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Characterization of Novel Histone H2B Mutants Associated with Chromosome Segregation Defects in *Saccharomyces cerevisiae*

Characterization of Novel Histone H2B Mutants Associated with Chromosome Segregation Defects in *Saccharomyces cerevisiae*

A thesis submitted in partial fulfillment
of the requirement for the degree of
Master of Science in
Cell and Molecular Biology

By

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Winona State University

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The University of Arkansas

Abstract

Histones are small basic proteins that associate with DNA to form the basic unit of chromatin, the nucleosome. Histones H3 and H4 form a tetramer that is bound by two H2A-H2B dimers to form the histone octamer, to which approximately 146 bp of DNA wrap around to form the nucleosome. High resolution structural information and recent advances in the understanding of histone post-translational modifications have illuminated the many regulatory functions chromatin exerts in the cell, from the transcriptional control of gene expression to chromosome segregation. However, the specific role that histones play in these processes is not well understood. Previous work from this laboratory identified histone H2A mutants that cause chromosome segregation defects associated with altered centromeric chromatin. As a continuation of that work, this project focuses on the identification of histone H2B mutants that cause severe defects in ploidy maintenance and mitotic chromosome transmission. A library of plasmids containing mutagenized copies of the H2B encoding gene, *htb1*, was built in *E.coli* by PCR Mutagenesis. The library was introduced into an *S.cerevisiae* strain that carries deletions of the genomic loci that encode H2B. The yeast transformants were screened for phenotypes that would likely indicate chromosome segregation defects. Fourteen novel H2B mutants were identified that displayed clear defects in chromosome segregation. The location of the amino acid replacements on the nucleosome model suggests domain(s) within the histone octamer are important for chromosome segregation.

This thesis is approved for

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INTRODUCTION

The long-term goal of our research is to understand the specifics of various interactions that are involved in chromatin structure during the chromosome segregation process. Chromatin is made up of four types of core histones proteins as well as DNA. There are various post-translational modifications such as acetylation, ubiquitination and methylation occurring especially at the N- and C-terminal tail residues of histone core proteins which can affect gene regulation. "Some of the post-translational modifications (PTMs) include acetylation of lysines, methylation of lysines and arginines, phosphorylation of serines and threonines, ADP-ribosylation of glutamic acids, ubiquitination and SUMOylation of lysines, and biotinylation of lysines." (Iwasaki et al., 2011)

There are multiple factors which cross-interact at the centromere. Most interactions at the centromere seem to include at least one of the core histone proteins. It was suggested that H2A mutants with single amino acid replacements resulted in altered centromeric chromatin structure. This then could lead to defective microtubule attachment and/or loss in microtubule tension-sensing which directly leads to chromosome loss and increase-in-ploidy phenotypes. (Pinto and Winston, 2000)

The immediate goal of my research is to determine the relationship between amino acid mutations in the H2B histone protein to chromosome segregation defects and ploidy defects. The *htb1* mutant library plasmid was created in a *LEU2* plasmid using PCR Mutagenesis. Following the transformation of the library plasmids into the parent yeast strain, a preliminary phenotypic screen was performed. The initial phenotype screen included cold sensitivity, heat sensitivity,

benomyl sensitivity and canavanine sensitivity testing which allowed us to narrow down the potential mutant candidates to about twenty-two yeast strains. Benomyl is a microtubule depolymerizing drug. Also, fluorescence microscopy and flow cytometry analysis were performed on all these strains. In essence, the work presented here centers on the creation and characterization of novel histone H2B mutants in relation to its role in chromosome segregation.

BACKGROUND AND LITERATURE REVIEW

The *Saccharomyces cerevisiae*, or commonly known as the Baker's yeast is one of the most popular eukaryotic model organism used in biological sciences, especially in molecular biology. Its genome was completely mapped out and published in the year 1996, being the first eukaryotic genome ever published. The real-world contribution of *S. cerevisiae* to the rapid progress in the molecular biology field is comparable to the *Escherichia coli* contributions in leading the way in prokaryotic studies. A fully defined genome has always helped with the various manipulations that molecular biologists perform at the genome level.

S. *cerevisiae* is a fungus belonging to the phylum Ascomycota. *S. cerevisiae* has two mating types the "a" and the "α" mating types whereas its life cycle consist of two stages which is the haploid and diploid stages. Both diploid and haploid cells undergo mitosis for cell repair, development and growth. When mating occurs between a haploid "a" strain and a haploid "α" strain, the genetic material is doubled and the strain becomes a diploid. A diploid yeast cell under stressful conditions such as lack of glucose can undergo sporulation (meiosis) which in turn produces haploid spores.

There are a few good explanations as to why *S. cerevisiae* is very popular in molecular biology studies. First of all, *S.cerevisiae* has a short generation time (doubling time of two hours at 30^{o}C) which enables us to conduct genetic studies and look at the phenotypes and genotypes of filial generations without having to wait for a long time as in the case of other more morphologically complex eukaryotes. Secondly, deletion or addition of genes into the chromosome of *S.cerevisiae* by transformation is relatively easy via homologous recombination.

Besides that, haploid strains of yeast cells simplify the creation of gene knockout or addition as there is only one copy of each chromosome. Finally and most importantly, eukaryotic yeast cells' genome is, for the most part, evolutionarily conserved to higher metazoans like plants and animals. One notable difference between yeast genome and other metazoans is the relatively lower quantity of non-coding DNA in the yeast genome.

Crosses can be performed relatively easy between two parent haploid yeast strains which can later yield four haploids of different mix of genotypes. Crosses are performed precisely to yield haploids with specific genotypes which come in different sets that can be used in research. Other benefits of using S. *cerevisiae* include the fact that it is inexpensive and at the same time, a nonpathogenic organism.

S.cerevisiae genome contains a set of sixteen chromosomes in a haploid strain. *S.cerevisiae* entire genome is only ~0.5% of that the size of human genome. (White et al., 2001) Much of the yeast genome is also constitutively open for transcription as compared to only a small percentage of genome is actively transcribed at a time in higher metazoans. (White et al., 2001)

Chromosome number XII is the largest with about 1×10^6 base pairs in size whereas the smallest chromosome; chromosome number I contain only about 23×10^4 base pairs of DNA. The yeast genome also contains about six thousand open reading frames encoding polypeptides larger than 100 amino acids in length. Every 1.5- 2.0kb of yeast DNA averages one gene and genes repeated in function are often co-regulated although not necessarily clustered together.

Besides that, the classical genetic map of *S. cerevisiae* has over a thousand markers determined by tetrad analysis. One major point to note is that this yeast has extremely active

recombination system. There are also complex families of retrotransposons called "Ty elements" of which there are about fifty dispersed copies per haploid genome.

Many innovative studies which address questions on chromatin structure in living cells came from yeast genetics. One big reason for this is the fact that *S.cerevisiae* genome contains only two copies of core histone non-allelic genes per haploid genome for each of the four core histone proteins. (White et al., 2001) Compared to the simple yeast; "in metazoans cells, the genetic analysis of histone function is complicated by the fact that there are generally many copies of the genes for each major histones." (Smith et al., 1996)

***Saccharomyces cerevisiae* life cycle:**

Yeast cells alternate between haploid (n) and diploid (2n) phases. Both "a" and the "α" cells can reproduce mitotically as stable haploid cells (Figure 1) or they can engage in sexual reproduction and produce a single diploid nucleus as the outcome in a diploid yeast cell. Sexual reproduction occurs via communication of pheromones. Starvation induces sporulation of diploid cells to four distinct haploid spores in which case gene reshuffling becomes possible. When nutrients are abundant again, haploid cells bud and divided mitotically again. It is important to note that only diploid cells can undergo sporulation under stressful conditions. Haploid cells will die under stress.

Sex in yeast cells is determined by the mating type locus (*MAT*) on chromosome III. The two variants of *MAT* gene locus, *MAT*a and *MATα* are called idiomorphs since they are not alleles. Idiomorphs differ in sequence, size and gene content. (Butler et al., 2003) Therefore, the ability for a *MAT*a and *MATα* haploids to mate is determined by the genetic configuration of the

MAT locus. Mating type switching could occur in homothallic strains with intact *HO* gene. A haploid homothallic cell can switch genotype at the *MAT* locus (from *MAT*a to *MATα* or vice versa) by gene conversion. During gene conversion, the genetic information at this locus can be replaced with information copied from one of the two silent cassette loci, *HMLα* or *HMR*a. The initiation of this gene conversion process occurs with Ho endonuclease expression encoded by the *HO* gene on chromosome IV. (Butler et al., 2003) *HO* gene is deleted in heterothallic yeast strains.

Chromatin, nucleosome structure, and the core histones:

Nucleosomes are the fundamental repeating units of the eukaryotic chromatin. (Figure 2) Nucleosomes in *S. cerevisiae* are closely spaced with a linker length of 15-20bp (White et al., 2001). The linker length in higher metazoans is much longer averaging ~190bp. Another major difference between the yeast linker and linkers of higher eukaryotes is that no linker histones have been found to be associated with yeast chromatin. (White et al., 2001).

