that the spatially separated nucleosomal regions, TBS-I and -II, are necessary for Sgo1 (a key modulator for chromosome bi-orientation) mediated chromosome bi-orientation and that TBS-III is important for Htz1 function [Kawashima S et al., 2011]. Our double mutant has a H2A N115S mutation and this belongs to this TBS-I region in the aforementioned work. The double mutant that we created in this present study contains both of these H4 K44Q and H2A N115S mutations making it interesting to see what their concerted effect is on the nucleosome structure and chromosome segregation.

Recent high-throughput technologies, for example "ChIP-chip" and "ChIP-seq," gave rise to high-resolution maps for many histone modifications on the human genome [Yu H et al., 2008]. By chromatin immunoprecipitation (ChIP) it has been made possible to isolate short DNA segments associated with a modified histone. ChIP, together with the whole genome microarrays

(ChIP-on-chip) has been used by a group to map the distribution of 20 of the already known modifications of H3, H4, H2A, H2B and the H2A variant H2A.Z across all 16 *Saccharomyces cerevisiae* chromosomes [Millar and Grunstein, 2006; Kurdistani SK et al., 2004].

After acetylation, another major type of histone PTMs is the histone methylation. H3 and H4 are the predominant histones that are methylated and sequencing studies from many organisms indicate that multiple lysines in H3 are being preferred for methylation. In histone H3 lysine (K) methylation occurs on residues 4, 9, 27 and 36, and in H4 lysine methylation occurs on position 20. Moreover, lysine residues can be mono-, di-, or tri-methylated, adding yet another layer of complexity to the so called histone mark. Lysine methylation is also present at the H1 amino terminus [Van Holde, 1988; Strahl and Allis, 2000]. The active histone marks in histone H3 are H3K4me, H3K36me, and H3K9ac [Turner BM, 2005]. In contrast, H3K9me and H3K27me and hypoacetylated forms generally constitute transcriptionally silent regions of chromatin. Additional studies challenge this simple distinction with a more complex partitioning of genome marking by specific PTMs. For example, H3K9me3 is also associated with several actively transcribed genes [Loyola A et al., 2006; Turner BM, 2005]. Indeed, histone modifications have important roles in biological regulation. For example, acetylation of histone tails neutralizes the positive charge of lysines and significantly alters chromatin behavior. Also, specific lysine methylation on histone tails can increase the affinity of binding counterparts on a variety of regulatory proteins thought to act by modulating chromatin compaction [Reviewed by Henikoff and Shilatifard, 2011]. In another study, it has been demonstrated that histone H4 lysine-16 acetylation regulates cellular lifespan [Dang W et al., 2009], and methylation of H3 K79 and H4 K20 are the main modifications involved in DNA repair, hence, indispensible to the integrity of

the cell's genome [Williamson and Pinto, 2010]. Similar to the enzymes responsible to add or remove acetyl group, there are two types of enzymes to add or remove methyl groups from the amino acid residues in different histones. For example, Set1, a HMT (Histone Methyl Transferase), which methylates H3K4, associates preferentially with coding regions. Set1 interacts with RNA polymerase II (Pol II), which is phosphorylated at serine 5 of its C-terminal domain (CTD) during the start of transcription. Another HMT, Set2, which methylates H3 K36, is also associated with coding regions of genes, by interacting with Pol II, with its CTD phosphorylated at serine 2. H3 K79 is methylated by the HMT called Dot1p, and because the pattern of localization of histone H3 on lysine 79 appears to be broad, this modification is assumed to take place throughout the open reading frame [Reviewed by Millar and Grunstein, 2006; Shilatifard A, 2006]. To remove the methyl group from histones, various pathways also exist including enzymatic demethylation (carried out by histone demethylases), replacement of H3 by the variant H3.3, and clipping of the N-terminal tail [Bannister AJ et al., 2002].

The clearest function of histone phosphorylation is observed when phosphorylated histone H2A (H2AX) demarcates large chromatin domains around DNA breakage. But, multiple studies have also shown other crucial roles of histone phosphorylation associated with chromatin remodeling implicated with other nuclear processes. There is no the H2AX variant in yeast and the corresponding phosphorylation occurs on S129 of H2A. In histone H3, phosphorylation of serine 10 and 28, and histone H2B serine 32 have been associated with proliferative gene transcription such as EGF (Epidermal Growth Factor), a transcription related to cell proliferation. Besides, although H3 T3, S10, T11 and S28 have been found associated with chromosome condensation and segregation, phosphorylation of H3 S10 is likely the best-documented mark related to

chromatin condensation [Gurley LR et al., 1978; Wei Y et al., 1999]. It has been reported that H3 phosphorylation at Ser10 is governed by the activity of Aurora/Ipl1 kinases in competition with the phosphatase activity of the serine/threonine protein phosphatase 1 [Cimini D et al., 2003]. In yeast, serine 122 of histone H2A is phosphorylated together with the nearby H2A S129, which are similarly modulated in DNA damage [Reviewed by Rossetto D et al., 2012]. Another recent study showed that the phosphorylation of histone H3 T3 mediated by Haspin cooperates with Bub1-mediated histone H2A S121 phosphorylation to target the CPC to the inner centromere in fission yeast and human cells [Yamagishi Y et al., 2010]. Ubiquitylation of histones has also been reported. Rad6 protein, a ubiquitin conjugating enzyme attaches ubiquitin to selected lysines in histones. In yeast, Rad6 attaches ubiquitin to lysine 123 in the C-terminal tail of H2B and this ubiquitination seems to be essential for methylation of both H3 K4 nad H3 K79 and thus responsible for appropriate pattern of gene silencing [Turner BM, 2002]. Later, Geng and Tansey demonstrated that some of the known biological effects of ubiquitylation of H2B are exerted via polyubiquitylation, rather than a single ubiquitin group [Geng and Tansey, 2008].

Due to the fact that there is a plethora of different histone modifications, the various levels of cross talk that exist between and within cellular and histone signaling pathways are easily appreciable. For example, certain modifications together give rise to a same outcome: histone H3 S10 and S28 phosphorylations, for example, both have been detected in condensed chromosomes during mitosis and meiosis. Ubiquitylation of histone H2A has been found to have an inhibitory effect on Aurora B, the primary kinase for H3 Ser10 and Ser28 phosphorylation. Histone H2A needs to be deubiquitylated by the enzyme Ubp-M to allow M-phase progression by proper

chromosome condensation during mitosis initiation. Sometimes, modification of one residue prevent binding of another particular protein to an adjacent residue, such as phosphorylation of H3 S10 affects the binding of the HP1 protein to the stable methyl mark H3K9me during mitosis, while dephosphorylation of H3 S10 promote the binding of HP1 back to methylated H3K9 [Reviewed by Banerjee T et al., 2011]. Inhibition or depletion of the mitotic kinase Aurora B, which phosphorylates serine 10 on histone H3, causes retention of HP1 proteins on mitotic chromosomes, suggesting that H3 serine 10 phosphorylation is necessary for the dissociation of HP1 from chromatin in M phase [Fischle W et al., 2005]. Again, different transcription factors most likely target methyl transferase enzyme complexes to different promoters. The need for more than one enzymatic activity may reflect the need to wipe out one set of modifications before another can be deposited. For example, conflicting modifying signals impinge on lysine 9 of H3 where acetylation is stimulatory and methylation is repressive [Rea S et al., 2000 reviewed by Kouzarides, 2002].

Indeed, site-specific combinations of histone modifications correlate well with particular biological roles. For example, the combination of H4 K8 acetylation, H3 K14 acetylation, and H3 S10 phosphorylation is associated with transcription whereas methylation of H3 K9 and the lack of H3 and H4 acetylation is implicated with transcriptional repression in higher eukaryotes. Again, diacetylation of histone H4 at K4 and K12 is associated with histone deposition at S phase, whereas phosphorylation of histone H2A (S1 and T119) and H3 (T3, S10 and S28) appear to be hallmarks of mitotic chromatin condensation [Peterson CL et al., 2004]. The monoubiquitinated histone H2B is deubiquitinated by deubiquitinating enzymes, Ubp8 and Ubp10, for example, which can negatively regulate the methylase activities of Dot1p (the

enzyme that methylates H3K79) [Shilatifard A, 2006]. It has been hypothesized that the SAGA (Spt-Ada-Gcn5-acetyltansferase) complex exploits the rich structural complexity of chromatin to both regulate and adjust its acetyltransferase activity and later demonstrated that SAGA indeed takes part in cooperative acetylation [Li and Shogren-Knaak, 2008]. Altogether, the combinatorial and complex arrays of histone PTMs affect all chromosomal-related processes in the cell.

Centromere, kinetochore, Spindle Assembly Checkpoint (SAC) and Chromosomal Passenger Complex (CPC):

Faithful segregation of chromosomes during cell division depends on proper attachment of the sister chromatids with the microtubules at their centromeres through the proteinaceous kinetochores. Then sister kinetochores needs to be bioriented to the opposite poles of the spindle. Thus, the attachment needs to be broken again for proper mitotic exit and to prepare the new cell for a fresh S phase of the cell cycle before that cell divides again. This is why the chromosome biorientation (the ingression of sister chromatids at the metaphase plate) during the metaphase and before proceeding the anaphase is so important in cell division, the abnormality of which can give rise to abnormal proliferation as well as cell death. This phenomenon is extremely complex with the involvement of many regulators and modulators creating the unique structures, machinery, and mechanisms for cell division such as centromere, kinetochore, SAC (Spindle Assembly Checkpoint) and CPC (Chromosomal Passenger Complex).

Chromosome segregation takes place on a bipolar spindle-shaped structure that is built from microtubules, and chromosomes build a special structure to connect with these spindle

microtubules. These connections occur at the site of the primary constriction of condensed chromosomes, which was initially called the centromere. It is the region of chromosomal DNA that guides kinetochore assembly, a proteinaceous structure that associates with this DNA. The interactions between the kinetochore and spindle microtubules are at the heart of alignment and segregation of chromosomes on the spindle. Following breakdown of the nuclear envelope, kinetochores start to interact both laterally and in an end-on manner with spindle and by metaphase, all chromosomes become bi-oriented, with sister kinetochores exclusively connected to microtubules that emanate from opposite spindle poles. Here, the kinetochore monitors and ensures bi-orientation for every single chromosomes in the cell, the APC (Anaphase promoting complex) which was prevented thus far, now gets activated and the separated sister chromatids move to opposite spindle poles i.e. anaphase commences. Eventually, chromatids decondense, nuclear envelope reappears and two daughter cells are generated with exact copies of the replicated genome and thus genomic integrity is maintained [Cheeseman and Deshai, 2008]. The centromere is a specific domain on eukaryotic chromosomes that plays an essential role in the faithful chromosome segregation during both mitotic and meiotic cell divisions. Analysis of *S. cerevisiae* centromeres revealed that each entromere has a highly conserved 8-base-pair (bp) region known as centromere DNA element (CDE) I, and a 25-bp region of partial dyad symmetry known as CDE III. These two elements are separated from one another by a 78-86 bp long DNA region that is >90% A+T-rich, termed CDE II. The centromeric DNA is complexed with chromatin so that 220 to 250 bp core that is centered around CDE III is resistant to nuclease digestion of the chromatin DNA and is believed to be because of the interactions between CEN DNA, CEN-specific DNA-binding proteins, and histone proteins. Mutation experiments have shown that certain conserved bases, such as the central cytidine residue in CDE III, results in

centromere dysfunction. Likewise, deletion of CDE III yields a nonfunctional CEN. In contrast, when CDE I is deleted, the centromere retains partial mitotic function and changing CDE II bases results in decreased CEN function in a gradual manner [Saunders M et al.,1988]. Later, analysis of in vitro mutations generated in centromeric DNA has determined an essential role for specific base pairs in CDE III where these base pairs seem to be critically involved in the binding of CBF3, a multimeric complex. Also, analyses of a substantial number of deletions and insertions have established the necessity of A+T richness in CDE II element without conservation of a particular primary sequence. Together, these results hinted that the conformation or structural deformation of this element contributes to centromere function [Schulman and Bloom, 1993]. Although the budding yeast centromere is extremely short (125 bp) compared to those of other eukaryotes, the kinetochore that assembles on this DNA displays a rich molecular complexity [Cheeseman IM et al., 2002]. It has been demonstrated that a single CenH3 nucleosome forms the minimal unit of centromeric chromatin necessary for kinetochore assembly and proper chromosome segregation. Just like all other parts of the genome, the DNA of CENs (centromeres) is organized into chromatin, with the exception of having conserved CEN-specific histone H3 variant (CenH3, Cse4 in budding yeast) in place of canonical one, the conservation of which across eukaryotes and its strict CEN localization in all species identify it as the epigenetic CEN identifier. It has been reported the presence of heterochromatin in centromeric chromatin ensures a high density of cohesion recruited to centromeric regions, and could have additional roles in centromere architecture and the prevention of merotely, where a single kinetochore is attached to microtubules from both spindle poles, and also might act as a trigger for kinetochore assembly [Pidoux and Allshire, 2005]. Recent evidence indicates that Cse4 binds to the centromeric protein Scm3, which displaces histones H2A and H2B from the

octameric Cse4 nucleosomes and forms an unusual hexameric nucleosomal core in vitro and most probably in vivo. Even more surprisingly, an *S. cerevisiae* minichromosome-based in vivo assay reveals a right handed wrapping of centromeric DNA around the CENP-A containing nucleosome, which is presumably only enabled by a 'hemisome' configuration of the nucleosomal core [Ishii K, 2009; Meraldi P et al., 2006]. Other studies showed that CenH3, and its interacting partner, Scm3, both are essential for chromosome segregation and are evolutionarily conserved from yeast to humans. Restricting the localization of budding yeast CenH3, Cse4, to centromeres and balanced stoichiometry between Scm3 and Cse4, contribute to faithful chromosome segregation [Mizuguchi G et al., 2007]. Also, like other eukaryotic centromeres, budding yeast centromeric histone H4 is hypoacetylated, alteration of which affects chromosome segregation [Sullivan and Karpen, 2004]. Studies of regional centromeres have revealed a unique pattern of centromeric and pericentromeric histone H3 and H4 modifications. Centromeric histone H3 is methylated at lysine 4 (K4), which is typically associated with active transcription, and the pericentromeric histone H3 is dimethylated at lysine 9 (K9), which is usually associated with heterochromatic regions [Sullivan and Karpen, 2004; Choy JS et al., 2012].

In contrast to the complex CEN arrangement of higher eukaryotes, *Saccharomyces cerevisiae* contains a short, defined CEN that associates with a single microtubule making budding yeast an ideal system to study the essential components of centromeric chromatin necessary for proper chromosome segregation [Fitzgerald Hayes M et al., 1982; Furuyama and Biggins, 2007]. Despite the differences between yeast and metazoan centromeric DNA (125 bp versus megabases of centromeric DNA respectively), the kinetochores are organized around

centromeric nucleosomes that contain specialized histone H3-like proteins (yeast Cse4p or its metazoan homologue CENP-A). However, the inner kinetochore proteins do not associate directly with microtubules or the microtubule-binding components of the outer kinetochore. To resolve this situation, the “central kinetochore” proteins mediate the linkage between the inner and outer kinetochore proteins. Electron microscope studies of vertebrate kinetochores revealed that microtubule plus ends make end-on attachments with the kinetochore [Meluh PB et al., 1998; Ortiz J et al., 1999; Cheeseman IM et al., 2002]. Kinetochores can initially bind to microtubules in any configuration, but proper chromosome segregation requires that each pair of sister kinetochores ultimately attach to microtubules from opposite spindle poles (bi-orientation). Indeed, kinetochore-microtubule attachments must be carefully regulated where incorrect attachments are destabilized, while correct attachments are stabilized. Thus, all kinetochores eventually reach the correct attachment state in a trial-and-error process, with destabilization providing a fresh opportunity to bi-orient before segregation [Cimini D et al., 2001; Nicklas RB, 1997, Lampson and Cheeseman, 2011]. Accurate chromosome segregation is essential to ensure genomic stability because the aneuploidy that results from segregation errors gives rise to embryonic defects and may contribute to the development of cancer. The eukaryotic kinetochore serves some central roles during mitosis. First, it serves as the site of attachment of microtubules to the chromosome. Second, it contains motor proteins that orchestrate the movements of chromosomes during mitosis and finally the kinetochore serves as the site of assembly for the proteins of SAC (Spindle Assembly Checkpoint) machinery, which ensures proper attachment and assembly of chromatids before anaphase commences [Biggins and Walczak, 2003]. Each kinetochore is composed of a variety of conserved multi-protein complexes that form multiple connections between centromeric DNA on the “inner” side to microtubules at the “outer” edge of

the kinetochore. Different mechanisms of spindle assembly and kinetochore–microtubule attachment have been reported, among them the traditional mechanism is the ‘search-and-capture’ model of spindle assembly in vertebrate cells where a mono-oriented chromosome oscillates near the spindle and a microtubule from the opposite pole is captured by the sister kinetochore, so that the chromosome is now bi-oriented and can congress toward the metaphase plate. Another mechanism is the self assembly of the spindle around chromosomes by having microtubules nucleated in the vicinity of chromosomes [Cheeseman and Desai, 2008; Biggins and Walczak, 2003].