Besides its principal role as the packaging element of DNA, the nucleosome is also the primary determinant of DNA accessibility. (Luger et al., 1997) In events such as transcription, DNA-binding factors need DNA to be disassociated from histones. The histone octamer is bound to DNA at multiple sites and therefore, histone disassociation would occur in semi-cooperative stages. The disassociation starts at the beginning of entry and exit points of DNA, and then proceeds into H2A-H2B dimer and eventually into H3-H4 tetramer. This pattern of histone disassociation was determined by measuring accessibility of restriction enzyme sites in nucleosome cores. ATP-dependent nucleosome-modification complexes such as the yeast

SWI/SNF complex are required for opening up chromatin and making it accessible to transcription factors. (Luger et al., 1997)

The nucleosome is a highly conserved nucleoprotein complex composed of core histone proteins and 145-147 base pairs of DNA wound in approximately 1.8 left-handed superhelical turns around the surface of the histones. (Smith et al., 1996) Its relative molecular mass is 206 Kd. (Luger et al., 1997) Despite the overall conservation of the nucleosome structure; individual nucleosomes acquire their own characteristics depending on the actual DNA sequence incorporated, which can have functional relevance. (Luger et al., 1997) For example, there is a statistical preference for DNA minor groove to face histone octamer at (A + T) – rich sequences while G.C base pairs are preferred when the major groove faces inwards. The latter is explained by G.C base pairs tendency to bend into major groove. (Luger et al.,1997).

Chromatin is the organizational structure that makes up the genetic information inside the nucleus of all eukaryotic cells. (Figure 3) Depending on the chromatin structure, which can be altered dynamically, all processes related to chromosome function can be regulated, including transcription, replication, recombination and DNA repair (White et al., 2001). X-ray crystallography data from various organisms have confirmed that the structure and function of this important structure is evolutionarily conserved between single cell eukaryotes such as the *Saccharomyces cerevisiae* and metazoans, including humans.

The histone proteins in chromatin function to package DNA in an ordered scheme of folding and compaction. At the first level of order, 1.65 tight superhelical turns of 147 bp of DNA are wrapped around a histone octamer to form a nucleosome core particle (beads on a string structure or euchromatin). Thousands of nucleosomes are compacted further into higher

organizational order leading to the chromatin fibers, which adopt the most condense state in the metaphase chromosome.

DNA packaging and compaction into chromatin is important for several reasons. Firstly, without compaction, the unwound DNA in chromosomes would be very long; so long that the DNA will not physically fit into the nucleus. Chromatin structure affects a whole range of processes that concern the DNA, the hereditary molecule. The different states of chromatin can promote or impede transcription, DNA replication, DNA repair, chromosome segregation among other processes. Furthermore, the dynamism of the chromatin structure is very complex and itself regulated by a host of different mechanisms.

As it was mention previously, core histone proteins are evolutionarily conserved from yeasts to higher order eukaryotes. This means there are minimal differences among the amino acid sequences of the histone proteins across species. The histone octamer consists of two copies of each of the four core histone proteins; H2A, H2B, H3 and H4. (Figure 4) Two histone pairs; either H2A and H2B or H3 and H4 forms tight dimers and subsequently organize about 30 bp of DNA. It is important to note that the histones form only H3-H4 and H2A-H2B heterodimeric pairs and not homodimers or other heterodimers, and this is a result from complementary internal packing. (Luger et al., 1997) Together, the octamer organizes about 146 bp of DNA which is referred to as a nucleosome. An interaction between the two H2A-H2B dimers happens at a small interface formed by the L1 loops of two adjacent H2A molecules.

All four core histone proteins can be divided into four regions; (1) central surface regions, (2) inner regions, (3) N-terminal tail which projects from the histone core and (4) C-terminal tail. The motif of all four histone proteins are highly similar, the histone fold consists of

three alpha helices connected by two loops; alpha1-loop1-alpha2-loop2-alpha3. (Figure 5) Also, a two-fold symmetry axis falls between the H3-H4 heterodimers and H2A-H2B heterodimers. The axis of symmetry intersects a single base pair in the center of the superhelix, dividing the 146bp DNA into 72bp and 73bp halves. Besides having a structural role, core histones also play significant part in gene regulation. Histones are known to "collaborate with transcription factors to provide for their own removal or structural modification, resulting in gene derepression." (Luger et al., 1997)

Although all eukaryotic core histones are extremely conserved across species, there are small but significant differences between the nucleosomes of yeast and higher eukaryotes' such as that of *Xenopus laevis*. Crystallography data reveals that the molecular surface of both *X. laevis* and *S.cerevisiae'*s nucleosome core particle (NCP) is characterized by a large acidic patch formed by seven amino acids from regions of H2A and H2B. Several basic regions provide the appropriate charge to create the interface with the surrounding DNA (White et al., 2001). However, the surface of the NCP in *S.cerevisiae* is more positively charged and therefore more basic compared to *X.laevis.* In addition, in *S. cerevisiae,* nucleosomes in neighboring planes stack on top of each other with their superhelical axes almost superimposed, while in *X.laevis* are more staggered (White et al., 2001).

The difference in crystal packing renders the *S.cerevisiae* -NCP crystals more fragile. White et al. propose that the dramatic changes in nucleosome-nucleosome interactions seen in yeast are a result of contacts involving the C-terminal helix (αC) of H2B (White et al., 2001). The αC helix essentially lies exposed on the surface of histone octamer in *S.cerevisiae*. However, it is important to note that all of the amino acids that are critically involved in the organization of DNA are conserved between both species (White et al., 2001).

Centromere, kinetochore, cell-cycle checkpoints and chromosome segregation:

The centromere is the part of the chromosome that links sister chromatids. Chromosomes segregation during mitosis and meiosis requires a physical connection between spindle microtubules and chromosomes. Spindle fibers attach to the centromere via proteinaceous structures called kinetochores during mitosis and meiosis. This attachment mediates the movement of chromosomes along the microtubules of the mitotic spindle (Cheeseman et al., 2002).

In eukaryotes, sister chromatids are connected to each other from S phase until the onset of anaphase. This allows for a relatively long time to pass after duplication is complete for chromosome segregation to begin. Sister chromatids are essentially connected to each other by a multi-subunit complex called cohesin. The proteins that form cohesin are highly conserved between yeast and humans. Moreover, the centromere serves as a *cis*-acting cohesion factor, which plays a significant role in establishing and maintaining of cohesion. (Kitagawa and Hieter, 2001)

The spindle fiber is composed of hundreds of proteins but its fundamental apparatus is the microtubules. Microtubules are essentially polymers composed of highly conserved subunits; the α- and β-tubulins which heterodimerize repeatedly to form the cytoskeletal fiber. The dynamicity of spindle microtubule ensures that an excess of microtubule ends are available for kinetochore capture during mitosis. The ends of spindle microtubules are available until every chromosome is attached. (Schulman and Bloom, 1991)

The most conserved structural element of *S.cerevisiae* centromeres is CDEIII, a 25bp DNA sequence. A single point mutation within CDEIII can disrupt entire centromere function.

These single point mutations which are able to disrupt centromere function also eliminate nuclease-resistance feature of centromere. Furthermore, the central CG base pair of CDEIII is conserved in yeast centromeres, and nucleotide substitutions cause a clear defect in centromere function. (Saunders et al., 1988) Thus, it is hypothesized that this highly conserved region is the primary contact site for binding or recognition of specific CEN proteins.

The centromere performs at least three critical roles during the cell division cycle. First, it provides the site of attachment for replicated sister chromatids (Glowczewski et al., 2000). Second, it serves to prevent premature sister chromatid separation during segregation (Brent and Finley Jr., 1997). Third, the kinetochore complex activates the spindle assembly checkpoint (SAC) pathway in the presence of spindle damage (Ortiz et al., 1999). It has been shown that kinetochore proteins can send a signal to the spindle checkpoint, which in turn will halt cell cycle progression to allow for repair and reattachment of chromosomes to the spindle (Kitagawa and Hieter, 2001). SAC functions to delay anaphase in response to either defective spindle organization or failure of chromosomes to attach to the spindle microtubules (MTs) via kinetochores. SAC essentially ensures the fidelity of chromosome transmission.

Individual kinetochore proteins belong to three distinct categories. Inner kinetochore proteins function at the interface with centromere; central kinetochore proteins function at the interface between the inner and outer kinetochore proteins, whereas outer kinetochore proteins functions at the interface with spindle microtubules. (Cheeseman et al., 2002)

Kinetochores must be assembled on sister chromatids facing opposite poles. Each kinetochore is then captured by MT's emanating from only one pole of the mitotic spindle. Sister chromatids that fail to obtain bipolar attachment will not segregate correctly at anaphase.