To prevent improper chromosome segregation, the spindle assembly checkpoint (SAC) signaling pathway is activated on unattached kinetochores to inhibit the metaphase-to-anaphase transition. The spindle checkpoint ensures the fidelity of chromosome segregation by ensuring amphitelic (bipolar or biorienting) attachment with microtubules and preventing syntelic (both sister kinetochores are attached to a single spindle pole), monotelic (only one of the sister chromatids is attached to a spindle pole) or merotelic (single kinetochore is attached to microtubules from both spindle poles) attachments by halting cell-cycle progression until all the chromosomes make proper bipolar attachments to the mitotic spindle and come under tension [Pinsky and Biggins, 2005]. The SAC is a surveillance system that can delay mitosis in response to either improper spindle organization, or an incorrect attachment of chromosomes to the spindle by halting metaphase-to-anaphase transition through communication with the machinery of ‘cyclosome’ or ‘anaphase-promoting complex’ (APC) so that anaphase will not occur. In eukaryotes, sister chromatids remain connected to each other from S phase until the onset of anaphase. This cohesion is essential for the separation of sister chromatids to opposite poles of the cell at the

correct time during mitosis, and it also allows sufficient time for proper chromosome segregation. A multi-subunit complex called cohesin is essential for connecting the sister chromatids. In *S. cerevisiae*, the cohesin complex consists of Smc1, Smc3, Scc1 (also known as Mcd1) and Scc3 during mitosis (although Rec8 replaces Scc1 in cells undergoing meiosis). All of these proteins are highly conserved between yeast and humans. Several reports demonstrated that the protease Esp1/ Separase cleaves Scc1 to induce sister chromatid segregation [Reviewed by Nasmyth K et al., 2000; Kitagawa and Hieter, 2001].

Gillett ES et al. in 2004 reported that proteins of the spindle checkpoint include the mitotic arrest defective (MAD) genes, *MAD1–3*, and the budding uninhibited by benzimidazole (BUB) genes *BUB1* and *BUB3*. Although mutations in almost all known kinetochore components engage the checkpoint, loss of function mutations CBF3 complex components (which consists of Ndc10p, Cep3p, Ctf13p, and Skp1p) and some subunits of the Ndc80 complex (which consists of Spc24p, Spc25p, Ndc80p, and Nuf2p) have the special property of abolishing the checkpoint. There are two main hypotheses regarding how kinetochore-microtubule attachment is monitored by the spindle checkpoint. The tension hypothesis proposes that the checkpoint monitors tension across paired sister kinetochores, whereas the attachment hypothesis suggests that the checkpoint monitors the occupancy of kinetochore-microtubule attachment sites. In budding yeast, Mad1p, Mad2p, Bub1p, and Bub3p are recruited to unattached kinetochores in *ndc80-1* cells and to kinetochores with monopolar attachments in *dam1-1* cells. Gillett ES et al., reported that they did not observe high levels of checkpoint proteins bound to kinetochores that have achieved bipolar attachment but lack tension and found their data most consistent with the attachment hypothesis. However, they also mentioned that it could be possible that lack of tension may cause the

transient binding of Bub and Mad proteins to kinetochores at levels that were below their limit of detection [Gardner RD et al., 2001; Stern and Murray, 2001; Rieder CL et al., 1995; Gillett ES et al., 2004].

It has been reported that a histone H3 mutation impairs the ability of yeast cells to activate the checkpoint in a tensionless crisis, leading to missegregation and aneuploidy. The defective tension sensing results directly from an attenuated H3-Sgo1p interaction essential for pericentric recruitment of shugoshin protein, Sgo1p, where reinstating the pericentric enrichment of Sgo1p alleviates the mitotic defects. Histone H3, and hence the chromatin, is a key factor transmitting the tension status to the spindle assembly checkpoint. Human and yeast shugoshin proteins collaborate with a specific form of protein phosphatase 2A (PP2A) to protect meiotic cohesin. A key player in correcting tension defects is the Aurora B kinase encoded by *IPL1* in budding yeast which destabilizes spindle-kinetochore attachment before anaphase nucleation, hence permitting correction of attachment errors. Luo J et al., in 2010 reported synthetic phenotypes when Ipl1p or one of its partners, Sli15p, was overexpressed in an *sgo1*$^{-}$ or H3 G44S mutant suggesting that increasing the detachment activity of Ipl1p/Sli15p brings about a molecular defect (i.e., spindle detachment) similar to that caused by benomyl treatment and lack of functional Sgo1p or intact H3 causes growth defects [Biggins and Murray, 2001, Luo J et al., 2010].

Additional research in this field has revealed the existence of an intricate signaling network created right on the kinetochore. It has been demonstrated that the SAC generates a cell cycle arrest by inhibiting the APC which ubiquitylates mitotic cyclins and pds5/securin to target them for proteasomal degradation to promote cell cycle progression and sister chromatid separation.

Erroneous microtubule attachment is destabilized by phosphorylation, mediated by Aurora B, of multiple microtubule-binding protein complexes at the kinetochore, such as the KMN network proteins and the Ska/Dam1 complex, while Plk-dependent phosphorylation of BubR1 stabilizes kinetochore-microtubule attachment by recruiting PP2A-B56 [Suijkerbuijk SJ et al., 2012]. Aurora B is a subunit of the chromosomal passenger complex (CPC), which also contains INCENP/Sli15, Survivin/Bir1 and Borealin/Nbl1 (also known as Dasra). The CPC is localized to the inner centromere during metaphase (which resides in chromosome arms during prophase) where it controls kinetochore-microtubule attachment and the SAC. The fact that spindle assembly checkpoint (SAC) signaling depends on kinetochore recruitment of the kinase Bub1 through Mps1-mediated phosphorylation of the kinetochore protein KNL1 (also known as Blinkin in mammals, Spc105 in budding yeast, and Spc7 in fission yeast) and recruitment of protein phosphatase 1 (PP1) to KNL1 is necessary to silence the SAC upon bioriented microtubule attachment indicates the presence of a phosphor-switch for proper chromosome biorientation. While Aurora B-dependent destabilization of erroneous attachments helps correct these improper attachments but also creates unattached kinetochores, which would activate the SAC [Biggins and Murray 2001; Pinsky et al., 2006; Tanaka TU et al., 2002; Funabiki and Wynne, 2013]. It would be worth noting here that CPC showed a potential role in tumorigenesis and as an anti-cancer therapeutic target as deregulated Aurora B was found to induce genomic damage [Kitagawa and Lee, 2015].

Two other important components of checkpoint, Bub1 kinase and Sgo1, act together to ensure efficient biorientation of sister chromatids during mitosis. Bub1 kinase is not required for most spindle checkpoint arrests, but is required for the response to reduced cohesion. Cohesion holds

replicated chromosomes together until every pair of sister chromatids is bi-oriented on the mitotic spindle, with the two sisters attached to microtubules that emanate from opposite poles of the spindle and generating tension by proper biorientation [Indjeian VB et al., 2005]. Bub1kinase domain acts to target Sgo1 to budding yeast centromeres and their functions become particularly important during spindle reassembly after antimicrotubule drug treatment, probably because the cells fail to respond to kinetochores under tensionless crisis, and that they are unable to correct syntelic attachments where both sister chromatids attach to microtubules from the same spindle pole [Fernius and Hardwick, 2007]. In budding yeast, Bub1 phosphorylate histone H2A (S121) and recruit the protector of cohesin, Shugoshin (Sgo1) to the inner centromere and, in the process, softens the chromatin spring in response to changes in microtubule dynamics [Haase J et al., 2012]. In fact, centromeric enrichment of Aurora B is regulated by positive feedback involving two histone kinases: Haspin kinase phosphorylated H3-pT3 promotes nucleosome binding of survivin, whereas Bub1 kinase phosphorylated H2A-S121 facilitates the binding of shugoshin, the centromeric CPC adaptor. Haspin colocalizes with cohesin by associating with Pds5, whereas Bub1 localizes at kinetochores. Thus, the intersection of these two histone phosphorylation marks define the inner centromere in yeast and human [Yamagishi Y et al., 2010].

In fission yeast, a Bub1 kinase-dead mutant, a histone H2A S121A mutant, deletion of Sgo2 (the sole mitotic form of shugoshin in fission yeast), and Cdk1-phosphorylation site mutants of Survivin defective in Sgo2-binding all showed comparable defects in chromosome segregation. Aurora B shows preferential accumulation on centromeres of unattached/misaligned chromosomes by a Plk1-dependent mechanism in untransformed cell lines, which found not to

be true for HeLa cells. Instead, removal of the CPC from the centromere depends on Cdk1-inactivation, the process that requires SAC silencing [Salimian KJ, et al., 2011; Kawashima S, et al., 2010; Tsukahara T et al., 2010; Hummer and Mayer, 2009; reviewed by Funabiki and Wynne, 2013]. Histone gene dosage has also implications with proper chromosome segregation. Cells subjected to overexpression or underexpression of either the H2A/H2B or H3/H4 gene pairs show an increase in chromosome loss and defective chromosome segregation [Meeks-Wagner and Hartwell, 1986].

However, not only the modification and balance of histones are important, but also histone proteins themselves can lead to phenotypes associated with defects in the chromosome cycle. For example, two independent mutants of H2A (S19F and G29D) in *S. cerevisiae* cause increase in ploidy and increase frequency of chromosome loss [Pinto and Winston, 2000], temperature sensitive mutations in the inner region of H2B causes defects in centromeric chromatin and chromosome segregation [Maruyama T et al., 2006]. Early deletion studies in *S. cerevisiae* demonstrated that the highly conserved N-terminal tails of H3 and H4 are essential for cell cycle progression. Megee PC at al., (1995) reported the requirement of the four most N-terminal lysine residues (domain A, positions 5, 8, 12 and 16) for normal nuclear division where mutant cells were arrested at G2/M. Kanta H et al., reported that Hda1 deacetylase complex affects histone function at the centromere and that Hda3 has a distinctive participation in chromosome segregation [Kanta H et al., 2006]. Cimini D et al., reported that histone hyperacetylation in mitosis prevents sister chromatid separation and produces chromosome segregation defects [Cimini D et al., 2003]. Another group showed that substitutions at histone H4 K91, K59, S47, and R92 and histone H3 K56 and K115 generate hypersensitivity to DNA-damaging agents,

linking the significance of the chemical identity of these modifiable residues to DNA metabolism [Hyland EM et al., 2005]. A screen of *Saccharomyces cerevisiae* histone alanine substitution mutants revealed that mutations in any of three adjacent residues, L97, Y98, or G99, near the C terminus of H4 led to slow growth, rapid polyploidy or aneuploidy, high rate chromosome loss and showed lower histone deposition, not only in the centromeric region, but also throughout the genome for the H4 mutants [Yu Y et al., 2011]. Recently, a global analysis of core histones reveals the nucleosomal surfaces required for chromosome bi-orientation where severe impairment of the centromere localization of Sgo1 was observed in some mutants such as H2A-I112A and H2A-E57A cells and the pericentromeric localization of Htz1, the histone H2A variant, was impaired in some mutants such as H4-L97A cells [Kawashima S et al., 2011]. Another recent study performed comprehensive structural analysis of mutant nucleosomes containing Lysine to Glutamine (KQ) substitutions in the H3 and H4 histone-fold domains and found that some of these amino acid substitutions altered local protein-DNA interactions and the interactions between histone residues within the nucleosome. Notably, the C-terminal region of H2A was significantly disordered in the nucleosome containing H4 K44Q [Iwasaki W et al., 2011].

Considering the fact from above discussion that both histone fold and histone tail regions of histone proteins can play important roles in chromosome segregation, we target both of these regions to incorporate in the double mutant of our study. In this regard, we combine single mutations from separate regions of two different histones close to other residues known to have chromosome biorientation defects and aim to study their combined effect in normal cell cycle progression.

Methods

A. Yeast strains and media

The yeast strains used are isogenic to FY2, originally derived from S288C [Winston F et al., 1995] and were developed following standard procedures [Rose MD et al., 1990; Guthrie and Fink, 1991]. The yeast strains made and used for this study are listed, along with their genotypes, in Table I. All yeast media, including Yeast Extract Peptone Dextrose (YPD), synthetic minimal (SD), synthetic complete (SC), omission media (SC-), media containing 5-fluoroorotic acid (5-FOA) drug selection media, and sporulation media were prepared using methods described by Rose MD et al. (1990). Benomyl plates were prepared by adding adequate amount of benomyl (Sigma, St. Louis) to hot YPD to final different concentrations of 5μg/ml, 10μg/ml, and 15μg/ml of benomyl. Canavanine plates were prepared by adding 60μg/ml of canavanine (Sigma, St. Louis) to synthetic complete medium lacking arginine (SC-Arg). Alpha-aminoadipate medium was prepared by adding alpha-aminoadipate (US Biological) to SD (Synthetic minimal dextrose medium) as described previously [Chattoo, BB et al., 1979]. Medium containing 5-FOA was prepared according to Guthrie and Fink, 1991. The 5-FOA was purchased from US Biological. *E.coli* containing plasmids was propagated in LB medium with 100μg/ml of ampicillin [Sambrook J et al., 1989].

B. Genetic methods including creation of a double mutant strain and yeast transformation

Genetic crosses were performed on YPD plates and the diploids were selected on SD. Then diploids were incubated in sporulation medium for 4-5 days and then digested with zymolyase. Tetrads were dissected under a microscope to obtain the progeny which were screened for the

phenotypes of desired genotypes. The progenies from first round of crosses were screened on SC-Lys, SC-Leu, SC-Trp, SC-Ura, YPD- Nourseothricin (YPD-Nat) and YPD-Hygromycin (YPD-Hyg) plates and the progenies from the second round of the crosses were screened on SC-Lys, SC-Leu, SC-Trp, SC-Ura, YPD-Kanamycin (YPD-Kan), YPD-NAT and YPD-Hyg plates and were also tested for temperature sensitivity at 37° Celsius.

Yeast cells were transformed with the appropriate plasmid DNA, containing *Sgo1*-2µm, for example, and also with PCR amplified DNA (PCR amplified with appropriate oligonucleotide primers) using the high efficiency Gietz Lithium Transformation Procedure [Gietz RD et al., 1995]. This transformation protocol was also used for gene disruption of the *HTA2-HTB2* loci by introducing the second copy deletion through homologous recombination of the pAG32 Hygromycin cassette with the loci of interest.

Temperature sensitivity analysis of yeast mutants was performed according to Rose MD et al., [Rose MD et al., 1990]. Benomyl sensitivity analysis of mutant strains was done following the procedure of Stearns T et al., [Stearns T et al., 1990]. Plasmid shuffling and gene disruption were performed using standard techniques [Rose MD et al., 1990; Guthrie and Fink, 1991].

C. Gene disruption in yeast cells

The desired strain to carry a deletion of the *hta2-htb2* locus was attempted to construct by PCR-mediated disruption using the Hygromycin (Hyg^R) resistance genes flanked by sequences homologous to the target genes following published procedures [Wach A et al., 1994; Goldstein and McCusker, 1999]. In this process, PCR products containing the Hyg^R resistance cassettes

were obtained using DNA primers with flanking regions of the *HTA2-HTB2* locus. A yeast strain was transformed with the resulting DNA obtained and purified from PCR and plated on medium containing hygromycin selecting for cells that had become resistant to the antibiotic due to homologous recombination between the PCR product and the targeted chromosomal locus [Guthrie and Fink, 1991]. To confirm the deletion by integration of the cassette to the targeted locus, PCR primers that hybridize outside and within the sequences that was targeted for recombination were used. Here, yeast colony PCR was performed to check the colonies directly from the transformation plate.