Misattachment of a single kinotochore is sufficient to activate the spindle assembly checkpoint and arrest cell cycle progression. (Gillett et al., 2004)

Bipolar attachments are necessary as they give rise to proper tension across paired sister kinetochores, which leads to the SAC silencing. In budding yeast, the primary signal for this checkpoint is the absence of an attachment between the centromere and the spindle. In addition to sensing microtubule defects, the yeast mitotic checkpoint also perhaps monitors tension on the kinetochore. Absence or a lack of tension can temporarily activate SAC even when kinetochore-microtubule connections are intact. (Cheeseman et al., 2002)

There have been several studies on examining the importance of histone in centromere structure and functions. The centromeric DNA is found to be organized around centromeric nucleosomes that contain specialized histone H3-like proteins (yeast Cse4p or its metazoan homologue CENP-A). These histone H3-like protein containing-nucleosomes are found only at centromeres. (Cheeseman et al., 2002)

A study was conducted on two *S.cerevisae* strains which gene for either histone H2B or H4 was repressed. It was shown that histone levels had a direct effect in the chromatin structure at the centromere. (Saunders et al., 1990) The centromere structure was found to be susceptible to micrococcal nuclease following histone repression. This research conclusively showed that histone proteins are involved in determining the centromere structure in chromatin, which when repressed, rendered the mutant cells incapable of chromosome segregation. (Saunders et al., 1990)

Apart from H2B and H4 mutants, H2A mutants also exhibit altered centromere chromatin. Analysis of chromatin structure of CENIII region by MNase digestion in *hta1-200*

and *hta1-300* mutants revealed clear difference in internucleosomal cuts compared to the wild type. Further, double mutant genetic studies performed established genetic interactions between histone H2A mutants and kinetochore components. The study went on to propose that the interaction between histone H2A present in nucleosomes flanking the centromere and kinetochore proteins is essential for normal centromere function. (Pinto and Winston, 2000)

***HTA1-HTB1* gene:**

All four core histones are essential for yeast cell viability. Proper histone levels are critical for all chromatin-mediated processes including transcription, chromosome segregation, DNA replication and DNA repair. In *Sacchromyces cerevisiae*, core histones proteins H2A and H2B are encoded by two gene pairs, *HTA1-HTB1* and *HTA2-HTB2*. *HTA1-HTB1* is located at chromosome IV whereas *HTA2-HTB2* gene is located on chromosome II.

Previous studies have indicated that when *HTA2-HTB2* gene is deleted, the *HTA1-HTB1* dosage compensates at the transcriptional level. This however, is not true for *hta1-htb1*Δ mutants as these mutants grow poorly or does not germinate as meiotic products. (Libuda and Winston, 2006)

Histone, histone modifications, histone mutations and chromosome segregation:

The nucleosome consists of two copies of four core histones H2A, H2B, H3 and H4 arranged as two H2A-H2B dimers and one H3-H4 tetramer, around which 146bp of DNA is wrapped around. Generally, each type of core histone is composed of a globular domain of

helical content and an extended N-terminal segment of 12 to 26 amino acids. (Morgan et al., 1991)

All four core histone proteins, being evolutionarily conserved, display high amount of identity in terms of histone octamer function at the level of histone-DNA interaction despite the differences in amino acid sequence and chromatin organization between yeast and higher eukaryotes. (White et al., 2001) Importantly, the amino acids that participate in protein-DNA interactions and the overall architecture of the histone octamer are unchanged between *S.cerevisiae* and *X.laevis*. (White et al., 2001)

The globular regions of the core histones contain about equal amount of acidic and basic amino acids. Majority of DNA-histone and histone-histone interactions involve the globular region which makes up the nucleosome core particle. As opposed to the globular regions, the N-terminal domain amino acid composition contains a relatively higher concentration of positively charged amino acid residues. (Morgan et al., 1991)

Both histone N- and C-terminal tails are believed to affect the folding of nucleosomes into higher-order chromatin structure. (Maruyama et al., 2006) Moreover, part of the N-terminal regions may interact with the DNA and further stabilize nucleosome structure even without having much role in maintaining core particle structure. (Morgan et al., 1991)

During DNA replication old histones are recycled from the parental DNA to the newly replicated DNA, and new histones are deposited in order to maintain the proper density of nucleosomes. (Yu et al., 2011) A variety of histone chaperones exist to carry out this function. Histone chaperones also serves to prevent nonspecific interaction between the basic histones and negatively charged DNA. (Yu et al., 2011)

Histone chaperones are categorized into two groups based on the kind of histone cargoes the carry. The central H3/H4 tetramer is loaded first on the DNA, followed by the deposition of H2A/H2B dimers on the nucleosome. Conversely, during nucleosome disassembly, H2A/H2B dimers are removed before the H3/H4 tetramer. (Yu et al., 2011)

The amino-terminal domains of core histone proteins are evolutionarily conserved highly basic polypeptide sequences that comprise 25%-33% of the proteins. They extend outward from the nucleosome core particle. The functions of the amino terminal domains have been investigated and evidence shows that they are responsible for many of the dynamic properties of eukaryotic chromatin. (Megee et al., 1995)

Unquestionably, post-translational modification (PTM) is an integral part of histone structure and function. Historically, histone PTMs are thought to only occur at the N-terminal tails but recent studies have shown PTMs also occur in histone fold regions, although to a lesser extent. PTMs may change the interaction of a histone protein with DNA or with other chromatin-associated proteins. Different downstream cellular processes such as transcription may require these covalent modifications. (Rice and Allis, 2001)

The most targeted residue for PTM in all core histones is the lysine amino acid residue. Lysine residues can be acetylated, methylated or ubiquitinated. "In *S.cerevisiae* H4 histone, K16 is the most favored site of acetylation, with 80% of all H4 molecules, and most or all monoacetylated H4 acetylated at K16." (Millar et al., 2006)

Both histone acetylation and methylation are reversible. Eukaryotic organisms encode enzymes such as histone acetyltransferases (HATs) and histone methyltransferases (HMTs), which add modifications as well as enzymes such as histone deacetylases (HDACs) and histone

demethylases (HDMs), which remove them. Lysine acetylation neutralizes the positively charged lysine and may alter histone-DNA and nucleosome-nucleosome interactions. (Millar et al., 2006) Methylation does not change the charge of lysine residue at physiological conditions. However, both methylation and acetylation of lysine residues can create binding sites for chromosomal proteins. (Millar et al., 2006)

"Human histones H3 and H4 contain 11 lysine residues in their histone fold region and 8 of those residues are known to be targets for acetylation." (Iwasaki et al., 2011) The study further reported that some of those amino acids substituted went on to alter local protein-DNA interactions and interactions between amino acid residues within the nucleosome, based on the model provided by the x-ray crystallographic data of nucleosome core particle. (Iwasaki et al., 2011)

A relatively recent study has utilized the development of antibodies that recognize individual histone modifications to map specific histone modifications to unique DNA sequences by chromatin immunoprecipitation (ChIP). This study used the ChIP method to map the distribution of 20 of the known modifications of H2A, H2B, H3, H4 and variant H2A.Z across all sixteen *S.cerevisiae* chromosomes. The comparison of individual modifications in this study showed histone modifications can occur concurrently. Similar patterns of modified and unmodified sites are found on biologically correlated genes. (Millar et al., 2006) The ChIP–on–chip experiments also revealed that nucleosome abundance is lower at active promoters than at inactive promoters.

Another type of PTM, the ubiquitination, is a known epigenetic marker in a host of chromatin-based events. Previously, it was shown that the yeast ubiquitin-conjugating enzyme

Rad6 is involved in the methylation of histone H3 at lysine 4 through ubiquitination of H2B at Lys 123. (Sun and Allis, 2002) They concluded by referring the 'trans-tail' regulation of histone modification as part of the "histone code hypothesis." It was later revised that K123 of histone H2B was in fact, not monoubiquitinated but extensively polyubiquitinated with at least two Ub groups. The polyubiquitination of H2B also appeared "to occur within the context of chromatin and is not associated with H2B destruction." (Geng and Tansey, 2008)

H2B-Ub at K123 participates in an assortment of processes including establishment of cell size, meiosis, gene activation, gene silencing and histone H3 methylation. At active genes, H2B ubiquitination is essential for methylation of histone H3 at lysine residues K4 and K79. (Geng and Tansey, 2008)

Histone H2B ubiquitination seem to associate specifically with RNA Polymerase II elongation during transcription. Apparently, H2B ubiquitination is regulated by the carboxy-terminal domain (CTD) of RNA Pol II and likely play a role in transcription elongation. It was demonstrated that "Rad6-mediated Ub-H2B is reduced by Pol II CTD mutations and completely abolished by the inactivation of the Ser5 CTD kinase that promotes the initiation of elongation." (Xiao et al., 2005) It is hypothesized that series of ubiquitination and deubiquitination in histone H2B may provide directionality to the transcription process. (Geng and Tansey, 2008)

There is a proposition that "distinct histone modifications, on one or more tails, act sequentially or in combination to form a 'histone code' that is, read by other proteins to bring about distinct downstream events." (Strahl and Allis, 2000) Any DNA-templated processes such as replication, repair, recombination and chromosome segregation could potentially be these "downstream events." The view of a histone 'language' was initially suggested since there was

remarkable diversity and biological specificity associated with distinct covalent histone modifications. The 'language' that may be encoded on histone tail domains was termed 'histone code' which gets read by other proteins or protein modules. (Strahl and Allis, 2000)