D. Gene sequencing

Sequencing of the mutated *HTA1* genes was performed by preparing the samples from genomic DNA and amplifying the mutant alleles by PCR, then sending them to Eurofins genomics, where Sanger's dideoxy method was used to do the sequencing [Sambrook J et al., 1989]. Yeast DNA was extracted according to procedures from Hoffman and Winston [Hoffman and Winston, 1987]. The primer used was oIP 382. The "sequence mix" was made up of 2µl primer, 8µl dH2O and 3µl DNA of 400ng final concentration.

E. Plasmids and Oligonucleotides primers

The *E.coli* plasmids are listed in Table II and the oligonucleotide primers used are listed in Table III.

F. Flow cytometry

Cells were harvested and processed for flow cytometry as previously described with few modifications [Gerring SL et al., 1990]. Yeast cells at their mid-log phase (~$5x10^6$ cells/ml) grown in liquid YPD were pelleted and resuspended in 50mM Tris pH7.5 containing 95% ethanol and kept in 4° Celsius overnight. The cells were then centrifuged and air-dried. The pellet was then washed with 50mM Tris pH7.5 and subsequently treated with 1mg/mL RNase and left in 37°C water bath for ~ 2 hours or more. Proteinase K was added afterwards and the samples were further incubated at 50°C for one hour. Cells were stained in 50mM Tris pH7.5 containing 15µg/mL propidium iodide (Sigma), wrapped in aluminium foil and kept at 4° Celsius until the flow cytometry was done. Finally, before the flow cytometry analysis, the stained cells were sonicated at low setting on Branson 1510 sonicator for ~30 seconds.

G. Ploidy assay on canavanine

The chromosome copy number of chromosome V was assayed by observing the CAN1 gene expression as described previously [Schild D et al., 1981]. As canavanine resistance is conferred by recessive mutations in the *CAN1* gene, the frequency of Can^R mutants is much greater among haploids than among diploids, with two copies of chromosome V in other words. Here, the strains, freshly grown on YPD first, were replica plated onto SC-Arg plates either with or without canavanine, then the cells were mutagenized by UV irradiation (300 ergs/mm2), and plates were wrapped in black polybags and incubated at 30°C for 3 to 5 days. Appearance of papillae indicated haploid cells. Conversely, the absence of growth indicated the presence of more than one copy of chromosome V, the diploid cells, in other words.

H. Fluorescence microscopy

Fluorescence microscopy was performed to observe the movement of centromere of chromosome IV. This assay relied on the intrinsic ability of green fluorescence protein (GFP) to fluoresce and the specific binding of Lac repressor to the Lac operator to visualize an array of 256 tandem repeats of the Lac operator [Straight AF et al., 1996] where this repeat was integrated at the *TRP1* locus located about 12 Kb to the right of the centromere of chromosome IV. Some strains contained the Lac operator integrated at the *LEU2* locus, located about 30 Kb to the left of the centromere of chromosome III. The Lac repressor is fused to the GFP gene and is expressed from the *HIS3* promoter in chromosome XV. The movement of GFP dots represents the movement of the centromere of either chromosome III or chromosome IV, which can be used to observe and analyze the chromosome segregation phenotype. Cells expressing green fluorescence protein (GFP) tagged centromeres of chromosome IV were visualized and imaged with a fluorescent microscope Axio Imager M1 (Zeiss). A minimum of 200 cells per time point was counted. The yeast cell morphology such as the unbudded, single budded or large budded states was noted.

I. Growth curves

To determine growth rates of both the single mutant and double mutant strains along with the wild type and to compare their growth, they were grown in fresh YPD. After overnight growth of all the mutant strains along with the wild types they were diluted to start with the cell density of 0.5×10^6 cells/ml and were allowed to grow at 30°C. An aliquot was taken from each culture every 2 hours for 12 hours. Each time the aliquot was taken, the cells were counted using the Countess Automated Cell Counter from Invitrogen, Thermo Fisher Scientific. Finally, the concentration of cells in cells/ml at each time point was plotted against time to draw the graph.

The doubling time was determined between intervals of linear growth for each strain, using the formula:

$$\text{Doubling time} = \frac{\text{duration} * \log(2)}{\log(\text{final concentration}) - \log(\text{initial concentration})}$$

The average of four doubling times with standard error of the mean was calculated for each strain.

J. Bacterial strains, plasmid isolation and PCR purification

E.coli strain DH5 alpha [F ‘80lacZ-M15-(lacZY A-argF) U169 endA1 rec A1 hsdR17 (rk-mk+) deoR thi1 supE44 Δgyr A96 relA1] was used for propagation and isolation of plasmids, according to standard procedures [Ausubel FM et al., 1987]. *E. coli* with desired plasmid was grown in LB containing 100 μg/mL of ampicillin as described previously [Sambrook J et al., 1989]. Plasmids were extracted using QIAGEN’s QIAprep plasmid miniprep kit and PCR product was purified by QIAGEN’s QIAquick PCR purification kit following their protocols.

Table I: Yeast strains and their genotypes

Strain	Strain	Genotype
FY 604		*MATα his3Δ200 leu2Δ1 trp1Δ63 ura3-52 (hta2-htb2)Δ::TRP1*
IPY 75		*MATa/MATα his3Δ200/his3Δ200 leu2Δ1/leu2Δ1 trp1Δ63/trp1Δ63 ura3-52/ura3-52 (hta2-htb2)Δ::TRP1/(hta2-htb2)Δ::TRP1*
IPY 1063		*MATa hta1-HTB1-Nat^R (hta2-htb2)Δ::Hyg^R his3-11, 115::lacI-GFP-HIS3 trp1-1::lacO-TRP1$^+$ ura3$^-$ leu$^-$*
IPY 1067		*MATa ura3-52 trp1Δ63 his3-205::HIS3-GFP-LacI leu2Δ1::LacO-LEU2 lys2Δ202 (hht1-hhf1)Δ::KanMX (hht2-hhf2)Δ::HHT-hhfs-URA3 <pIP220>*
IPY 1068		*MATa (hht1-hhf1)Δ::Kan^R (hht2-hhf2)::hhts-K42A-HHFS-URA3 ura3-52 trp1Δ63 his3-205::lacI-GFP-HIS3 leu2Δ1::LacO-LEU2 lys2Δ202 <pIP220>*
IPY 1075		*MATa ura3-52 trp1Δ63 his3-205::HIS3-GFP-LacI leu2Δ1::LacOLEU2 lys202 (hht1-hhf1)Δ::KanMX (hht2-hhf2)Δ::hhts-hhfsK44Q-URA3 <pIP220>*

Table I: Yeast strains and their genotypes (Cont.)

Strain	Strain	Genotype
RY 01	IPY 1182	*MATα hta1-N115D-HTB1-Nat^R (hta2-htb2)Δ::Hyg^R lys2Δ202 ura3$^-$ his3-205(or his3-11)::lacI-GFP-HIS3 trp1-1::LacO-TRP1$^+$ leu2$^-$*
RY 02	IPY 1183	*MATα hta1-N115S-HTB1-Nat^R (hta2-htb2)Δ::Hyg^R lys2Δ202 ura3$^-$ his3-205(or his3-11)::lacI-GFP-HIS3 trp1-1::LacO-TRP1$^+$ leu2$^-$*
RY 03	IPY 1184	*MATα hta1-Q114R-HTB1-Nat^R (hta2-htb2)Δ::Hyg^R lys2Δ202 ura3$^-$ his3-205(or his3-11)::lacI-GFP-HIS3 trp1-1::LacO-TRP1$^+$ leu2$^-$*
RY 04	IPY 1185	*MATa HTA2-HTB2, hta1-N115D-HTB1-Nat ^{R} (hht1-hhf1)Δ::Kan^R (hht2-hhf2)::hhts-K42A-HHFS-URA3 leu2Δ1::LacO-LEU2 trp1Δ63 lys2Δ202 his3-205(or his3-11)::lacI-GFP-HIS3 <pIP220>*
RY 05	IPY 1186	*MATα HTA2-HTB2, hta1-N115D-HTB1-Nat ^{R} (hht1-hhf1)Δ::Kan^R(hht2-hhf2)::hhts-K42A-HHFS-URA3 leu2$^-$ trp1-1::lacO-TRP1$^+$ lys2Δ202 his3-205(or his3-11)::lacI-GFP-HIS3 <pIP220>*
RY 06	IPY 1187	*MATα hta1-N115D-HTB1-Nat^R (hta2-htb2)Δ::Hyg^R (hht1-hhf1)Δ::KanMX lys2Δ202 ura3$^-$ leu2Δ1::LacO-LEU2 his3-205(or his3-11)::lacI-GFP-HIS3 trp1Δ63 <pIP220>*
RY 07	IPY 1188	*MATa leu2$^-$ lys2Δ202 trp1-1::LacO-TRP1$^+$ his3-205(or his3-11)::lacI-GFP-HIS3*
RY 08	IPY 1189	*MATa HTA2-HTB2 hta1-N115D-HTB1-Nat^R (hht1-hhf1)Δ::KanMX (hht2-hhf2) Δ::hhts-hhfsK44Q-URA3 leu2Δ1::LacOLEU2 trp1Δ63 lys2Δ202 ura3-52 his3-205::HIS3-GFP-LacI <pIP220>*
RY 09	IPY 1190	*MATa HTA2-HTB2 hta1-Q114R-HTB1-Nat^R hht1-hhf1)Δ::Kan^R (hht2-hhf2)::hhts-K42A-HHFS-URA3 ura3-52 lys2Δ202 leu2$^-$ his3-205(or his3-11)::lacI-GFP-HIS3 trp1-1::LacO-TRP1$^+$ <pIP220>*
RY 10	IPY 1191	*MATa (hta2-htb2)Δ::Hyg^R) (hht1-hhf1)Δ::Kan^R leu2Δ1::LacO-LEU2 trp1-1::Laco-TRP1$^+$ lys2Δ202 his3-205(or his3-11)::lacI-GFP-HIS3 <pIP220>*
RY 11	IPY 1192	*MATα HTA2-HTB2 hta1-Q114R-HTB1-Nat^R (hht1-hhf1)Δ::KanMX ura3-52 (hht2-hhf2)Δ::hhts-hhfsK44Q-URA3 leu2$^-$ trp1-1::LacO-TRP1$^+$ his3-205(or his3-11)::lacI-GFP-HIS3 <pIP220>*
RY 12	IPY 1193	*MATa HTA2-HTB2 hta1-N115S-HTB1-Nat^R (hht1-hhf1)Δ::Kan^R (hht2-hhf2)::hhts-K42A-HHFS-URA3 ura3-52 trp1Δ63 his3-205::lacI-GFP-HIS3 leu2Δ1::LacO-LEU2 lys2Δ202*
RY 13	IPY 1194	*MATα (hht1-hhf1)Δ::KanMX ura3-52 lys2Δ202 leu2$^-$ (hht2-hhf2)Δ::hhts-hhfsK44Q-URA3 trp1-1::Laco-TRP1$^+$ his3-205(or his3-11)::lacI-GFP-HIS3*
RY 14	IPY 1195	*MATa hta1-N115S-HTB1-Nat^R (hta2-htb2)Δ::Hyg^R ura3-52 lys2Δ202 (hht1-hhf1)Δ::KanMX (hht2-hhf2)Δ::hhts-hhfsK44Q-URA3 leu2Δ1::LacOLEU2 his3-205::HIS3-GFP-LacI trp1Δ63 <pIP220>*

Table II: Plasmids

Plasmid Name	Relevant Genotype	Cloning Vector/ Restriction Site	Reference
pIP153	*SGO1*, *LEU2, 2µm*	YEplac181	Pinto et al., (unpublished)
pIP209	*SGO1*, *TRP1, 2µm*	YEplac112/ *SpH*I, *Sal*I	Pinto et al., (unpublished)
pSAB6	*HTA1*, *URA3*		Pinto and Winston, 2000
pIP220	*HHT1-HHF1*, *LYS, CEN*	pRS317/ *Sal*I, *Not*I	Pinto et al., (unpublished)
pAG32	*HTA2-HTB2 ⊿::Hyg^R, CEN*		Pinto et al., (unpublished)

Table III: Oligonucleotide primers

Name	Sequence	Purpose
oIP280	5’ AAC GTC AAA GCT GTA GTACG 3’	5’ confirmation of (hta2-htb2) Δ
oIP281	5’ GAA TGC TCG TGT AGT GAA CC 3’	3’ confirmation of (hta2-htb2) Δ
oIP345	5’ GAC ATA CCA GTC TTC CTC ATA TGA CCT ACT TTA AAA CCC C- TCA GGG GCA TGA TGT GAC T 3’	5’ for deletion of *HTA2-HTB2*
oIP346	5’ CCT TCC AAT CAT ATC TGG ACA AGT AAC AGA ACC CTA ATG TTA - AGC TCG TTT TCG ACA CTG GAT 3’	3’ for deletion of *HTA2-HTB2*
oIP382	5’ CTG GTC TTG TTGAAC CAT CA 3’	5’ Re-integration confirmation

Table III: Oligonucleotide primers (Cont.)

oIP383	5’ CTC GTT TCT GAT AAA CCA GG 3’	3’ Re-integration confirmation
oIP384	5’ GGTCATATGTCAGGGGCATGATGTGACT 3’	To clone at 3’ end of HTA1-HTB1 locus
oIP385	5’ GGGATGCATAGCTCGTTTTCGACACTGGAT 3’	To clone at 5’ end of HTA1-HTB1 locus
oIP430	5' GAGAGCCTAGCTGTAATATATC 3’	upstream of oIP-280 in HTA2-HTB2
oIP431	5’ GAGATGCAATAGGTCAGGCTCT 3’	Oligo in the middle of Hygromycin cassette

Table IV: Characteristics of the H2A mutants obtained from phenotypic analysis (Only the strains with obvious phenotypes have been included)

Benomyl sensitive	Sensitive to Canavanine + UV	Heat sensitive (37°C)	Cold sensitive (13°C)
H2A G5G, Q114R	H2A S19P	H2A G45D	H2A G5G, Q114R
H2A N100S, H113Y	H2A G29D, D92G	H2A V50D	H2A S19P
H2A N115D	H2A G45D	H2A N115S	H2A Q85R
H2A N115S	H2A V50D		H2A N100S, H113Y
H2A I88V, H113Q	H2A G68D		H2A A104T
			H2A N115D

Results

Characterization of H2A mutants

HTA1 mutant library was created previously in our lab with an error-prone, low-fidelity PCR approach [I. Pinto, unpublished]. In the process of creating the H2A mutants, the parent yeast strain IPY1063 with both its chromosomal copies of *HTA1-HTB1* and *HTA2-HTB2* genes knocked out and replaced with drug cassettes, was transformed with the mutant *hta1* library. Following transformation, the *URA3* rescue plasmid was shuffled out from yeast strains by replica-plating transformants on 5-FOA plates (Figure 2). After this point, the sole source of histone H2A protein in all of the transformants was the mutant *hta1* gene from the mutant library. The selected mutants with putative chromosome segregation defects, based on phenotypic characterization, were integrated at their original *HTA1-HTB1* locus. In addition, this IPY1063 strain was engineered with a GFP-tagged centromere that allowed the visualization of chromosome segregation in these cells. I started with some of these H2A mutants located both at the DNA entry/exit point in the nucleosome and also in the histone fold to characterize them further.

Preliminary screening of the mutants showed impaired growth at different conditions. The mutant H2A strains selected for this phenotypic study were: H2A A104T, H2A G5G, Q114R, H2A N115D, H2A I88V, H113Q, H2A N100S, H113Y, H2A S19P, H2A Q85R, H2A N115S, H2A V50D, H2A G45D, H2A G68D, H2A Q114R, H2A A8T, H2A R18K, and H2A G29D, D92G along with the wild type (WT) IPY1063. All of these mutants were replica plated on YPD

medium at 30°C (physiological temperature) as control, 37°C (heat sensitivity) and 13°C (cold sensitivity) to assess temperature phenotype. Also, the mutants were replica-plated on YPD containing benomyl (a microtubule depolymerizing drug) and SC-Arg containing canavanine. SC-Arg was used as a control for the canavanine assay. Benomyl affects polymerization of microtubules [Gupta K et al., 2004; Stearns T et al., 1990], while canavanine is used to assess increase in ploidy phenotype. Strains that have problems in chromosome segregation are usually more sensitive to the presence of a drug that affects the function of microtubules. Strains with relatively weak growth at these conditions were chosen as good candidates for creating desired double mutants combining the mutant H2A strains with either H3 or H4 previously characterized mutations. Some H2A mutant strains exhibited weak growth on 13°C, 37°C, benomyl and canavanine media (See Table IV with mutants' phenotypes). Each strain was scored from a 1 to 5 scale; where 1- no growth at all, 2- very little to no growth, 3- slow growth, 4- growth close to but little less than the wild type, and 5- growth same as the wild type. Some of these mutants showed weak growth (Score 1 or 2) in at least one of the various screens; YPD at 13°C, YPD at 37°C, benomyl and canavanine growth medium.

Some of the selected H2A mutants mentioned above which showed weak growth in at least one of the different phenotypic analyses were selected to measure their DNA content by flow cytometry. Surprisingly, although the mutants showed growth defect and drug sensitivity, none of the H2A mutants showed any obvious ploidy defect. The H2A mutants carrying the following mutations H2A G5G, Q114R, H2A N115D, H2A I88V, H113Q, H2A N100S, H113Y and H2A N115S showed clear sensitivity to benomyl while mutants H2A V50D, H2A G45D and N115S showed sensitivity to heat (sensitivity to 37°C temperature), and H2A A104T, H2A G5G,

Q114R, H2A N115D, H2A N100S, H113Y, H2A S19P, H2A Q85R showed sensitivity to cold (sensitivity to 13°C temperature). Finally, H2A S19P, H2A V50D, H2A G45D, H2A G68D, H2A G29D, D92G showed some ploidy problem in the canavanine assay, although it could not be confirmed by flow cytometry. This indicates that although they didn't show any obvious ploidy phenotype, taking temperature sensitivity into consideration, it might be possible that they were having difficulty traversing the cell cycle.

The following H2A mutant strains were sent for sequencing to confirm their sequence at the integrated locus: H2A G5G, Q114R, H2A N115D, H2A I88V, H113Q, H2A N100S, H113Y, H2A S19P, H2A N115S, H2A V50D, H2A G45D, H2A G68D, H2A Q114R, and H2A G29D, D92G. The results indicated that all strains carried the correct integrated mutant alleles.

In order to analyze the ploidy status of the mutants and determine whether they have a cell cycle defect that affects the integrity of their genome, the DNA content of all of the H2A mutant strains was determined by flow cytometry. Interestingly, most of the mutants did not show any obvious ploidy defect (Figure 3).

Suppression of H2A mutants' benomyl sensitivity by *SGO1* 2 µm

Recently, there has been evidence of interaction of *SGO1*, which has a role in localizing CPC to centromere, with histone residues. It has been reported that H3G44S has an impaired tension sensing ability, most likely due to the improper recruitment of Sgo1p to the kinetochore, and overexpression of Sgo1 was able to suppress the mutant phenotype [Luo J et al., 2010]. Indeed, Sgo1p is an important member of the checkpoint protein families that sense tension at the

kinetochores during chromosome segregation. In a previous study in our lab, it was found that the mutants H3K42 (A/Q), H3G44 (A) and H4K44 (Q) positioned either at the same position in H3 (H3G44A) or near to that position in the nucleosome (H3K42 and H4K44) were sensitive to Sgo1 overexpression in terms of their benomyl sensitivity. In other words, these mutants' benomyl sensitivity was suppressed by high copy Sgo1 expression whereas the other mutations including H4L97A and H4G99A that were tested in the same study not positioned at the DNA entry/exit site, rather positioned towards the nucleosome center, showed no suppression of benomyl sensitivity by Sgo1 overexpression. This clearly indicates a relevance of the residues located at DNA entry/exit point, and the DNA entry/exit point itself, with Sgo1 interaction and so is necessary for error free tension sensing during chromosome segregation. [Pinto et al., unpublished]. We therefore, in line with this, tested our H2A mutants for the suppression of their benomyl sensitivity by *SGO1* overexpression introducing a plasmid that expresses *SGO1* 2µ in high copy. Consistent with previous studies, we found that the H2A mutations H2A Q114R, H2A N115D, H2A H113Q, H2A H113Y, and H2A N115S showed clear suppression of their benomyl sensitivity by Sgo1 overexpression (Figure 4). This is the same location where another residue of histone H2A has been found to have a role related with Sgo1 function. The residue H2A S121 was found to be associated with the recruitment of Sgo1 to the kinetochore to be able to localize the chromosomal passenger complex at its appropriate location and together with H3T3 define the inner centromere and thus associated with proper chromosome segregation process [Yamagishi Y et al., 2010, Kawashima S et al., 2010]. Later we use this observation to select our single mutations of H2A and H3 or H4 to create a double mutant to analyze in the current study. The other H2A mutations H2A V50D, H2A G45D, H2A G68D that we tested for

comparison of benomyl sensitivity by high copy *SGO1* 2µm showed almost indistinguishable phenotype (Figure 5).

Creation of a double mutant between H2A mutants (H2A Q114R, H2A N115D, H2A N115S) and H3 (H3 K42A) or H4 (H4 K44Q)

Based on the results of the phenotypic analysis of drug sensitivity, temperature sensitivity, ploidy, and flow cytometric analysis and also the suppression of benomyl sensitivity by high copy *SGO1*, three H2A mutants containing mutations in H2A Q114R, H2A N115D and H2A N115S were selected to cross with H3 mutant containing mutation in H3 K42A and/or H4 mutant containing mutation in H4 K44Q to create a double mutant to observe the combined effect of the two mutations. This was particularly interesting because of the locations of all the mutations mentioned above at the DNA entry/exit point of the nucleosome and would be interesting to see whether one mutation can compensate for another or makes it worse in the respective double mutant.

Crosses were performed between a parent H3 or H4 mutant and a parent H2A mutant strain to create the desired double mutant. Few rounds of crosses were required to get the desired double mutant strain because of selecting on appropriate marker to screen the progenies and to match mating type in the first place to perform the cross. Because our parent H3 or H4 mutants were of mating type “a”, we performed our first round of cross to get a parent H2A strain of a “α” mating type. Thus the desired genotype in the progeny of first round of crosses was Hyg (+) i.e. second copy of H2A deleted, Nat (+) i.e. first copy was replaced with the mutated H2A, Lys (-) i.e. to be able to select on Lys later and no wild type H3 or H4 copy on plasmid and Ura (-) i.e. second

copy of H3 or H4 is wild type. The progenies were screened on SC-Lys, SC-Leu, SC-Trp, SC-Ura, YPD-Nat and YPD-Hyg, MAT a-met15Δ mating lawn and MAT α -met15Δ mating lawn plates.

Summary of the first round of crosses

Objective: The objective of the first round of crosses was to get a Lys- and Ura- strain from each H2A mutants: Representative example: RHx2= a parent (N115D-IPY 1063) X α parent (EBx1-16A)

Genotypes:

a parent: N115D-IPY 1063

MATa hta1-N115D-HTB1-Nat^{R} (hta2-htb2)Δ::Hyg^{R} his3-11,115::lacI-GFP-HIS3 trp1-1::LacO-TRP1^{+} ura3^{-} leu^{-}

α parent: EBx1-16A

MATα lys2Δ202 ura3-52 or (ura3Δ0) (hht2-hhf2)Δ::hht2-K42A-URA3^{+} trp1-1::LacO-TRP1^{+} his3-205 (or his3,11)::lacI-GFP-HIS3 leu2*Δ0 (leu2-3)*

Desired phenotype of genotype from this cross: *MATα, Hyg^{R+}, Nat^{R+}, Lys^{-}, Ura^{-}*

Outcome: From the first round of crosses we got the desired genotype containing strains as we wanted.

With the desired strain from the first round of crosses (parent H2A ready to cross with the parent H3 or H4), second round of crosses was performed with the parent H3 or H4 strain. The progenies were screened on SC-Lys, SC-Leu, SC-Trp, SC-Ura, YPD-Kan, YPD-Nat and YPD-Hyg plates. Two more YPD plates were also used; one for observing growth at 37 degree Celsius (to assess temperature sensitivity) and another at 30 °C (as a copy of the master plate). The

desired genotype of the progenies from this cross is: Hyg^{R+}, Nat^{R+}, Kan^{R+}, $Ura^{+,}$ Lys^{-}, His^{+}, Leu^{+} or Trp^{+} (never both); where Kan^{R+} means the first copy of H3 or H4 is deleted. And Leu^{+} or Trp^{+} means the centromere of either chromosome III or IV is GFP tagged. However, no offspring from the second round of crosses gave us the desired genotype containing both the mutations from H2A and H3 or H4 except one (with the H2A N115S and H4 K44Q) with the expected marker except that it was a Lys^{+}. This was not unexpected as we wanted so many things from in our progenies and the crosses were very complicated. Interestingly, we got many of the progenies from the second round of crosses having all the desired genotypes and the markers to select with, except that those were always Hyg^{R-} i.e. the second copy of H2A was not deleted, and hence wild type (WT), on the other hand. So, we attempted to introduce the second copy deletion of *hta2-htb2* gene locus in those offspring with the hygromycin cassette but never got a proper transformant or integration in the proper region of the genome, in other words. I will elaborate the possible reason/s behind this later and also in the discussion chapter.

Summary of the second round of crosses

Objective: The objective of the second round of crosses was to incorporate either H3 or H4 mutations together with H2A mutations: Representative example: RHx5 = a parent (IPY 1068) X α parent (RHx2-5B)/ IPY 1182

Genotypes:

a parent: IPY 1068

MATa (hht1-hhf1)Δ::Kan^{R} (hht2-hhf2)::hhts-K42A-HHFS-URA3 ura3-52 trp1Δ63 his3-205::lacI-GFP-HIS3 leu2Δ1::LacO-LEU2 lys2Δ202 <pIP220- HHT1-HHF1-Lys2 $^{+}$>

α parent: RHx2-5B/IPY 1182

MATα hta1-N116D-HTB1-Nat^{R} (hta2-htb2)Δ::Hyg^{R} lys2Δ202 ura3^{-} his3-205(or his3-11)::lacI-GFP-HIS3 trp1-1::LacO-TRP1^{+} leu2^{-}

Desired phenotype of genotype from this cross: Hyg^{R+}, Nat^{R+}, Kan^{R+}, Ura^{+}, Lys^{-}, His^{+}, Leu^{+} or Trp^{+} (never both)

Outcome: From the second round of crosses we got only one strain with all the desired genotype except that it was a Lys+, so later we went from YPD to α-amino-adipate medium to lose the Lys plasmid from the strain and thus shuffled out the rescue plasmid leaving all the sources of histone H2A and H4 protein as our mutated H2A (H2A N115D) and H4 (H4 K44Q) genes. Also, we obtained many of the progenies with all the desired characteristics except the second copy of H2A was not deleted and so attempted to transform those to introduce the second copy deletion of *hta2-htb2* gene with hygromycin cassette. However, not a single transformant got the integration of the cassette in the proper genomic locus. Even if few colonies from a transformation got the integration in the proper place that diploidized where the other copy of the H2A gene became WT and we never got what we wanted from this transformation.

With the intention of getting the desired double mutants without this transformation procedure we pursued another cross, third round of crosses, to minimize the demand of the number of expected characteristics in the progenies. We tried this third round of crosses from the progenies of the second round of crosses reducing the number of expected characteristics to have in the progenies this time, which is matching the same genotype at least for some characteristics in both the parents of the cross, or to be homologous at least for few loci, in other words.

Summary of the third round of crosses

Objective: The objective of the third round of crosses was actually the same as the objective of the second round of crosses which was to eventually create desired double mutant strains for H2A-H3 (H2A Q114R, H2A N115D, H2A N115S with H3 K42A) and/or H2A-H4 (H2A Q114R, H2A N115D with H4 K44Q) without the need of the one combination (H2A N115S and H4 K44Q) that we already obtained from the second round of crosses: Representative example: RHx17= a parent (RHx2-5B-3D) X α parent (RHx2-5B-5C)

Genotypes:

a parent: RHx2-5B-3D

MATa HTA2-HTB2 hta1-N116D-HTB1-Nat^R (hht1-hhf1)Δ::Kan^R (hht2-hhf2)::hhts-K42A-HHFS-URA3 leu2Δ1::LacO-LEU2 trp1Δ63 lys2Δ202 his3-205::lacI-GFP-HIS3 <pIP220-HHT1-HHF1-Lys2 $^+$>

α parent: RHx2-5B-5C

MATα hta1-N116D-HTB1-Nat^R (hta2-htb2)Δ::Hyg^R HHT1-HHF1 (hht2-hhf2)::hhts-K42A-HHFS-URA3 leu2$^-$ trp1Δ63 lys2Δ202 <pIP220- HHT1-HHF1-Lys2 $^+$>

Desired phenotype of genotype from this cross: Hyg^{R+}, Nat^{R+}, Kan^{R+}, Ura$^+$, Lys$^-$, His$^+$, Leu$^+$ or Trp$^+$ (never both)

Outcome: The third round of crosses didn't give us any sporulation of the diploids whatsoever, so we stopped here with the crosses.

We rather concentrated on the transformation of suitable candidates from the second round of crosses but the difference in our transformation approach this time from the previous approach was that we used different primers to amplify the hygromycin cassette (pAG32) containing the second copy of *H2A* deletion. Previously, for the transformation to obtain the same result as I discussed above, we used primers oIP345 and oIP346, which gave us 42 bp flanking region at both sides of the cassette, which didn't produce the expected outcome. So, this time we used oIP280 and oIP281, which gave us around 200 bp flanking sequence at both sides of the cassettes. However, even this approach didn't produce the desired outcome, which is the proper integration of the cassette in the genome, leaving the one double mutant from the second round of crosses as our desired double mutant to carry out further analysis along with the associated single mutants and wild type obtained from the crosses. They are as follows: (For genotypes of the strains, refer to Table I)

(IPY 1183) RH X 3-7D (Plate-2): H2A single mutant containing H2A N115S (Figure 6)

(IPY 1194) RH X 13-4C (Plate-a): H4 single mutant containing H4 K44Q (Figure 7)

(IPY 1195) RH X 13-2D (Plate-b): Double mutant containing H2A N115S and H4 K44Q (Figure 8)

(IPY 1188) RH X 7-2D (Plate-b): WT for everything (True WT)

(IPY 1190) RH X 8-10D (Plate-a): WT for H2A and H4 mutant only (WT parent)

IPY 75: Diploid as a control for flow cytometry

Analysis of the double mutant H2A N115S H4 K44Q

The double mutant that we have obtained from the crosses is: *hta1*-N115S *hhfS*-K44Q. We have analyzed the double mutant along with the relevant single mutants of H2A, H4 and also representative wild type/s from the crosses. We performed phenotypic assays on the above strains to observe presence of phenotypes related with chromosome segregation defects, as well as general cell behavior. A growth curve experiment to assess their relative growth, sensitivity assay to benomyl and growth temperature, flow cytrometry analysis and canavanine assay to observe ploidy, and microscopy to monitor GFP tagged centromere of chromosome IV to assess cell cycle halt and abnormal chromosome segregation were performed. Finally, suppression of benomyl sensitivity by overexpression of Sgo1 was carried out to know whether the double mutant may have an interaction with Sgo1 and relation with abnormal chromosome segregation.

Phenotypic analysis

The double mutant, the respective single mutants and the two WTs (one true WT and another comparable WT) were tested for growth in benomyl or benomyl sensitivity and temperature sensitivity (heat sensitivity at 37°C and cold sensitivity at 13°C). In regular YPD at 30°C, both the double mutant and the single mutants showed slow growth compared to the wild type strains. In benomyl, (benomyl 5µg/ml), all of the mutants showed sensitivity, and the double mutant appears to be slightly more sensitive than the single mutants. In heat sensitivity assay i.e. the growth at 37°C, the H2A mutant and the double mutant were the most sensitive ones. The H4 mutant showed a very mild sensitivity. Finally, at 13°C to assess the cold sensitivity of the mutants, the H4 and the double mutants were the most sensitive and the H2A mutant behaved almost like the wild type (Figure 9).