Chromatin condensation in apoptotic cells has been linked to phosphorylation of H2BS10 in yeast and human cells. (Ahn et al., 2006) In a related study, modification of K11 in H2B N-terminal region was found to be a significant in yeast apoptosis. H2B K11Q mutants were resistant to cell death elicited by H_2O_2 while H2B K11R mutants that mimic deacetylation promote cell death. Further, it was also shown that acetylation of histone H2B at K11 inhibits phosphorylation of an adjacent site S10, catalyzed by Ste20 kinase. From these observations, it was deduced that there exist a regulatory network of "acetylation-phosphorylation unidirectional crosstalk which serves to integrate input signals to the tail of yeast H2B, controlling a switch from cell proliferation to cell death." (Ahn et al., 2006)

Genome-wide studies have shown that acetylation at many sites, especially those in H3, shows positive correlation with transcription activity. However, this is not true for all sites. For example, acetylated histone H4K16 located in the middle of coding regions of genes is negatively correlated with transcription. (Millar et al., 2006) Conversely, deacetylation is not only associated with gene repression but is also involved in gene activation. For example, Hos2, a histone deacetylase, is recruited preferentially to active genes where it is required for optimal transcription. (Millar et al., 2006)

H3 histone phosphorylation at serine 10 has been associated with immediate-early gene activation such as the activation of the *c-myc*. Moreover, H3 histone phosphorylation at both serine 28 and serine 10 has been shown as a must for proper segregation. (Strahl and Allis, 2000)

Expression studies in *S.cerevisiae* have shown N-terminal domain of H2B has a role in genome-wide transcription. In the study aforementioned study, lysine residues K6, K11, K16, K17, K21 and K22 in the N-terminal domain of H2B were systematically mutated and characterized. The mutations resulted in changes in genome-wide protein expression. It was concluded that known sites of lysine acetylation in this domain are required for transcriptional activation of a number of genes involved in NAD biosynthesis and vitamin metabolism. (Parra et al., 2006)

However, it was also reported that residues 30-37 of the H2B N-terminal domain, which is called the H2B repression (HBR) domain, is required for transcriptional repression of a large subset of yeast genome. Moreover, the deletion of HBR domain was found to cause an increased sensitivity to DNA damage by UV irradiation. (Parra et al., 2006)

In a separate study involving histone H2A mutants, it was found that a new class of mutations causes transcriptional defects at the SNF/SWI-dependent gene *SUC2*.The complete deletion of N-terminal tail of histone H2A resulted in a decrease in *SUC2* transcription. These mutants were also phenotypically distinct from *snf/swi* mutations. (Hirschhorn et al., 1995)

The SNF/SWI complex is essential for transcription activation of large number of diversely regulated genes by overcoming chromatin-mediated gene repression. Most importantly, this study showed that, unlike *snf/swi* mutants, the chromatin structure at the *SUC2* promoter in these H2A mutants was in an active conformation thereby confirming the H2A mutations were interfering with transcription activation independent and possibly downstream of the SNF/SWI activity. (Hirschhorn et al., 1995)

In an *S.cerevisiae* alanine substitution mutant screen, three adjacent residues L97, Y98 or G99 near the C terminus of H4 led to mutant phenotypes including slow growth, polyploidy, aneuploidy and high rate chromosome loss. The suspect for these phenotypes is improper kinetochores assembly caused by problems in histone deposition pathway throughout the genome for the H4 mutants. Poor histone deposition causes a lower nucleosome density on the chromatin and hence, poor growth. This ultimately contributes to the observed genome instability and growth defects. (Yu et al., 2011)

Additional analyses of histone mutants have shown that histones play functional roles in sporulation and nuclear division. (Smith et al., 1996) Another set of mutational studies conducted showed histone H4 plays a role in genomic integrity of *S.cerevisiae.* The research suggested that the N-terminal domain of histone H4 is required for mitotic progression in *S.cerevisiae*. Mutations that delete the N-terminal domain or alter four conserved lysine residues within N-terminal domain caused delay during G2 + M phases of cell cycle. (Megee et al., 1995) Moreover, N-terminus of H4 has also been shown to be essential for transcriptional repression of the silent mating type loci.

Histone H4 allele *hhf1-20* has been shown to confer defects in core centromere chromatin structure and mitotic chromosome transmission. (Glowczewski et al., 2000) The novel temperature sensitive H4 mutant, *hhf1-20*, at semipermissive temperatures has a 60-fold increase in the frequency of chromosome loss. The same study further established that Cse4p-histone H4 interactions are crucial for centromere function in *S.cerevisiae*. (Glowczewski et al., 2000)

In order to investigate which cellular processes are dependent on histone H2B, a research team blocked histone H2B mRNA synthesis and examined chromosome segregation, replication

and transcription in asynchronously growing yeast. In wild type cells, histone synthesis and assembly of nucleosomes onto newly replicating DNA occurs during S-phase, followed by chromosome segregation in mitosis. (Han et al., 1987) In this study, the cells with H2B mRNA blocked were arrested in mitosis, with a cell division cycle (*cdc*) phenotype. Also, chromatin structure and nuclear segregation were altered. (Han et al., 1987)

They reasoned that chromosome segregation defect observed was a direct result of H2B depletion as chromosome structure changes were observed. Basically, fewer intact nucleosomes were observed after cell cycle arrest. Additionally, phenotypes in cells arrested resulting from histone H2B repression was found out to be irreversible. However, a full round of DNA replication did occur after histone repression in arrested cells. Yeast cell division cycle (*cdc*) mutants essentially arrest at a specific point in the cell cycle under nonpermissive conditions due to stage-specific defect. For instance, some *cdc* mutants are defective in DNA replication and therefore arrest at S phase during interphase.

Histone H2B mutations in inner region of the protein have been reported to affect centromere function, silencing and chromosome segregation. Histone protein roles in chromatin dynamics was analyzed by using three temperature-sensitive (*ts*) *Schizosaccharomyces pombe* strains containing amino-acid substitutions in the *htb1* gene. The mutations occurred in highly conserved, non-helical residues in histone H2B which have been implicated in DNA-protein or protein-protein interactions in the nucleosome. (Maruyama et al., 2006) The three mutant *htb1* strains were *htb1-72* (G52D), *htb1-223* (P102L) and *htb1-442* (E34K).

Allele *htb1-72* (G52D) caused disruption of gene silencing in heterochromatin region and chromosome lag in anaphase. The second allele, *htb1-223* (P102L) caused structural aberrations

in the central centromere chromatin and unequal chromosome segregation in anaphase. The third allele, *htb1-442* (E34K) however, exhibited little defect. The study also reported that monoubiquitinated H2B is documented to be greatly unstable in *htb1-223* mutant allele. (Maruyama et al., 2006) Overall, the study concluded histone H2B and uH2B play important roles in centromere-kinetochore functions.

Not much is known about how individual histones contribute mechanistically to mitotic progression and regulation. An important factor for errorless chromosome segregation is that prior to anaphase, poleward pulling force must be generated on the spindles attached between chromosomes and spindle pole bodies. Tensionless crisis has been shown to lead to missegregation and aneuploidy. (Luo et al., 2010)

A study has shown a histone H3 mutation that impairs the ability of yeast cells to activate the spindle assembly checkpoint in tensionless crisis. This study demonstrated that histone H3 is a key molecule in transmitting the tension status to the spindle assembly checkpoint. (Luo et al., 2010)

Two *S.cerevisiae hta1* mutants originally isolated in a screen for defective *SUC2* gene transcription were further revealed to retain increase-in-ploidy and cold sensitivity phenotypes. These *hta1* mutants were *hta1-200* and *hta1-300* responsible for S20F and G30D amino acid residue changes, respectively. Both these mutations occurred in evolutionarily-conserved amino acid residues. The study went on to provide strong evidence that histone H2A is required for proper chromosome segregation by playing a role in centromere function. Some of the evidence presented in support for this conclusion was:

1. The *hta1* mutants are unable to maintain the haploid state, forming diploids soon after germination.
2. The *hta1* mutants have a delayed cell cycle at G_2-M.
3. These mutants have an increased rate of chromosome loss.
4. The *hta1* mutants have an increase-in-ploidy phenotype as a result of unipolar segregation of sister chromatids.
5. The *hta1* mutations show genetic interactions with mutations in genes encoding kinetochore proteins.
6. The *hta1* mutants have an altered chromatin structure over centromeric DNA.

Taken together, all the evidence presented supports the notion that histone H2A plays a critical role in centromere function. Any perturbation in centromeric chromatin structure might result in aforementioned problems. (Pinto and Winston, 2000)

The studies presented here essentially serves as a continuation of this work. This project focuses on the identification and characterization of histone H2B mutants that cause severe defects in ploidy maintenance and mitotic chromosome transmission.