Growth curve

The growth curve was created starting fresh cultures in YPD. Aliquots from each culture were taken and were counted every 2 hours for 12 hours. The growth curve revealed that the double mutant grew almost with the same rate as the H2A N115S. The growth of the H4 mutant was the slowest among all, and the differences observed in the growth curve were consistent with the calculated doubling times for each strain. Interestingly, the data indicates that the presence of the H2A N115S allele in the double mutant supersedes the slower growth rate of the H4 K44Q strain (Figure 10).

Canavanine Assay

All the mutants along with the two wild type strains (as control haploids), a control diploid strain, and the double mutant (passed three times on YPD) were replica plated from YPD to SC-Arg containing canavanine, and SC-Arg, (used as a control). The H2A and the double mutant showed clear haploid phenotype with similar growth like the control haploids in the canavanine plate after UV exposure, whereas the H4 mutant clearly showed diploid phenotype as it didn't show any growth just like the diploid control (Figure 12).

Flow cytometry

We performed flow cytometry on all the strains after at least three passages of growth on YPD after the cells had lost the plasmid carrying the wild type H3-H4 genes, to allow for complete replacement of histones in the mutant cells. As we had observed earlier, the H2A N115S mutant remained haploid, while the H4 K44Q strain clearly diploidized, showing 2C and 4C peaks. However, the double mutant containing both H2A N115S and H4 K44Q remained haploid

(Figure 11). This suppression by H2A N115S indicates that there might be something with the incorporation of the H2A mutation that makes the adverse effect of the H4 mutant go away and prevent the double mutant from diploidizing. Thus, we found the results consistent with the canavanine assay and our flow cytometry data confirmed the ploidy phenotype of the strains.

GFP-CEN assay

Fluorescence microscopy revealed the green fluorescence protein (GFP) as bright shiny dots, which was used to monitor the movement of the centromere of chromosome IV. Bud morphology was also observed under this microscope. The H4 mutant strain showed clear diploidization with many cells with two GFP dots indicating improper chromosome segregation and ploidy problem as reported in a previous study in our lab [Pinto et al., unpublished]. The cells were large budded in most cases as well, also indicating the delayed G2/M phase in cell division or arrest at this point of the cell cycle. The H2A mutant behaved very similar to the wild type strain. The double mutant displayed a pattern of chromosome segregation similar to the H2A N115S mutant and the wild type, but showed some remnants of diploid cells and some aneuploidy. However, the cells were large budded in a good number of cases indicating delay in cell cycle stages and accumulation of proteins (Figure 13). A large-budded morphology is characteristic of cells arrested or delayed in traversing the G2-M phase of cell cycle. This is characteristic of cell division (cdc) mutants that are blocked in cell division but continue protein synthesis, and has been previously observed in other H2A mutants [Pinto and Winston, 2000].

Suppression of benomyl sensitivity by *SGO1* overexpression

By transforming all the mutant strains and the wild type with high copy *SGO1* 2µm plasmid, we performed dilution spotting assay with 10 fold serial dilutions in 96 well microtiter plate taking independent single colonies from both, strains transformed with the vector only and with the *SGO1* plasmid, streaking them for single colonies and growing them in respective liquid media (SC-Trp for the double mutant and SC-Leu for the other mutants as well as the wild type). We picked two colonies from each vector only plate and two colonies from the *SGO1* plasmid containing plate and did the same for all the mutant strains (H2A, H4 and the double mutant) as well as for the wild type. Interestingly, all of the mutant strains showed clear suppression of benomyl sensitivity by Sgo1 overexpression (Figure 14). These data suggest that not only the particular H2A, H3, or H4 residues, but the whole region of DNA entry/exit site of the nucleosome may play role in establishing the association of Sgo1p with the pericentromere, and that the increase in ploidy seen in the H4 mutant may have an origin independent of the interaction between Sgo1 and the nucleosome.

Discussion

In a previous study in our lab, a histone H2A (*HTA1*) mutant library was created where mutations were generated by error prone PCR. The mutated gene was inserted into a yeast episomal plasmid by gap repair process, and eventually was integrated into the genome of the parent yeast strain IPY 1063, which carries both its chromosomal copies of *HTA1-HTB1*and *HTA2-HTB2* genes knocked out and replaced with drug cassettes. Moreover, the *URA3* rescue plasmid was shuffled out by replica-plating transformants on 5-FOA plates leaving the mutant *hta1*gene as the sole source of histone H2A protein. This strain also carried a GFP-tagged centromere to visualize chromosome segregation [Pinto et al, unpublished]. The current study further characterized some of these H2A mutants based on the initial phenotypic screen. After characterizing them for potential chromosome segregation defects, we set out to create double mutant strains combining a histone H2A mutation from our characterized mutants together with H3 or H4 mutations from previously characterized mutants in our lab [Pinto et al., unpublished]. The goal was to analyze their combined effects on the double mutants in terms of chromosome segregation phenotypes.

New H2A mutants emphasize roles of histones in chromosome segregation

The mutant H2A strains, after initial screening for severe observable traits, selected for further phenotypic study were: H2AA104T, H2AG5G, Q114R, H2AN115D, H2AI88V, H113Q, H2AN100S, H113Y, H2AS19P, H2AQ85R, H2AN115S, H2AV50D, H2AG45D, H2AG68D, H2AQ114R, H2AA8T, H2AR18K, and H2AG29D, D92G along with the wild type IPY 1063. Benomyl is a microtubule-depolymerizing drug [Stearns T et al., 1990] and mutants having

problems in chromosome segregation are usually more sensitive to this and other drugs that affect the function of microtubules. Cananvavine allows us to detect increase in ploidy. Some of the selected H2A mutants showed weak growth in at least one of the different phenotypic analyses including sensitivity at 13°C, 37°C, benomyl and canavanine assay. The DNA content of those strains was determined by flow cytometry analysis. It was interesting to note that although the mutants showed growth defect and drug sensitivity, none of the H2A mutants showed any obvious ploidy defect. In the outcome of phenotypic analysis of the H2A mutants, the strains with the mutations H2AG5G, Q114R, H2AN115D, H2AI88V, H113Q, H2AN100S, H113Y and H2AN115S showed clear sensitivity to benomyl, H2AV50D, H2AG45D and N115S showed heat sensitivity (sensitivity to 37°C temperature), H2AA104T, H2AG5G, Q114R, H2AN115D, H2AN100S, H113Y, H2AS19P, H2AQ85R showed cold sensitivity (sensitivity to 13°C temperature), and H2AS19P, H2AV50D, H2AG45D, H2AG68D, H2AG29D,D92G showed some ploidy problem in the canavanine assay. It is important to note here that although some mutants seem to have ploidy problems in canavanine assay, this could not be confirmed by flow cytometry, all of the H2A mutants selected for analysis under the current study behaved like haploid by their 1C and 2C peaks and never diploidized even after passing them through several generations. However, that doesn't mean that the cells were behaving normal. Given the fact that some of them clearly showed drug sensitivity to benomyl and some temperature sensitivity, it might be possible that although they are progressing through the cell cycle, they are doing it with more difficulty.

It has been previously shown that histone residues located in the DNA entry/exit site of the nucleosome have implications in chromosome segregation defects and have relation with *SGO1*

function in this purpose. For example, H3G44S residue's impaired tension sensing has been correlated with the lack of Sgo1association with the nucleosome. Indeed, overexpression of Sgo1 was able to solve the tension-sensing problem in this mutant [Luo J et al., 2010]. Our lab also found that Sgo1 is implicated with the DNA entry/exit site residues in correcting the improper tension sensing of mutations in H3K42, H3G44 and H4K44 [Pinto et al., unpublished]. Not only the H3 and H4 residues of DNA entry/exit site have been found to have implication with chromosome segregation defects but also the residues from H2A at the same region have been shown to behave in similar fashion. For example, a study found H2AS121 to be associated with Sgo1function and chromosome segregation together with H3T3 [Yamagishi Y et al., 2010, Kawashima S et al., 2010]. Another recent study demonstrated the importance of this region of nucleosome to be associated with chromosome bi-orientation defects, which again showed suppression of chromosome segregation phenotype by Sgo1 overexpression. It has been proposed that Sgo1 may help the localization or recruitment of the CPC to the kinetochores to ensure kinetochore bi-orientation [Kawashima S et al., 2011]. Keeping in mind these recent discoveries showing the necessity of the DNA entry/exit region of the nucleosome for proper chromosome segregation and also their implication with Sgo1 function, we tested our H2A mutants of this region for the suppression of benomyl sensitivity, a chromosome missegregation phenotype, by Sgo1 overexpression. In our study, H2A mutations close to DNA entry/exit point of the nucleosome: H2AQ114R, H2AN115D, H2AH113Q, H2AH113Y, and H2AN115S showed suppression of their benomyl sensitivity by high copy *Sgo1* 2µm plasmid. This was consistent with previous studies in our lab where H3 K42A and H4 K44Q were positioned in the DNA entry/exit point and showed suppression of benomyl sensitivity whereas H4L97A and H4G99A positioned towards the nucleosome center, and hence don't belong to the DNA

exit/entry region, showed no suppression of benomyl sensitivity by *Sgo1* overexpression, consistent with the conclusion that the DNA exit/entry region is necessary for faithful chromosome segregation by proper tension sensing during the process [Pinto et al., unpublished]. With our additional data for the H2A mutants we have now expanded our understanding of this region of the nucleosome by establishing that mutations in residues H2AH113, Q114 and N115 appear essential for *Sgo1* function, and that not only replacement of those amino acids with alanine, as described by Kawashima S et al. (2011) for H113 and N115, can cause the same phenotype. Thus, it appears that a regional disruption of the H2A structure is likely to affect Sgo1 binding, either because the whole region participates in the association with Sgo1, or because the phosphorylation of H2A121S, required for Sgo1 recruitment (Kawashima S et al., 2010), is altered by the H2A mutations in the neighboring amino acids.

The double mutant carrying the H2AN115S and H4K44Q mutations reveals new insights into nucleosome function in proper cell cycle progression

The H2AN115S residue, positioned in the C-terminal tail of histone H2A, in our double mutation falls in the region of DNA entry/exit point of the nucleosome. This mutation didn't show any obvious ploidy defect either in the canavanine assay or by flow cytometry. However, this single H2A mutant showed clear sensitivity to benomyl. We showed in the current study that this benomyl sensitivity was suppressed by Sgo1 overexpression, which led us to analyze it further, in combination with another histone mutation in the same region of the nucleosome. The other single mutant H4K44Q, a residue located in the L1 loop of histone H4, is positioned in close proximity to the H3K43 and H3G44 mutants, falls in the DNA entry/exit point of the nucleosome [Luger K et al., 1997]. This single mutant containing H4K44Q replacement showed

clear sensitivity to benomyl and clear and stable diploidization in canavanine assay which was also supported by flow cytometry data, and again showed clear suppression of benomyl sensitivity by high copy *SGO1* 2µm plasmid expression which is consistent with the previous finding in our lab [Pinto et al., unpublished]. It was our intention to test various H2A mutants in combination with H3 and H4mutants; however, after many attempts through various crosses we were unable to obtain them. Although it is not possible to predict the reason of this outcome from negative results, it is conceivable that the particular combinations of H2A and H3 or H4 mutations are not viable (H2AQ114R, H2AN115D, H2AN115S with H3K42A, and H2AQ114R, H2AN115D with H4K44Q), and that is the reason we could not generate those strains. This notion was supported by our efforts to create the double mutant strain in a few cases where we obtained the double mutant with the desired genotypes, except that they all had the wild type of the *HTA2-HTB2* gene locus, the second gene copy encoding for H2A and H2B. We attempted to delete this copy directly by recombination with the hygromycin resistance gene, and although we obtained many Hyg^R colonies, none of them carried the insertion at the *HTA2-HTB2* locus. Moreover, the only time the integration was correct happened in strains that had already diploidized, and therefore had a wild type copy and a deleted copy at the *HTA2-HTB2*, in other words, they were the *HTA2-HTB2/hta2-htb2Δ::*Hyg^R heterozygotes.

The double mutant strain H2A N115S-H4 K44Q surprisingly didn't display a synthetic phenotype. Not only the strain didn't have worse phenotypes than the individual mutants, but it appears that the combination was making the strain better as was seen from the ploidy analysis through canavanine assay, flow cytometry data, and GFP-CEN analysis chromosome IV. With respect to temperature sensitivities, the double mutant displayed the phenotypes of both single

mutants; increased temperature sensitivity at 37°C, like the H2AN115S mutant, and increased cold sensitivity at 13°C, like the H4K44Q mutant. Thus, it would appear that these characteristics, usually related to nucleosome structure and/or stability, are intrinsic to each amino acid replacement and the newly established interactions after combining both mutations are not altering those properties.

The growth curve revealed that the double mutant grew almost with the same rate as the H2A mutant, showing an improvement over the single H4K44Q mutant. This result can be an indirect effect over the whole metabolic activities of the cell, and not exclusively related to the mitotic phenotypes, however, it is interesting to note that the growth rate resembling the H2AN115S mutant correlates with the haploid behavior of the double mutant, as we discuss next. The result of flow cytometry was consistent with the canavanine assay for ploidy analysis, showing that the double mutant remained a haploid and didn't diploidize as the single H4K44Q mutant, even after extensive growth on YPD to ensure that wild type histone had been maintained in the cell. This surprising suppressor phenotype was confirmed by monitoring the movement of the centromere of chromosome III or IV (depending on the markers) using the GFP-CEN assay, where the data clearly showed that the majority of the cells were cycling in a haploid state. However, there were many large budded cells indicating the G2-M phase delay of the cell cycle, which is a characteristic of cell division (cdc) mutants where cells continue to synthesize proteins without being divided. These results indicate that the increase in ploidy phenotype is independent of additional cell cycle problems that cause the delays in all three histone mutants. Finally, irrespective of the ploidy status, all of them showed benomyl sensitivity and also clear suppression of the benomyl sensitivity by high copy *SGO1* 2µm plasmid expression. While the

double mutant and the H2A single mutant didn't diploidize, this suppression phenotype suggests that the histone mutations alter the association of Sgo1 at the centromeric region by either affecting its interaction with pericentric chromatin directly, or by inhibiting the association of other components of the CPC at the centromere, in an Sgo1-independent manner. Nonetheless, these data implies a tension defect in the histone mutants that can be relieved by the presence of high levels of Sgo1.

The behavior of the double mutant can be better understood by considering the structure of the nucleosome. It can be explained from the location of the residues in the nucleosome where they can participate in histone-DNA, histone-histone and histone-nonhistone protein interactions. Indeed from past crystallographic data and structural analysis of the position of the mutant residues, we can draw some possible conclusions of the combined effect of the two mutations present in our double mutant strain.

Luger K et al., described that the single arginine in the L1 loop of each H3, H4 and H2A (H3-R83, H4-R45, H2A-R42) extends into the DNA minor groove and each makes hydrogen bonds to a threonine hydroxyl group in the adjacent L2 loops of H4, H3 and H2B (H4-T80, H3-T118, H2B-T85) respectively. This prevents arginine side-chain to make bond with cytosine or thymidine, or the N3 of adenine or guanine. Surprisingly, H4-R45 hydrogen-bonds to the O_2 of a thymidine base even maintaining the interaction with threonine [Luger K et al., 1997]. The H4 R45 is adjacent to the H4 K44Q mutation which might influence this very interaction. It has also been reported that mutations in yeast histones H3 and H4 that partially overcome the loss of the SWI/SNF complex, the so called chromatin remodeling factors, are found predominantly in the

H4L1 loop and the adjacent H3L2 loop where the buried H3-R116, H4-R45 residues in the minor groove are effected, and H3-T118, which is hydrogen-bonded to both H4-R45 and a DNA phosphate group and another residue H3 V43 are also affected [Luger K et al., 1997]. While the interactions stated above were from *Xenopas laevis* nucleosome, taking their sequence similarity with *Saccharomyces cerevisiae*, specially where the mutations in our discussion are located, this observation appears significant; even when the path of H4 N terminal amino acid residues and H2A C terminal amino acid residues are slightly different in the nucleosome of these two species [White CL et al., 2001]. Moreover, it has been demonstrated that sequence differences cause changes in molecular surfaces and histone-histone interaction [White CL et al, 2001] supporting the indication that our double mutations with H2A N115S can make such interactions suitable for suppressing chromosome segregation phenotypes expressed by the single mutation H4K44Q. In another study, Muthurajanet UM et al., showed that individual point mutations in the structured regions of histones H3 and H4 could partially restore the in vivo effects of SWI/SNF inactivation in yeast where local protein–DNA interactions were disrupted and weakened in a subtle and complex manner rather than prominent global effects. They showed that the disruption of only two to six of the 120 direct histone-DNA interactions within the nucleosome has a pronounced effect on nucleosome mobility and stability [Muthurajan UM et al., 2004]. In this study SIN mutant nucleosomes were crystallized to observe the altered interaction pattern ("SIN" mutations in *Saccharomyces cerevisiae* alleviate transcriptional defects that result from the inactivation of the yeast SWI/SNF complex [Kurumizaka and Wolffe, 1997] where they divided the mutations in several classes based on the function of the affected residues in nucleosome structure and showed that class I mutations affect either H3 T118 or H4 R45 where H4 R45 was changed to cysteine, alanine, glutamate or histidine, and H3 T118 to isoleucine. They went on to

elaborate that class I mutations resulted in a loss of selected protein-DNA interactions. In fact, class I mutants (affecting residues H4 R45 and H3 T118) displayed the most severe Sin phenotype, next to H4 V43I [Kruger W et al., 1995] where mutation of H4 R45 to cysteine or alanine resulted in an 'empty' minor groove with no additional distortions in the DNA, concluding that the side chain of H4 R45 contributes greatly to this particular protein-DNA interaction interface by making van der Waals contacts with the minor groove, loss of which results in the Sin phenotype. All these can make possible the interactions of protein-protein and protein-DNA necessary for chromosome biorientation in a favorable or unfavorable way. In this context, it appears that in our double mutant H2A N115S can alter the negative interactions of H4K44Q considering the fact that this H4 single mutation is located right beside one of the strongest Sin phenotype mutant residues, H4 R45.

Also, the H4 K44 residue reportedly interacts with the Set2 methyltransferase, which di- and trimethylates the H3K36 residue in *S. cerevisiae*. Iwasaki W et al., demonstrated that the H2A C-terminal loop region (H2A I111−K118) is completely disordered in the H4 K44Q mutant NCP (Nucleosome core particle) and the Set2 binding surface is perturbed in the mutant NCP [Iwasaki W et al., 2011] which can very well have implication with altered chromatin structure and hence abnormal chromosome segregation and our introduced H2A N115S may alleviate this problem and make the double mutant better in terms of chromosome segregation phenotype. Although Set2 has not been implicated in chromosome segregation, a PTM at H4 K44 by Set2 or any other histone modifier could regulate binding of proteins required for centromere function. It will take further study to determine whether the mutant phenotypes we have described are due to the histone-histone (H2A-H4 or H2A-H3, for example) histone-DNA or histone-protein (Sgo1 or

other components of CPC, for example) interactions, or a combination of them. This work provides a new framework to continue the analysis of the nucleosome structure as an essential component of the chromatin required for normal centromere-kinetochore attachments during cell division.

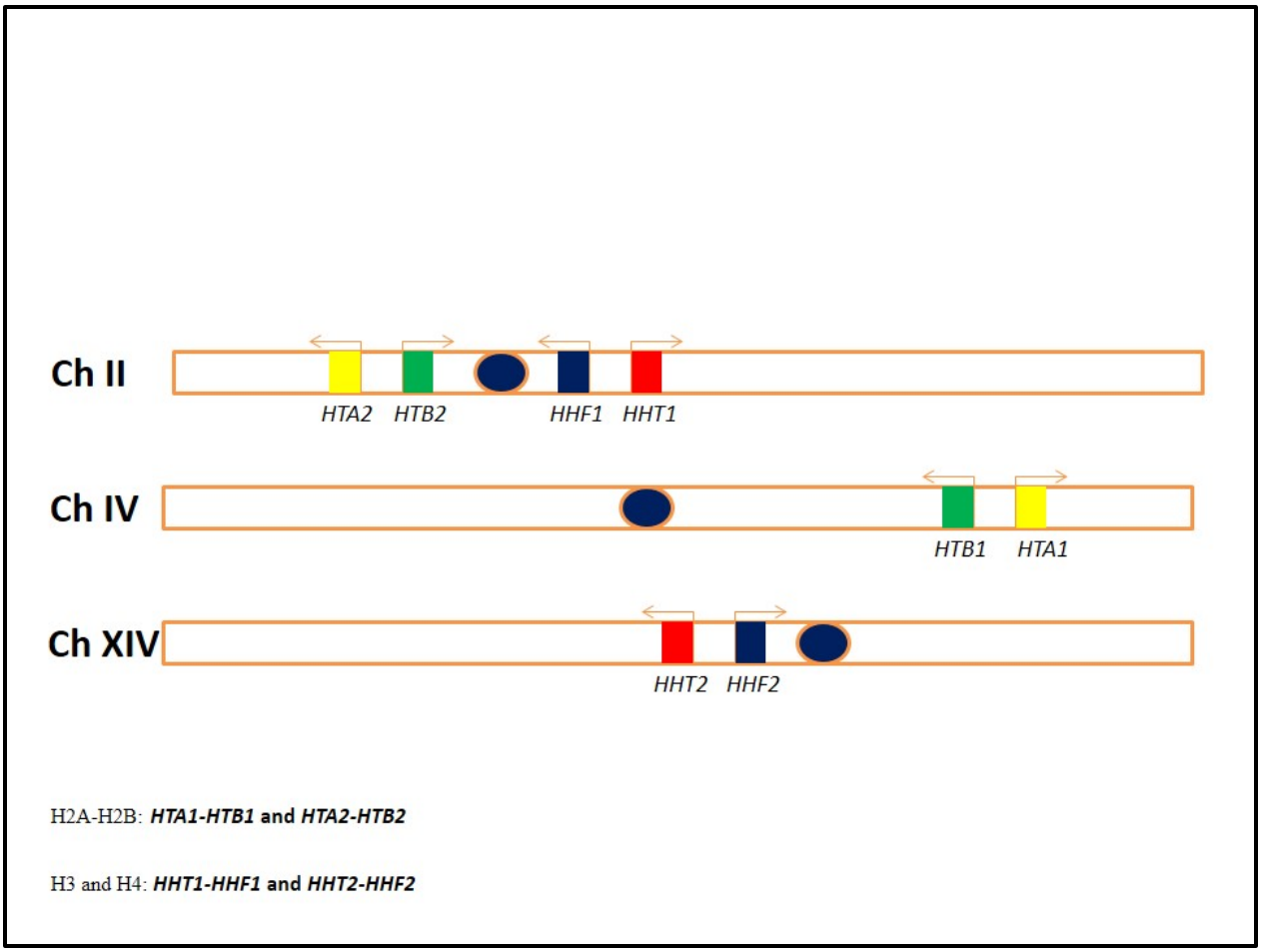


Figure 1: The location of histone genes in *S. cerevisiae* genome

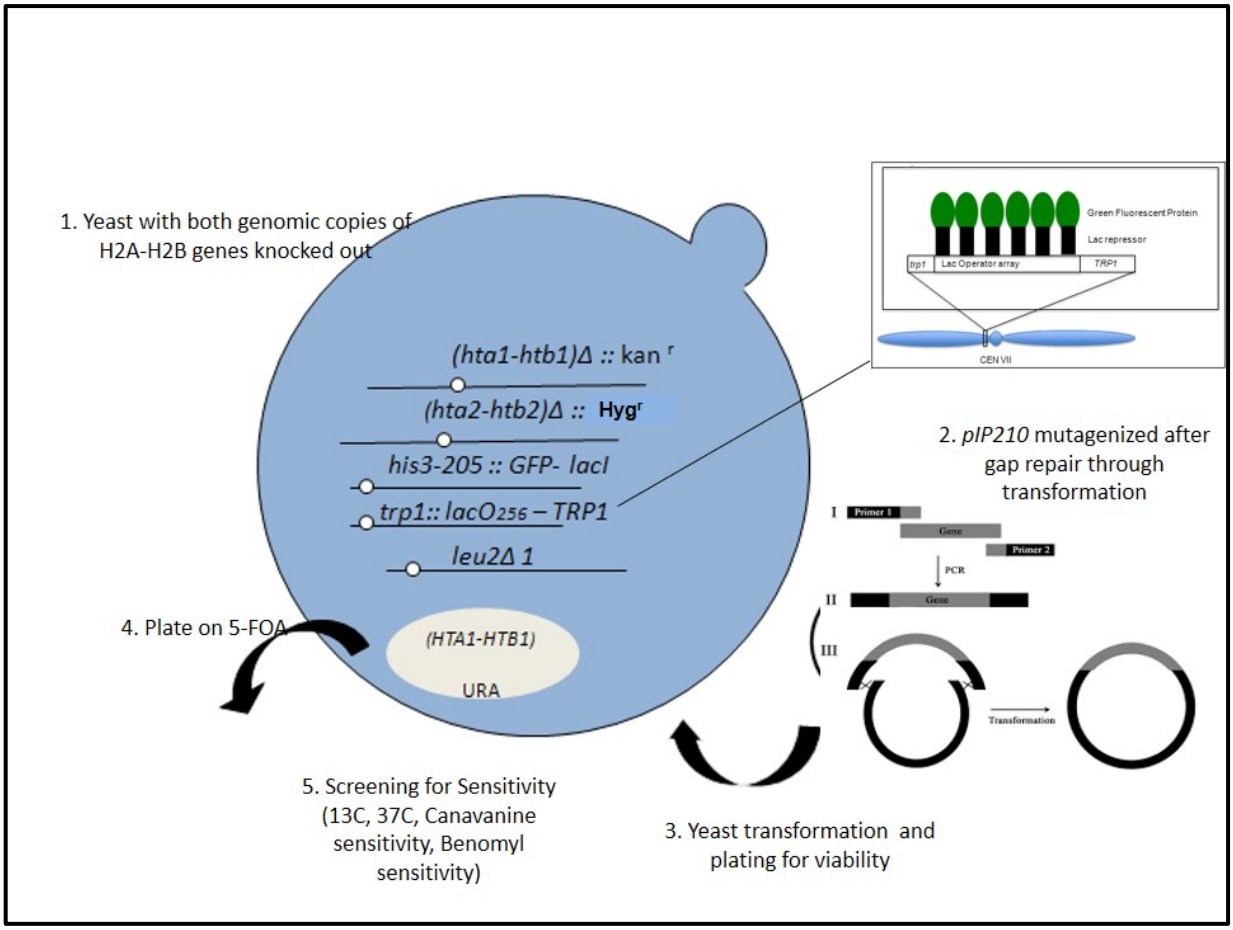


Figure 2: Gap repair and plasmid shuffle for *HTA1* mutagenesis

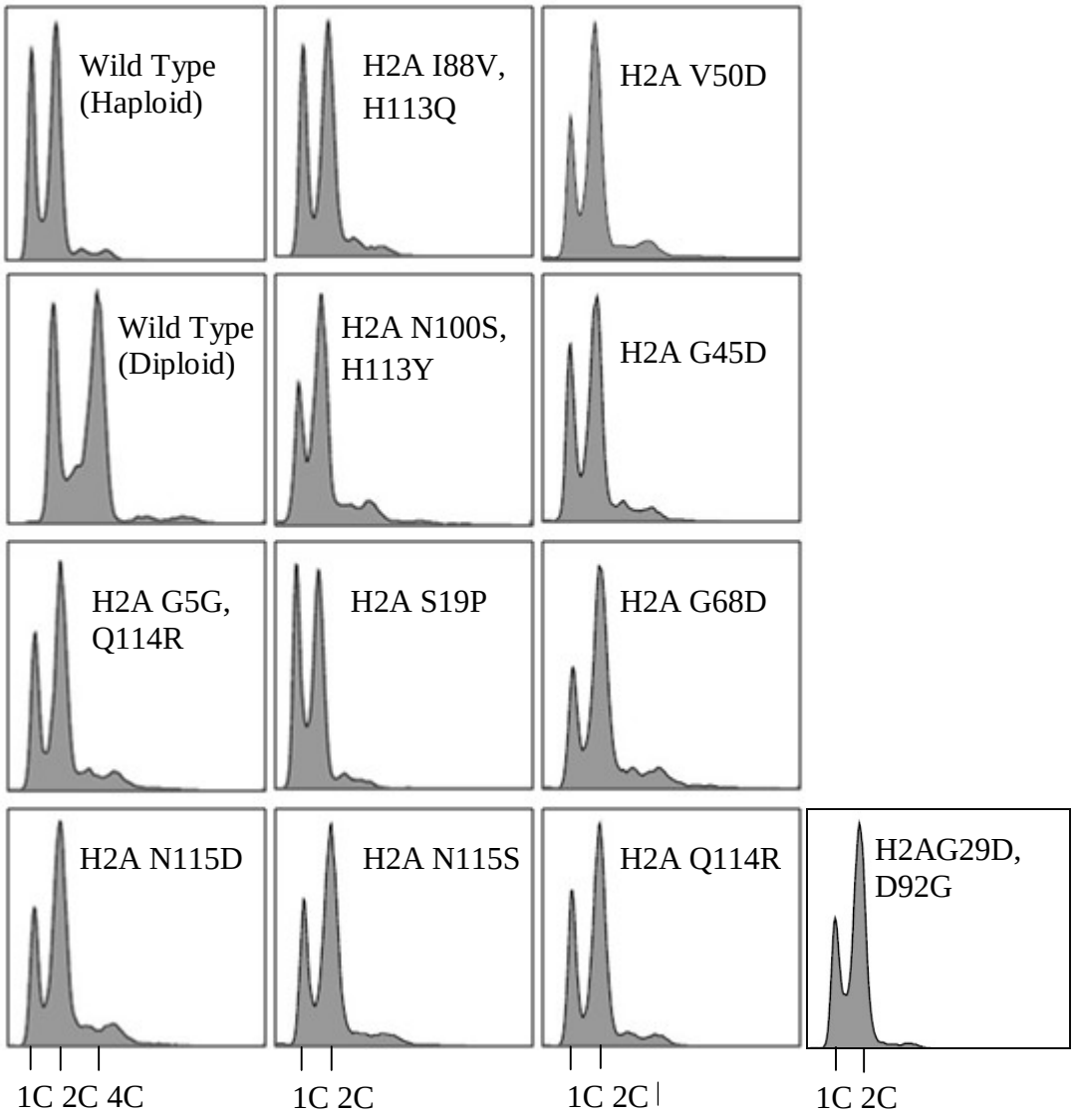


Figure 3: Flow cytometry analysis of the H2A mutants. The presence of 1C and 2C peaks indicate haploid state and the presence of 2C and 4C peaks indicate diploid state.

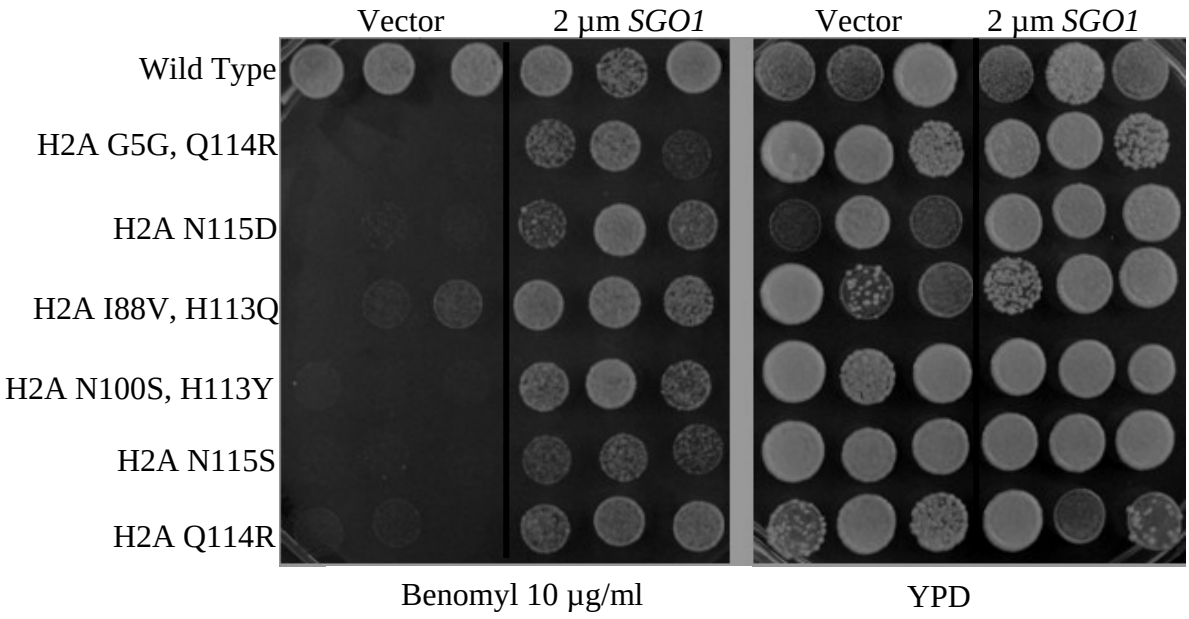


Figure 4: Suppression of benomyl sensitivity of H2A mutants by *SGO1* overexpression. 3 independent colonies from transformation plate with vector and 3 independent colonies from transformation plate with *SGO1* 2 µm plasmid were spotted on benomyl plate and YPD as control. The benomyl plate shows clear suppression.