MATERIALS AND METHODS

Saccharomyces cerevisiae and E*scherichia coli* strains:

The *S.cerevisiae* strains used in this study are listed in Table I. All strains are isogenic to FY2, derived from S288C unless otherwise indicated (Winston et al., 1995). Yeast strain construction and other genetic manipulations such as plasmid shuffling and gene disruption were performed using standard techniques (Rose et al., 1990; Guthrie and Fink, 1991). *E.coli* strain DH5 alpha [F ‘80lacZ-M15-(lacZY A-argF) U169 endA1 rec A1 hsdR17 (r_k-m_k+) deoR thi1 supE44 Δgyr A96 relA1] was used for cloning, propagation and isolation of plasmids.

Oligonucleotides primers:

The oligonucleotide primers used in these studies are listed in Table III.

Media for growth and phenotypic analysis:

Standard yeast medium such as the Yeast Extract Peptone Dextrose (YPD) was prepared by adding 2.0% Bacto™ Agar to liquid YPD. (Rose et al., 1990) Other media such as such as synthetic dextrose minimal medium (SD), synthetic complete and dropout medium (SC) and 5-fluoro-orotic acid (5-FOA) drug selection medium were prepared using methods described by Rose et al. (1990). The 5-FOA was purchased from US Biological.

E.coli containing plasmids carrying the β-lactamase gene was propagated in LB (Luria-Bertani) medium with 100µg/ml of ampicillin (Sambrook et al., 1989). Benomyl medium was prepared by adding Benomyl (Sigma) to hot YPD to a final concentration of 15µg/mL. Canavanine plates were prepared by adding 3µg/mL of canavanine sulfate (Sigma) to dropout medium lacking arginine (Rose et al. 1990).

Temperature sensitive yeast mutant analysis was conducted according to Rose et al. (1990) Benomyl sensitivity mutant analysis was carried out following the procedure of Stearns et al 1990.

Ploidy assay:

The copy number of chromosome V was assayed by monitoring the CAN1 gene as described in Schild et al. (1981). Since 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 or among strains with two copies of chromosome V. In this assay, strains were replicated onto SC-Arg plates either with or without canavanine, the cells were mutagenized by UV irradiation (300 ergs/mm^2), and plates were incubated at 30°C for 3 to 5 days. Appearance of papillae indicated haploid cells. Conversely, the absence of growth indicates more than one copy of chromosome V.

DNA Cloning and other DNA manipulation procedures:

All the restriction enzymes used are either from Promega or New England Biolabs. They were used in accordance to the manufacturers' recommended conditions. Digested DNA was separated by gel electrophoresis using 0.8% agarose gels submerged in TRIS-Acetate buffer (Sambrook et al. 1989). The individual bands were excised and purified using the GeneClean II System Kit (Bio101). DNA ligations were performed according to methods outlined in Sambrook et al. (1989). T4 DNA Ligase enzyme was purchased from Promega. Competent DH5 alpha *E.coli* was used for transformation of ligated plasmids as outlined in Sambrook et al. 1989.

Gene disruption in yeast cells:

The strain carrying a deletion of the *hta1-htb1* and *hta2-htb2* loci was constructed by PCR-mediated disruption using either the kanamycin (Kan^r) or nourseothricin (CloNat) resistance genes flanked by sequences homologous to the target genes following published procedures (Wach et al. Yeast. 1994 Dec; 10(13):1793-808 and Goldstein AL, McCusker JH.Yeast. 1999 Oct; 15(14):1541-53). In brief, PCR products containing the Kan^r or CloNat resistance cassettes were obtained using DNA primers with flanking regions of the *HTA1-HTB1* and *HTA2-HTB2* loci. A yeast strain was transformed with the resulting DNA and plated on medium containing kanamycin or CloNat, selecting for cells that had become resistant to the antibiotic as a result of homologous recombination between the PCR product and the targeted chromosomal locus (Guthrie and Fink, 1991).

Confirmation of the deletions was done by PCR using primers that hybridize outside the sequences targeted for recombination. The deletion of both loci that encode histone H2B, *HTA1-HTB1* and *HTA2-HTB2*, was done sequentially in a yeast strain that carried the plasmid pSAB6, which contains the *HTA1-HTB1* genes and the *URA3* gene as a selectable marker. The presence of the plasmid kept the cells alive after the second locus was deleted.

Yeast Transformation:

Yeast cells were transformed with plasmid DNA using either the One-step Yeast Transformation method (Chen et al. 1992) or the high efficiency Gietz Lithium Transformation Procedure (Gietz et al. 1995). The latter was used for gene disruption of the *HTA1-HTB1* and *HTA2-HTB2* loci by homologous recombination and for introduction of the mutagenized H2B library.

PCR Mutagenesis:

PCR Mutagenesis was performed in order to create the *HTB1* mutant library. Mutagenic PCR was performed as previously described (Denise et al. 1992) with slight modifications. The template DNA used was pIP164 (*HTA1-HTB1 LEU2*) diluted to 10 ng/µl. The PCR reaction contained the following components: (1) 10 ng DNA template; (2) 10X Platinum Taq Buffer (Invitrogen); (3) 10mM each dGTP, dCTP and dTTP; (4) 2mM dATP; (5) Platinum Taq Polymerase (Invitrogen); (6) 50 mM $MgCl_2$; (7) 10 mM $MnCl_2$. The reaction was repeated three more times with alternating 2mM dCTP, dGTP and dTTP, with the other three types of dNTPs at 10 mM. The final volume for each reaction was 100 µL. Oligonucleotide primers used were oIP 276 and oIP 350. The reaction was preheated at 94°C for initial denaturation step followed by 30 cycles. The 30 cycles consist of 3 sequential steps which are: (1) 30 seconds at 94°C; (2) 30 seconds at 52°C; (3) 4 minutes at 74°C. The fifth step is 10 minutes at 74°C and after that, 4°C indefinitely. QIAquick PCR Purification Kit was used to purify all four reactions.

Fluorescence microscopy:

Fluorescence microscopy was used to monitor 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 et al. 1996) This repeat was integrated at the TRP1 locus, which is located about 12 Kb to the right of the centromere of chromosome IV. 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 chromosome IV which can be used to analyze the chromosome segregation phenotype.

The cell nuclei of all the strains were also stained with fluorescent dye 4', 6-Diamidino-2-phenylindol-dihydrochloride (DAPI; Boehringer Mannheim GmbH, West Germany) used at 1:30 dilution. Yeast cells were pelleted and fixed with 95% ethanol and resuspended in buffer PBS pH 7.2 before stained with DAPI. Cells were viewed on a Zeiss AXIO Imager.M1 fluorescence microscope. The yeast cell morphology such as the unbudded, single budded or large budded states were noted alongside the strains' nuclear morphology. Quantitation of the DAPI signal was performed on at least 200 cells from each strain.

Gene Sequencing:

The sequencing of the mutated *HTB1* genes was performed by the University of Arkansas DNA Resource Center using the Sanger dideoxy-mediated chain termination method (Sambrook et al. 1989). The primer used was oIP 276. The mutated *HTB1* gene was cloned into *LEU2* plasmid (template type) of about 8 Kb size. The "sequence mix" was made up of 2µl primer, 8µl dH_2O and 3µl DNA of 400ng concentration.

Flow Cytometry:

Cells were processed for flow cytometry as previously described in Gerring et al., 1990 with few modifications. Yeast cells at log phase (OD_{600} = 0.3-0.4) grown in liquid YPD were pelleted and resuspended in 50mM Tris pH7.5 containing 95% ethanol and fixed overnight at room temperature with slow shaking. The cells were then centrifuged and let to air dry. The pellet was washed with 50mM Tris pH7.5. Then, the cells were treated with 1mg/mL RNase and left in 37°C water bath for overnight. Proteinase K was added and incubated at 50°C for one hour. Cells were stained overnight at 4°C in 50mM Tris pH7.5 containing 15µg/mL propidium iodide

(Sigma). Before flow cytometry protocol, the stained cells were sonicated at low setting on Branson 1510 sonicator for 5 seconds.

RESULTS

PCR Mutagenesis is used to create a mutant *HTB1* library.

In order to create the *HTB1* mutant library, we used a PCR method which amplifies the 395bp of the *HTB1* gene template with an error-prone, low-fidelity PCR approach. In the end, a collection of random point mutations within the *HTB1* gene is generated. The gene template was a 2.6 kb long section of DNA containing the *HTA1-HTB1* gene and PCR primers used were oIP 276 and oIP 350. These forward and reverse primers were designed to amplify only the *HTB1* gene, which is 395bp long.

There were four independent mixes or PCR reactions (reaction tubes); each with a different mix of oligonucleotides, which provided a biased mix for each dNTP.

Following the PCR product purification, the mutated (amplified) *htb1* gene product was ligated into the *LEU2* vector plasmid pRS415 to create a mutant *htb1* gene library. (Figure 6) Both vector plasmid and insert *htb1* gene were digested with *SpeI* and *EagI* restriction digestion enzymes after which they were ligated using T4 DNA ligase. These library plasmids were selected, amplified and purified in *E.coli* and the DNA used for yeast transformation.

The parent yeast strain IPY 1009 with both its chromosomal copy of *HTA1-HTB1* and *HTA2-HTB2* genes knocked out and replaced with drug cassettes, was transformed with the mutant *htb1* plasmid library. After the transformation, the *URA3* rescue plasmid was shuffled out from yeast strains by replica-plating transformants on 5-FOA plates. (Figure 7) After this point, the sole source of H2B protein in all the transformants was the mutant *htb1* gene from the library of mutant plasmids.