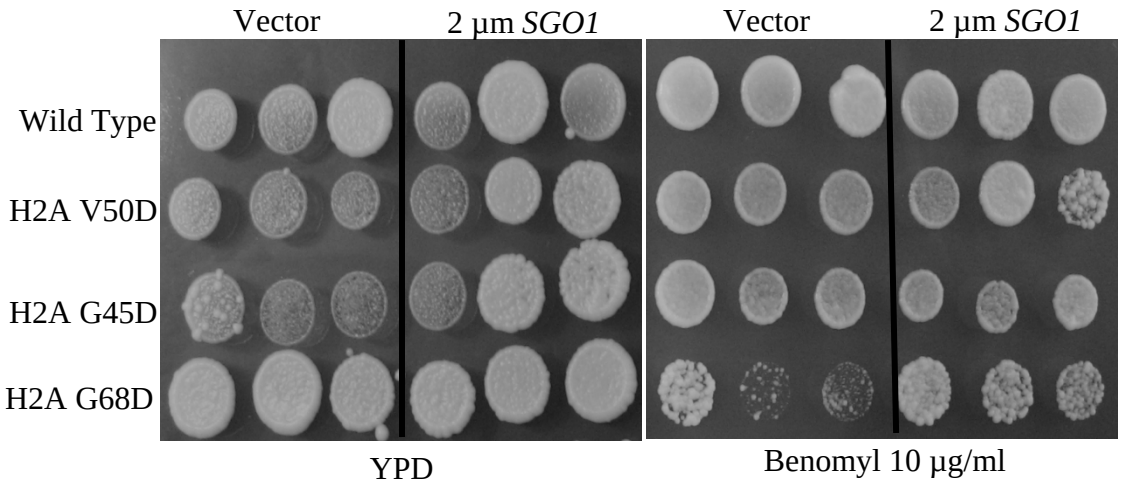


Figure 5: Benomyl sensitivity of H2A mutants in the presence or absence of *SGO1* overexpression. 3 independent colonies from transformation plate with vector and 3 independent colonies from transformation plate with *SGO1* 2 µm plasmid were spotted on benomyl plate and YPD as control.

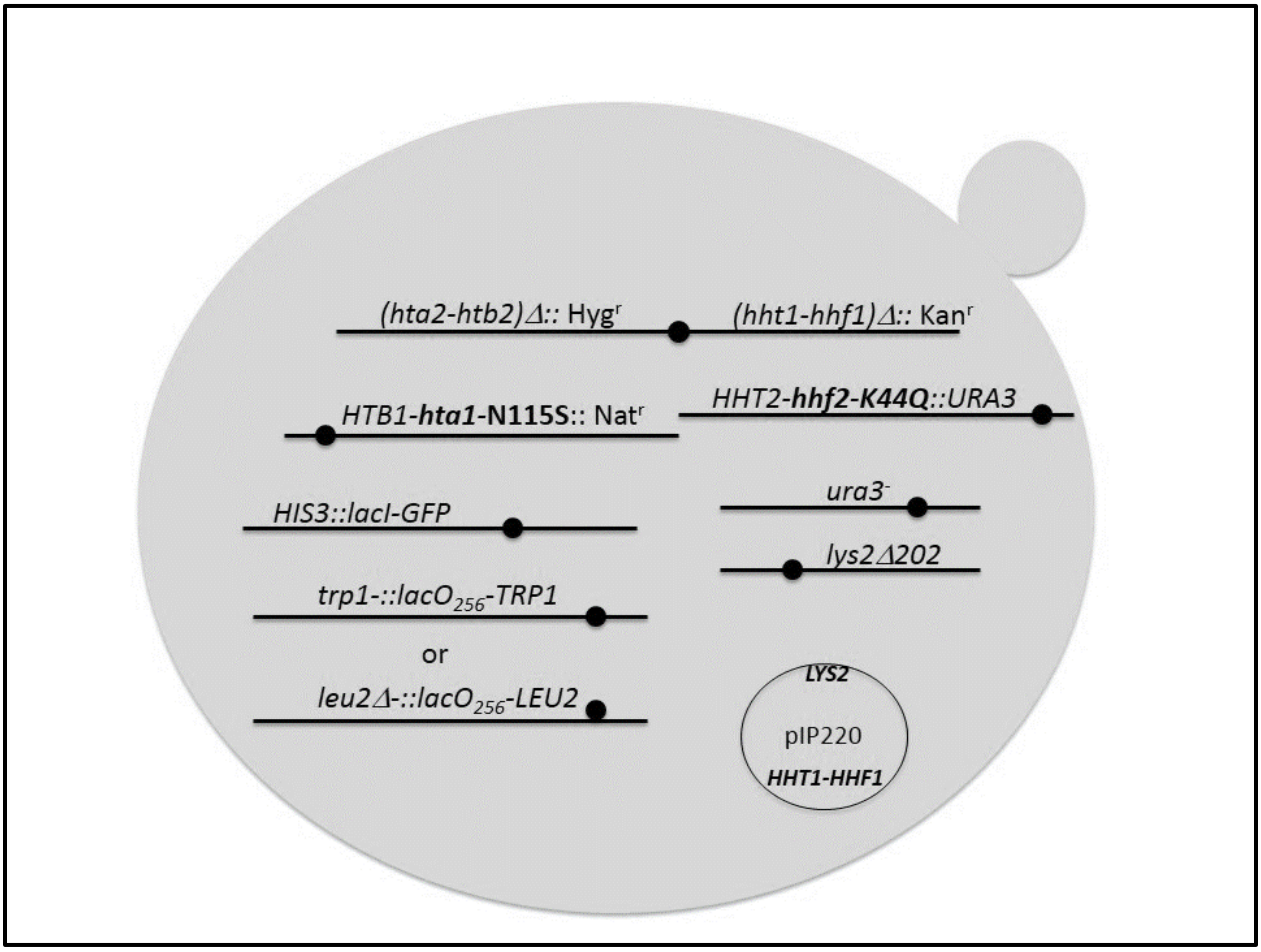


Figure 6: Genotype of the double mutant H2A N115S-H4 K44Q

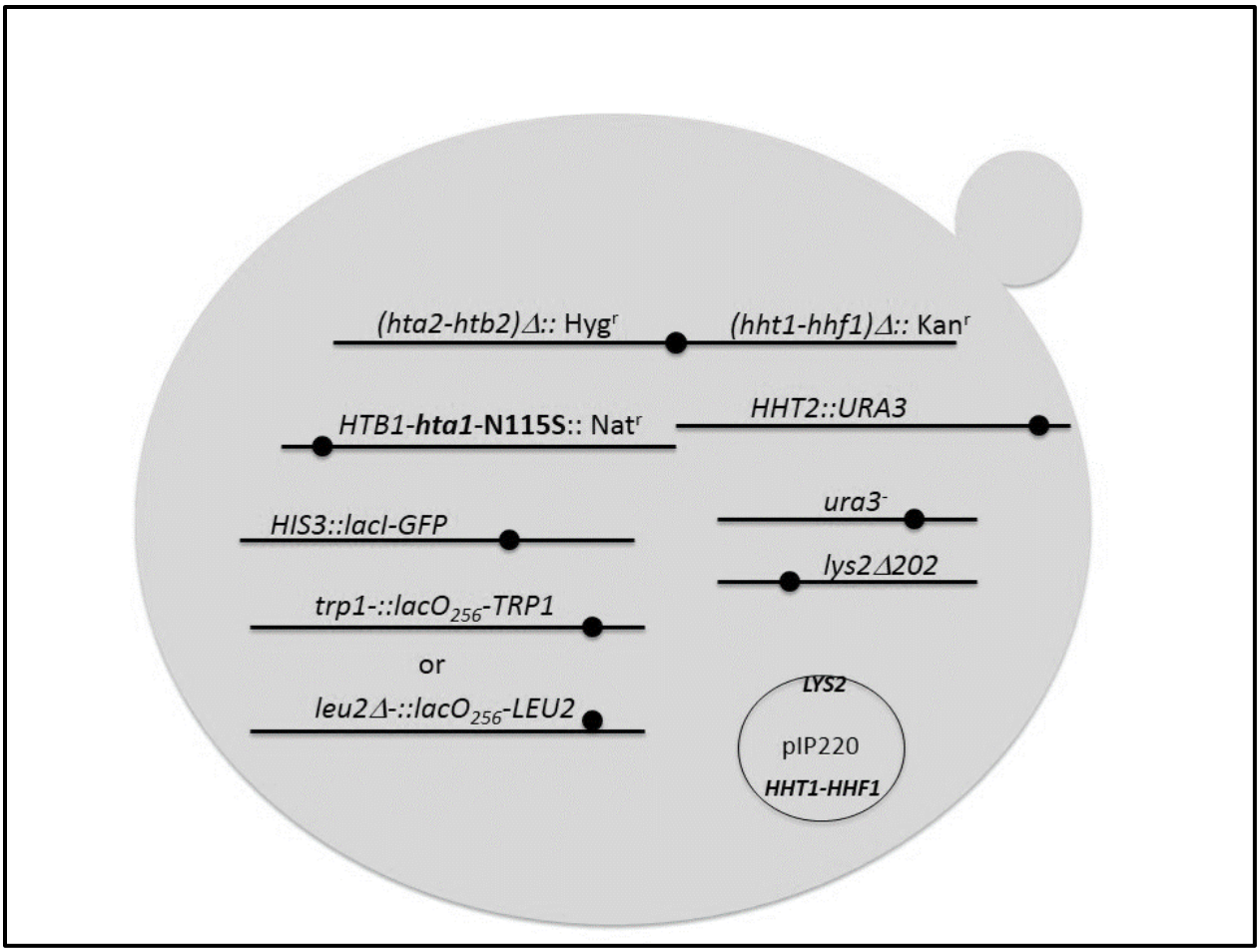


Figure 7: Genotype of H2A single mutant H2A N115S

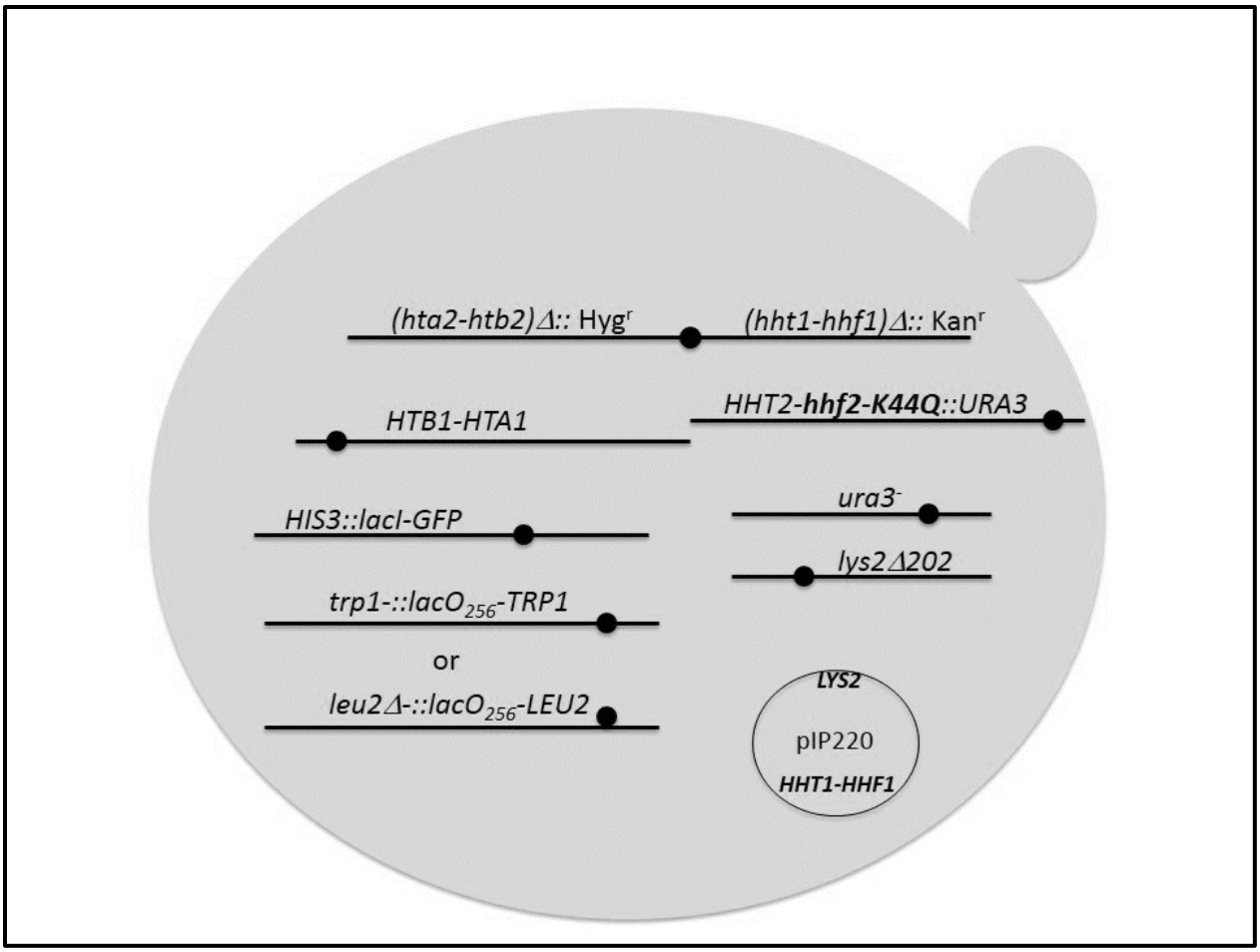


Figure 8: Genotype of H4 single mutant H4 K44Q

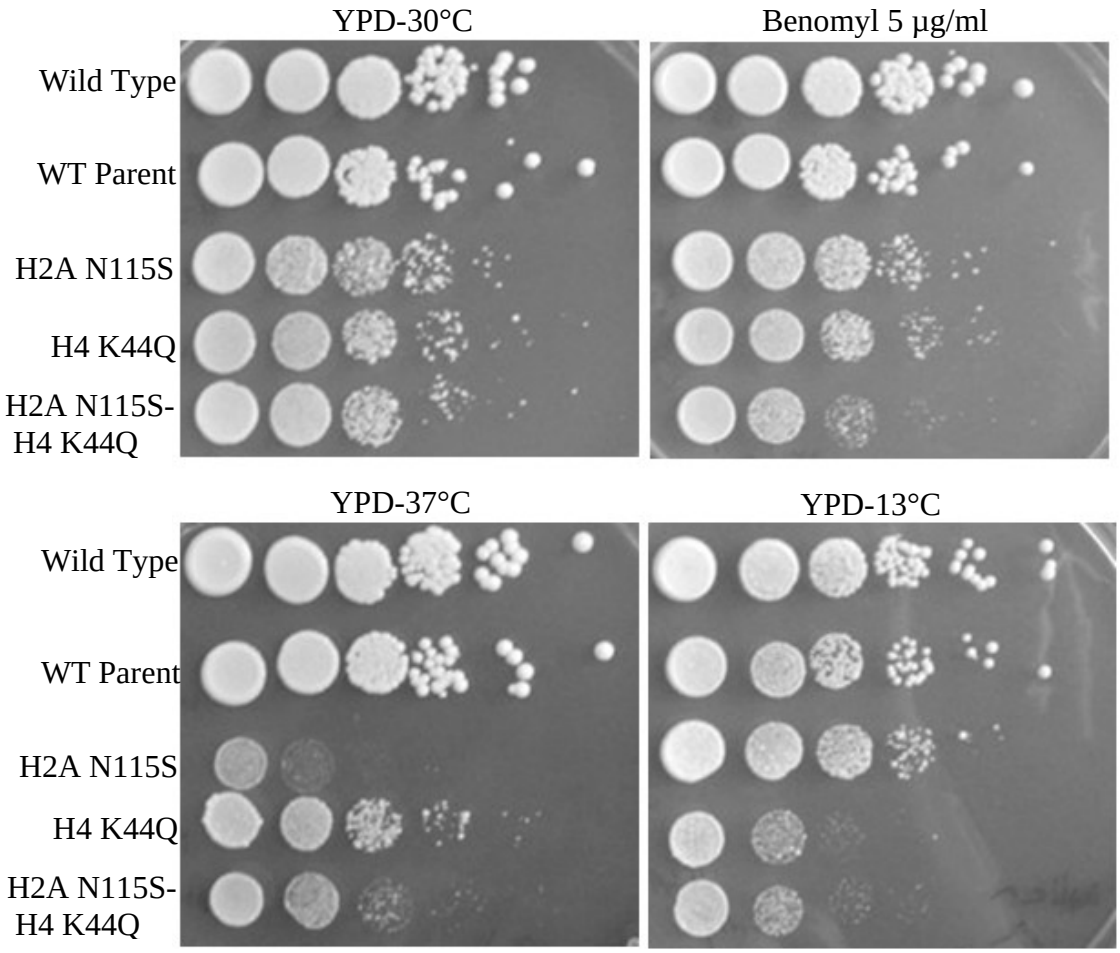
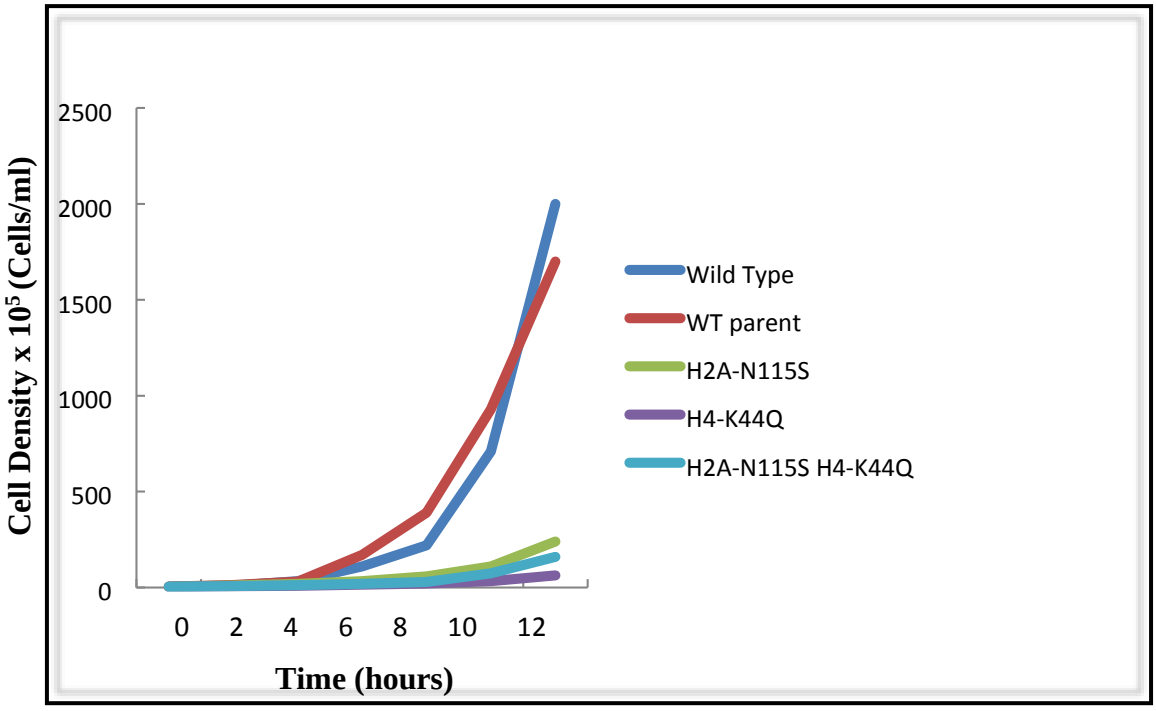


Figure 9: Temperature and benomyl sensitivity of the single and double mutants. Strains were grown at 30°C for 3 days, 37°C (heat sensitivity) for 2 days and 13°C (cold sensitivity) for 7 days to assess their growth at various temperatures. Also shown at top right, the benomyl sensitivity of the mutants where all the strains were allowed to grow in 5 µg/ml of benomyl in YPD for 3 days.



Strain	Doubling Time (hours)
Wild Type	1.4 ± 0.2
Wild Type parent	1.8 ± 0.2
H2A N115S	2.2 ± 0.2
H4 K44Q	3.0 ± 0.4
H2A N115S H4 K44Q	2.4 ± 0.4

Figure 10: Growth curve of the single and double mutants

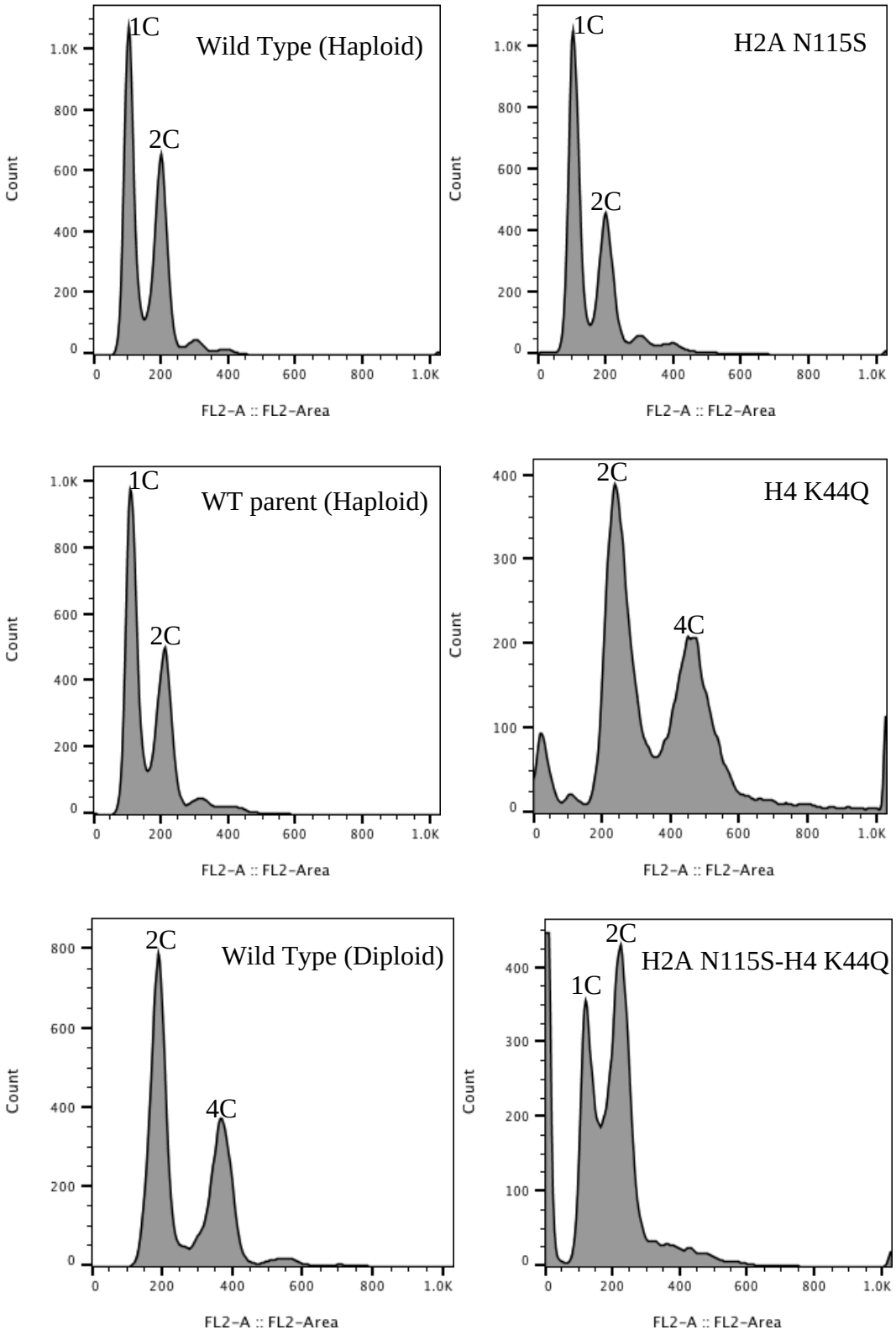
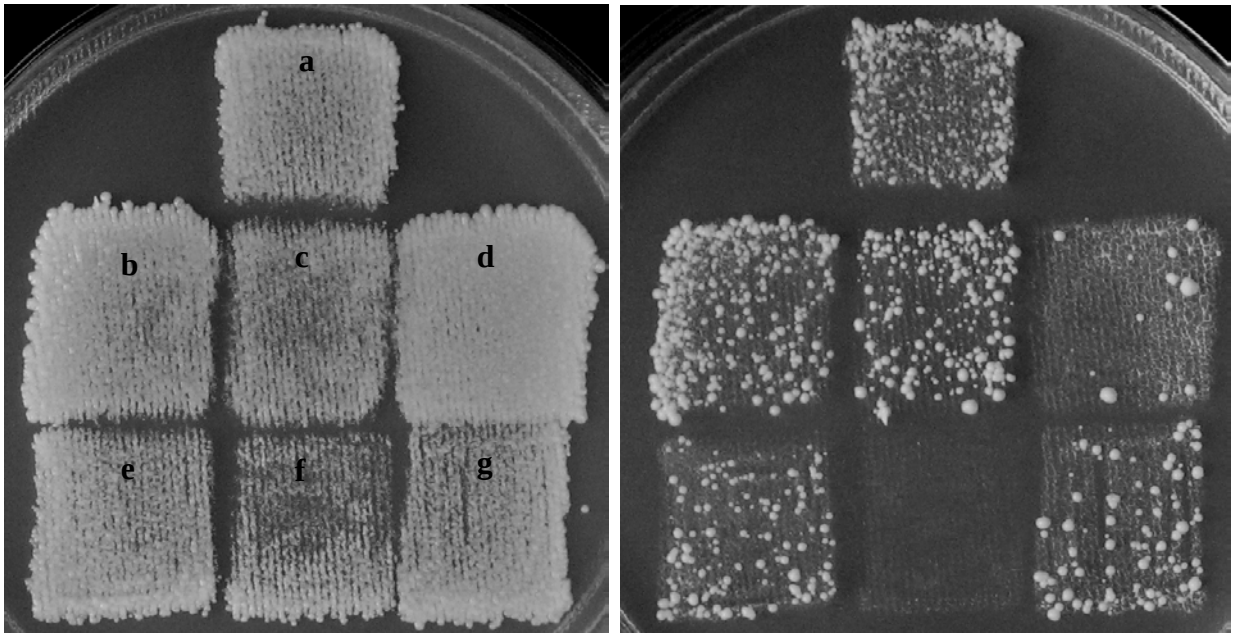


Figure 11: Flow cytometry result of the single and double mutants.



SC-Arg + UV SC-Arg + CAN + UV

a. H2A N115S-H4 K44Q (After three passages in YPD)
b. Wild type (Haploid)
c. Wild type parent (Haploid)
d. Wild type (Diploid)
e. H2A N115S
f. H4 K44Q
g. H2A N115S-H4 K44Q

Figure 12: Canavanine assay of the mutants. Overnight cultures were replica plated on SC-Arg plates with or without CAN (Canavanine), irradiated with UV, and incubated for 5 days at 30°C. Only the single mutant H4 K44Q behaved as diploid indicating an increase in ploidy phenotype.

							
Wild type	53	47		<1			
hta1-N115S	54	36	3		7		
hhf1-K44Q	6	2	1	30	27	20	14
hta1-N115S *hhf1*-K44Q	52	30	2	5	9		2

Centromeres were visualized by the lacI-GFP associated with the lacO array present at CEN III or CEN IV in the indicated strains, grown on YPD. A minimum of 200 cells was counted for each strain. Categories indicate cell morphologies associated with their mitotic stage, visualized under light microscopy and overlapped with the GFP fluorescence.

Figure 13: GFP-CEN assay of the mutants

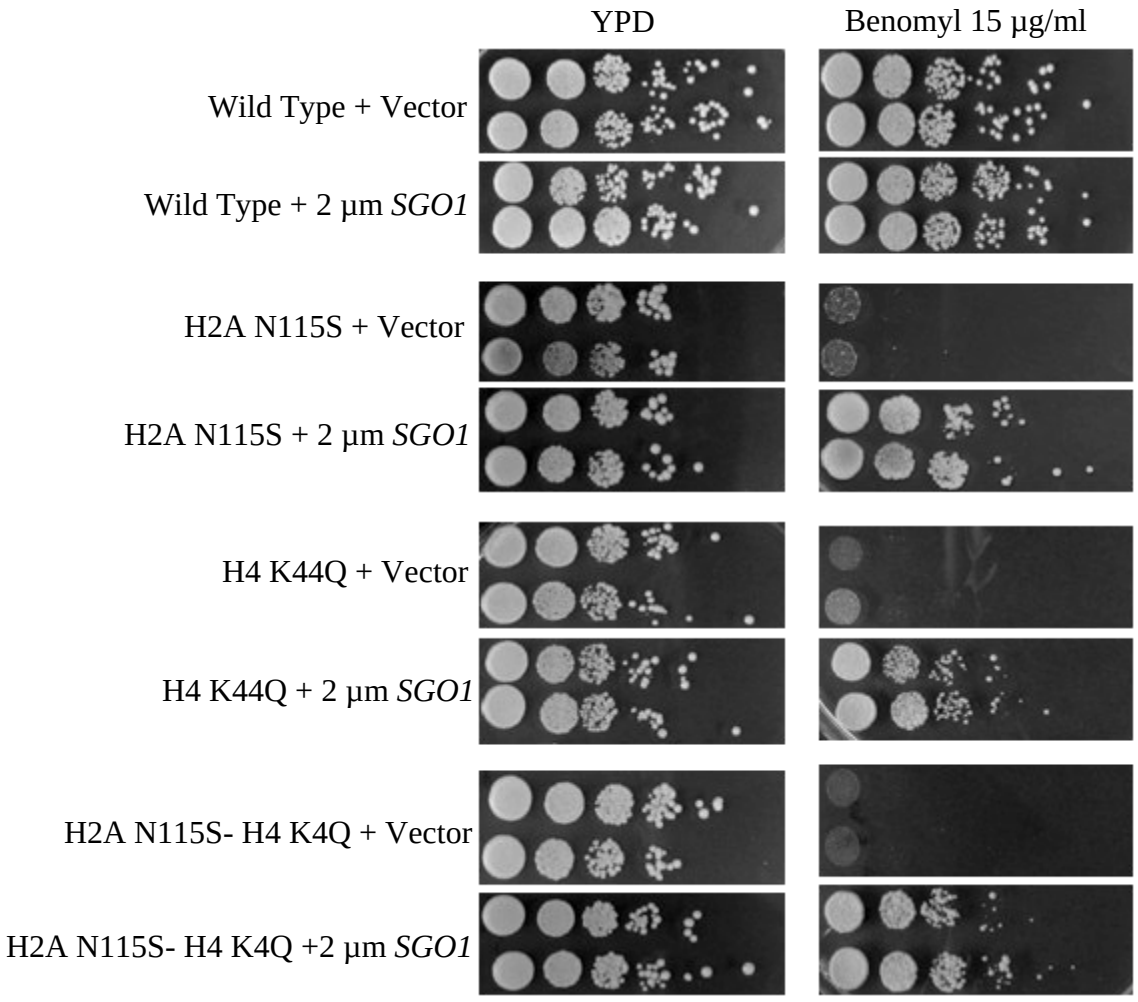


Figure 14: Suppression of benomyl sensitivity of the mutants by high copy *SGO1* 2 µm plasmid expression. All of the mutant strains showed clear suppression.

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