Preliminary screening of yeast transformants shows impaired growth at various conditions.

Two rounds of yeast transformations yielded about 2700 yeast transformant strains. All 2700 colonies were replicated plated on YPD medium at 30°C (physiological temperature) as control, 37°C (heat sensitivity) and 13°C (cold sensitivity). Additionally, the colonies were also replica-plated on benomyl containing medium (a microtubule desensitizing drug) and canavanine medium. SC-Arg was used as a control for the canavanine testing. Strains with relatively weak growth at these conditions were chosen for further studies.

From the 2700 colonies, we narrowed down to about 126 strains which exhibited weak growth on 13°C, 37°C, benomyl and canavanine media. (Figure 8) Each strain was scored from a 1 to 5 scale; 1-no growth at all, 2- very little to no growth, 3 –slow growth, 4-growth at a little lesser than wild type, and 5- growth same as wild type. Strains scored at 1 through 3 were more desirable as these strains' phenotype traits had been previously correlated with strains having ploidy defects. The selected 126 strains was still a big group of potential candidates for fluorescence microscopy, therefore we narrowed it down to an even smaller group; 22 strains. (Figure 8) These strains were #11, #12, #13, #14, #19, #21, #23, #31, #35, #37, #78, #82, #87, #89, #119, #134, #141, #143, #144, #149, #150 and #154. All these strains showed weak growth (Score 1 or 2) on at least one of the various screens; 13°C, 37°C, benomyl and canavanine growth medium.(Figure 9)

To these 22 candidate strains, we added a group of previously, partially-characterized *htb1* mutant strains; SM1, SM2A, SM3, SM7, SM13A and SM14. (Pinto et al., Unpublished). These six *htb1* mutant strains were obtained originally from an unrelated *htb1* mutant screen concerning transcription defect mutants. The *HTB1* gene of these strains was initially in plasmid

vector pJH 55, a *HIS3* plasmid. Preliminary screening and flow cytometry work had been performed on these strains by a former student in the lab. We isolated the *htb1* gene from these SM mutant strains and ligated them into the *LEU2* plasmid vector. We repeated the preliminary phenotype screening and flow cytometry on these six strains. Additionally, we performed the DAPI staining and DAPI quantitation on them.

GFP tag analysis and DAPI quantitation shows segregation defects.

All 22 strains which were positively identified as strains with the most severe phenotype at the preliminary screening were observed under the microscope. Fluorescence microscope images reveal the green fluorescence protein (GFP) dots, which can be used to monitor the movement of the centromere of chromosome IV. Apart from the positions of GFP dots, this assay also helped us determine the bud morphology.

Beside the analysis of sister chromatids by GFP tagging of chromosome IV, nuclei of the strains including the six SM strains were visualized with 4', 6-Diamidino-2-phenylindol-dihydrochloride (DAPI). Quantitation of the DAPI signal was done on at least 200 cells from each strain. (Figure 10)

Strain #11 displayed cells with large-budded morphology. DAPI quantitation showed significant increase in total count of large-budded cells (82%) compared to wild type (13%). (Figure 10) A large-budded morphology is characteristic of cells arrested or delayed in traversing the G2-M phase of cell cycle. (Pinto and Winston, 2000) The anomalies were mostly large budded cells with the undivided nucleus located at the neck of the bud in the mother cell and the daughter cell not having a nucleus (47% compared to 2% in wild type). (Figure 10)

Strains #150, SM2A and SM13A also seem to share the same type of morphology as strain #11; large-budded cells with single nuclei in one cell and zero nuclei in the daughter cell. It was quantified using DAPI fluorescence that the 3 strains respectively contained 43%, 46% and 45% of large budded cells with single nucleus.

Strains #11, #14, #87 and #143 (Figures 11b, 11c, 11f and 11g) exhibit large-budded cells; one of which has two GFP dots whereas the other daughter cell has no GFP at all. Two

GFP dots represents the result of unipolar segregation of sister chromatids. These findings were corroborated by the flow cytometry results (Figure 12a) while DAPI quantitation shows strain #11 to contain 10% cells with such defect compared to none in wild type (Figure 10). The same culture used for fluorescence microscope viewing was used in the flow cytometry for all 4 strains. All 4 strains were found to have increase-in-ploidy phenotype, most likely as a direct result from unipolar segregation of sister chromatids.

The third most common type of large budded cells observed in these strains show bipolar segregation with both daughter cells having equal nuclear content. However, the large bud morphology is characteristic of cell division (*cdc*) mutants that are blocked in cell division but continue protein synthesis. (Pinto and Winston, 2000) For example, strains #14, #21, #78, #87, #154, SM3, SM7 and SM14 all show three to four times more such defect compared to the wild type strain in DAPI quantitation. (Figure 10)

Another interesting morphology observed primarily in strains #78 and #150 and to a smaller extent in #154 is a developing projection out of the large budded cells. (Figures 11e, 11h and 11i) These projections are similar to the "shmoo" which usually grows on yeast cells in response to mating pheromones. Cells of mating type-a respond to α-factor pheromone by growing projection towards the source of α-factor. Likewise, cells of mating type-α cell respond to a-factor. The irregularity in this case however is present in cells of mating type a. The parent cell is also heterothallic, with the *HO* gene deleted. Therefore, the cells are not capable of mating type switching.

Mutations in histone *HTB1* gene can cause increase-in-ploidy.

Strains #11, #14, #21, #87 and #143 all seem to have some problem with maintaining the normal haploid state. (Figure 12a)

Cells grown in 5ml YPD were chosen at the logarithmic growth phase and processed for flow cytometry. 22 strains were selected for the protocol; #11, #12, #13, #14, #19, #21, #23, #31, #35, #37, #78, #82, #87, #89, #119, #134, #141, #143, #144, #149, #150 and #154. The X-axis represents fluorescence intensity whereas the Y-axis represents number of cells counted. (Figures 12a and 12b)

From these 22 candidates, cells in strains #14, #87 and #143 showed distinct peaks at 2n and 4n number of chromosomes indicating diploidization. (Figure 12a) Cells in strain #11 showed a very slight tendency to diploidize which is evident by a low but still significant peak at 4n. (Figure 12a) This suggests that although not as strong a phenotype as the other 3 candidates (#14, #87 and #143), strain #11 is still significantly more at risk for diploidization compared to a wild type haploid strain.

Strain #21 on the other hand, showed slightly shifted peaks in flow cytometry data. (Figure 12a) These shifted peaks in the X-axis indicate cells are bigger in size. For example, the data shows n peak shifted slightly to the right (2n count of DNA) on the X-axis. As mentioned earlier, cells with chromosome segregation defects (mitotic check point failure) continue protein synthesis processes even after mitosis has stalled and segregation of chromosomes is not occurring. Protein build-up is the reason for the size of cells to be significantly bigger. When flow cytometry was repeated in this strain, a peak of about 300 cells which had a 0 n peak was evident. (Pinto et al., Unpublished) These are essentially cells with aneuploidy problems,

aberrant fragmented cells which could have resulted in chromosome loss through faulty chromosome segregation process.

Fourteen mutation sites in the *HTB1* gene

DNA from all 22 strains was sequenced. The *LEU2* plasmids containing the 395bp of *htb1* gene were extracted from the yeast strains and introduced into *E.coli* for amplification using both chemical and the electroporation methods. The plasmids were then extracted from *E.coli* using QIAprep® Spin Miniprep Kit.

Once we obtained the plasmids containing the *htb1* gene from all 22 strains, we prepared the DNA to be sequenced and had it sent to the University of Arkansas, DNA Resource Center for DNA sequencing. Primer used was oIP 276. Table 4 outlines the DNA sequencing results. Essentially, 8 out 22 of the strains sequenced had one or two mutations in the ORF of the HTB1 gene which had effects on the amino acid sequence of the H2B protein. These 8 strains were #11, #14, #21, #78, #87, #143, #150 and #154.

In combination with the previously partially-characterized six "SM" *htb1* mutants, fourteen individual strains of H2B mutants were identified and characterized (Table 4). Eleven of these fourteen mutations in H2B were in residues that show complete identity with human histone H2B type 1-K. (Figure 13)

POLYVIEW 3D imaging software highlights particular mutated amino acid residues in histone octamer-DNA complex.

POLYVIEW 3D is a web-based tool for macromolecular structure visualization and analysis. In particular, it provides a wide array of options for automated structural and functional analysis of proteins and their complexes. This software is available online for free and is maintained by University of Cincinnati, Cincinnati OH.

The source of nucleosome core particle (NCP) structural data was obtained from the RCSB Protein Data Bank (PDB). The PDB ID for the yeast NCP used for the 3D-imaging work was "1ID3". "Chain color and rendering" and "residue highlighting" options were entered accordingly. Chains A and E are red, representing H3; chains B and F are blue, representing H4; chains C and G are yellow, representing H2A and finally, chains D and H are green, representing H2B (our protein of interest). The DNA is shown as a white double helix around the histone octamer while the mutation residues are shown in magenta.

From the Polyview3D-imaging work (Figure 14), we visualized the location of each mutation within the nucleosome core particle. This analysis focused on the following questions: (1) whether a particular mutant residue physically interacts with other histone protein(s) (2) Whether there is physical protein- DNA interaction involving the mutated residue in H2B (3) Type of secondary protein structure where the mutation is located. These questions helped us infer potential answers as to what importance do these specific mutant residues hold to chromosome segregation process. How does a single mutation in H2B protein manage to destabilize the genome enough to cause chromosome segregation and/or ploidy problems?

All four core histones are essentially made up of a flexible N-terminal region, a histone fold region (consisting of alpha helices and loops) and a C-terminal region. The 3D-Imaging provided us with visual representation of the locations of the various mutant residues generated in this work. (Figure 14) Since the software is based on crystallography data, residues on the flexible tail of N-terminal domain were not resolved to a specific locality. Therefore, residues K16 and K22 are not shown. S42 is on the α1 helix; T55, L65, E74 and K82 are on α2 helix; L83M, N87 and K88 are on loop 2; K89 and I92 are in α3 helix; K111 is on α-C terminal helix and A120 is on C-terminal tail. (Figure 14) The mutated residues were essentially scattered throughout the entire length of the H2B protein except for loop 1 in the histone fold region (Figure 15), where no mutation was identified.

DISCUSSION

In previous studies performed on two mutant alleles of histone H2A; *hta1-200* and *hta1-300,* it was shown that these mutations caused chromosome segregation defects and ploidy instability phenotypes. These *hta1* mutants were found to be unable to maintain their haploid state, forming diploids soon after germination. Besides that, the two mutants were also showed to have an increased rate of chromosome loss. The research conclusively showed that histone H2A is required for normal chromosome segregation by playing a role in centromere function. (Pinto and Winston, 2000)

Histone mutants with chromosome segregation defects are also known to display cold sensitive, heat sensitive, benomyl sensitive and canavanine sensitive phenotypes as did the *hta1-200* and *hta1-300* H2A mutants. The objective of this work was to analyze the contribution of histone H2B to chromosome segregation. To this end, a library of random mutations introduced in the *HTB1* gene by PCR was generated and used for in vivo screening of the same phenotypes used to characterize the H2A mutants. After the preliminary screening of about 2700 transformants, 22 strains with the most clear phenotypes for the four sensitivities aforementioned were chosen for further studies, with the goal of identifying amino acid residues associated with chromosome segregation defects in histone H2B.

The 22 strains were then subjected to fluorescence microscopy. Essentially, the movement of the GFP dot represents the movement of the centromere of chromosome IV which can be used to analyze the chromosome segregation phenotype under the microscope. There is one GFP fluorescence signal (green dot) in each dividing wild type (haploid) yeast cell during chromosome segregation. Strains which have defects in chromosome segregation will show more

than one GFP fluorescence signal (undivided chromosomes) in each cell. These cells may be diploid or polyploids.

Aside from the GFP-tag analysis of sister chromatids, the DNA of the cells was also stained with the fluorescent dye DAPI, which allows the microscopic visualization of the cell nucleus. (Figure 16) The yeast cell morphology such as the unbudded, single budded or large budded states were noted alongside the strains' nuclear morphology. All 22 strains were subjected to flow cytometry, which provides the information of DNA content. Finally, plasmids containing the possible mutant *HTB1* gene were isolated and their DNA sequenced. All 22 strains were sequenced to find out whether there is a mutation in the *HTB1* open reading frame. When there was a nucleotide change observed, it was then determined whether the DNA mutation is responsible for any amino acid residue change.

From these 22 strains, eight strains had mutations in the *HTB1* gene which were responsible for mutated H2B histone protein. These eight strains were strains #11, #14, #21, #78, #87, #143, #150 and #154. To this pool of eight novel *htb1* mutants, six previously, partially-characterized *htb1* mutant strains (SM1, SM2A, SM3, SM7, SM13A and SM14) were added to further their characterization. (Pinto et al., unpublished) Preliminary screening on these six strains had shown some correlation with chromosome segregation defect phenotype. DAPI staining was performed on these six additional mutants as well as the eight strains from the *htb1* mutant screen. At this point, we had a total of 14 novel *htb1* mutant strains.

Of these 14 strains identified and correlated with causing chromosome segregation phenotypes, 11 mutations involve amino acid residues that are identical to their counterpart in human histone H2B. These 11 amino residues are K16, K22, S42, T55, E74, L83, N87, K88, I92,

K111 and A120. The other 3 amino acid residues; L65, K82 and K89 belong to the same group (with positively charged amino acid side chains) as their human counterpart. (Figure 13)

Characterization studies of novel histone H2B mutants

All fourteen novel mutations encoded amino acid changes that were scattered throughout the entire length of the H2B protein, encompassing the N-terminal tail region, histone fold region and the C-terminal region of the protein. Within the histone fold region, only loop 1 domain did not have any mutations. (Figure 15) Overall, there were 17 unique nucleotide mutations with 14 transition events and 3 transversion events.

Ten out of the fourteen amino acid residues that displayed chromosome segregation defects were found to be in the histone fold domain, either on the surface or buried deeply within the histone octamer. The other four amino acid residues were located in the N-terminal tail and C-terminal tail with two mutations per domain. From the Polyview 3D images (Figure 14), the mutated amino acid residues in the histone fold region that appear on the surface of histone H2B include S42, T55, K88 and K89 whereas L65, E74, K82, L83, N87 and I92 are all buried deep within the histone core octamer. Generally, mutations buried deep within the histone core may locally affect the stability of histone-histone interaction, thus contributing to an overall change in nucleosome stability. (White et al., 2001) Moreover, there are plenty of interactions between side chains of amino acid residues that could be perturbed even with only a single amino acid mutation. The amplified global effect of change in a single nucleosome can be manifested through an overall change in chromatin stability as H2B is a global protein. Destabilization is especially feasible when mutated amino acid residues are involved in providing specific folding to the overall protein structure as protein folding is essential to its functional properties.

Additionally, these histone H2B mutants could potentially change pockets of interaction (microdomains) where enzymatic interactions take place. One such instance is the K16 residue, which is a known site for acetylation, a modification that can be reversed by the Hda1 histone deacetylase complex. Disruption at this site, for example, causes problems with the transcriptional activation of a number of essential genes. (Parra et al., 2006)

Unlike mutations buried within the histone octamer, surface mutations such as the K89E will most likely disrupt the DNA-histone interaction. The negatively charged glutamic acid residue for example, will repel the phosphate groups which make up the DNA sugar-phosphate backbone. Steric hindrance resulting from this repulsion may possibly alter the route of the DNA molecule over the histone octamer.

The plasmid isolated from strain #11 was found out to have a mutation in the coding region of *HTB1* gene at position 223 from the ATG start codon where a guanine was changed to an adenine, a transition. This nucleotide change resulted in amino acid residue change; E74K which resides in the α2 helix in the histone fold region. Glutamic acid was changed to a lysine residue. Both amino acids belong to the polar group but what is interesting is the fact that the change is from a negatively charged glutamic acid to the positively charged lysine. A change in charge especially from a positive to negative charge, or vice versa could potentially disrupt the nucleosome microenvironment at the particular location of the amino acid which was mutated. The secondary structure of the mutant H2B protein could have been functionally altered as a result of the mutation since the side chain interactions between the E74 residue and other proximal amino acid will get disrupted. Based on the Polyview 3D image (Figure 14), it seems that there could also be a histone-histone interaction between H2A and H2B histone fold regions at this particular location. The preliminary screening of strain #11 revealed that it exhibited weak

growth in 13°C, 37°C (heat sensitive), benomyl and canavanine sensitivities. Flow cytometry data suggests the strain is of haploid DNA content with a large proportion of cells delayed at S phase and the G2/M phase of the cycle (Figure 12a). The fluorescence microscopy images of GFP-marked centromeres clearly indicate chromosome segregation defects. (Figure 11b) In addition, DAPI quantitation data shows 82% of cells in strain #11 are large budded (compared to only 13% in wild type strain) with 47% of cells containing one daughter cell with one nuclei and the other daughter cell without any nuclei. (Figure 10) Together, these data suggest that the E74K mutation affects the integrity of the genome by causing chromosome segregation defects that lead to cell cycle delays (activation of SAC) and aneuploidy.

Plasmid isolated from strain #14 was identified with a mutation in the position 263 from the start codon within the ORF of the *HTB1* gene. The nucleotide change is from an adenine to cytosine, a transversion event. This mutation resulted in the change of amino acid residue N87T; asparagine residue to threonine residue; both of which happened to belong to the polar neutral (uncharged) group. Position 87 in the polypeptide is at loop 2. Plasmid isolated from strain SM1 also had a mutation at the same nucleotide position; 263 from an adenine to a thymine, also a transversion event. This time, the DNA mutation translates to a change of amino acid asparagine to isoleucine (N87I). Asparagine is polar neutral whereas isoleucine is a nonpolar (hydrophobic) residue in the aliphatic group. Polyview 3D image (Figure 14) shows that the 87th asparagine is in proximity to the to DNA, which may suggest an altered protein – DNA interaction. Strain #14 showed benomyl sensitivity whereas strain SM1 showed decreased growth at 13°C, 37°C, benomyl and canavanine sensitivity. Flow cytometry confirmed the diploid nuclei DNA content of strain #14 (Figure 12a) which was corroborated by the fluorescence microscope images. (Figure 11c) DAPI quantitation shows total of 69% of cells having large-budded morphology.

34% of total cells seem to exhibit unipolar segregation with only one daughter cell having nucleus, whereas the other does not have any. (Figure 10)

Plasmid isolated from yeast strain #21 was identified with two mutations in the open reading frame of *HTB1* gene. One mutation is at location 197; thymine to cytosine change (transition) which is responsible for L65S amino acid residue change. The other mutation is at position 278 which is also a thymine to cytosine change giving rise to I92T amino acid residue change. Preliminary screening of yeast strain #21 showed cells displaying weak growth at 13°C, 37°C and benomyl sensitivity. Flow cytometry data suggests haploid DNA content, ruling out a ploidy defect. (Figure 12a) Fluorescence microscopy data on this strain was interesting as it was the only strain which showed diffused GFP proteins within the nucleus of yeast cells. (Figure 11d) This could possibly be an outcome of a rearrangement of the *lac* operators used to detect the GFP signal in this particular strain; and not necessarily a result of the histone H2B mutations. DAPI quantitation shows total of 65% of cells in strain #21 having large-budded morphology. (Figure 10)

L65S represents a change from leucine to serine which is a substitution of a nonpolar residue with a polar residue. Serine's side chain has a hydroxyl group whereas leucine does not. This means that the new amino acid serine which occupies the microenvironment of L65 could make electrostatic interactions with nearby amino acid side groups of the H2B polypeptide. Quaternary structure between histone H2A and the mutant H2B could have also been changed since the position of L65 seems to be adjacent to H2A histone in the Polyview 3D image. (Figure 14)

I92T; the second mutation in strain #21 shows substitution of a nonpolar residue with a polar residue at the alpha helix 3 region within the histone fold. The amino acid mutation which substitutes polar residues for nonpolar residues and vice versa could potentially change the dynamics of DNA wrapping since the side groups of these amino acid residues are involved in numerous interaction be it with other histones or with the DNA.

Some of the interactions between histone and DNA include (1) hydrogen bonds between phosphates of DNA backbone and main-chain amide nitrogen atoms of amino acid near last turn of α1 and α2 helices; (2) electrostatic interaction between negatively charged individual phosphate groups and collective positive charge of helix dipole generated by α1 and α2 helices of all four core histones; (3) salt bridges and hydrogen bonds between side chains of basic amino acid and/or hydroxyl side chain containing- amino acids and phosphate oxygen on DNA and finally; (4) non-specific minor groove insertions of H3 and H2B N-terminal tails into two minor grooves each on the DNA molecule.(Luger et al., 1997)

Plasmid isolated from yeast strain #78 shows there is one mutation in the open reading frame of *HTB1* gene. The mutation is at location 68 from the ATG start codon which is an adenine to guanine change (transition) subsequently responsible for K22R amino acid residue change. Both lysine and arginine residues are positively charged amino acid residues. However, the interest for this particular mutant comes from the fact that K22 is situated in the N-terminal tail which is prone to post-translational modifications. We also know that the lysine residues often times undergo acetylation, methylation and/or ubiquitination. Therefore, this particular residue is definitely a potential substrate for post-translational modification such as acetylation and ubiquitination which can only occur to lysine residues.

Post-translational modifications are known to be able to alter accessibility of effector proteins to DNA, which in turn can regulate downstream gene regulation processes. The phenotypes from preliminary screening associated with strain #78 include weak growth at 13°C, 37°C, benomyl and canavanine sensitivities. Flow cytometry data suggest no ploidy defect as it shows haploid DNA content. (Figure 12a) Nevertheless, there is a clear indication of chromosome segregation problems judging from the fluorescence microscopy images as separation of cells were not matched by replication and equal division of the centromere DNA. (Figure 11e) DAPI quantitation shows total of 61% of cells in strain #78 having large-budded morphology. (Figure 10)

Plasmid isolated from yeast strain #87 revealed a mutation at position 335 which is a change of adenine to guanine nitrogenous base (transition). This in turn causes H2B protein expression to be altered at Lysine 111. The change of amino acid at position 111 is from a lysine residue to an arginine residue. Lysine 111 is located at the alpha helix domain in the C-terminal tail. It is possible that this lysine is a modifiable residue involved in post-translational modification events such as acetylation. The preliminary phenotype testing on strain #87 revealed it to have weak growth on benomyl plates and sensitive to canavanine testing (higher tendency to diploidize). This finding was backed by the flow cytometry data which also shows the nuclei DNA content of the strain to exhibit polyploidy. (Figure 12a) Fluorescence microscope findings also reveal segregation defects with multiple yeast cells showing the centromere (GFP signal) not being duplicated and segregated into dividing yeast cells. (Figure 11f) DAPI quantitation shows a total of 57% of cells in strain #87 having large-budded morphology compared to only 13% in wild type cells. (Figure 10)

Another strain found to be carrying a mutation at position 111 is strain SM13A where the Lysine 111 is changed to a glutamic acid residue. The nitrogenous base change responsible for the change in SM13A is an adenine to guanine transition at position 334. However, it is to be noted that strain SM13A carries two mutations; one being adenine to guanine change at nucleotide position 334 and the other, adenine to guanine change at nucleotide position 49, the latter nucleotide change being responsible for K16E.

As far as the amino acid change in strain SM13A is concerned (K111E), a lysine to glutamic acid residue change signifies a charge alteration from positive to negatively charged amino acid. As previously explained, this change in charge (positive to negative, and vice versa) could potentially play a big role in modification of the lysine 111 microenvironment via modified amino acid side group interaction(s).

The second mutation in strain SM13A; the change of adenine at position 49 in the open reading frame of *HTB1* gene to guanine is responsible for a lysine to glutamic acid residue change at position 16 (K16E) in the N-terminal domain of H2B protein. Lysine 16 is a known modifiable residue, where hypoacetylation occurs. The post-translational modification of lysine 16 has been shown to have a role in transcription regulation of genes involved in metabolism and normal cellular function. K16 in H2B protein is in fact hypoacetylated by the Hda1 histone deacetylase in subtelomeric heterochromatin regions. (Parra et al., 2006) Glutamic acid, a negatively charged amino acid residue substituted at Lysine 16 will impede acetylation and deacetylation processes of this critical residue and may ultimately cause problems in the genome-wide transcription regulation. Previous research has further indicated that multiple acetylated lysine residues in histone H2B including the K16 are required for transcriptional activation of genes involved in NAD biosynthesis and vitamin metabolism. (Parra et al., 2006)

Phenotypes testing on strain SM13A showed weak growth at 13°C, 37°C and canavanine sensitivity. These weak growth phenotypes at 13°C and 37°C could possibly be explained by the reduced transcriptional activity of the various genes involved in metabolism and normal cell function which is directly linked to modification of Lysine 16. DAPI quantitation shows total of 72% of strain SM13A cells having large-budded morphology, which is more than 5 times the amount of cells with the same morphology in the wild type cells. (Figure 10)

Plasmid isolated from yeast strain #143 was identified with a mutation at nucleotide 250 from the ATG start codon, where a thymine has been mutated to an adenine within the ORF of the *HTB1* gene. This transversion type mutation gave rise to L83M where a leucine amino acid residue was changed to a methionine residue at position 83. Leucine 83 is situated at loop 2 region between alpha helix 2 and alpha helix 3 in the H2B protein histone fold region. The majority of histone-histone interactions occur between histone folds. The Polyview 3D software image (Figure 14) indicates L83 to be a proximate residue to the H2A histone. This proximity could mean that there are protein-protein interaction occurring at this particular position between H2A and H2B which may be destabilized with methionine substitution into this position. Histone wrapping could be affected which may lead to unstable chromatin, globally which leads to chromosome segregation defects. It is to be noted that methionine is a rather uncommon amino acid in all four core histones. Preliminary screenings on strain #143 indicate weak growth on benomyl and canavanine media. Further, flow cytometry data confirms a ploidy defect with 2n and 4n peaks. (Figure 12a) Moreover, fluorescence microscope images of strain #143 show clear segregation defects with replicated chromosome material not divided equally between dividing cells. (Figure 11g)

DNA sequencing also revealed that the plasmid isolated from yeast strain #150 contained two mutations; adenine at position 166 change to guanine and adenine at position 248 change to cytosine within the open reading frame of the *HTB1* gene. Adenine to guanine change is a transition whereas the adenine to cytosine is a transversion event. Both these DNA mutations are responsible for H2B protein amino acid sequence change at positions T55A and K82T respectively. Also, both T55 and K82 residues are located in the alpha helix 2 region within the histone fold. Threonine to adenine change at position 55 represents a shift from polar to nonpolar (hydrophobic) side group. This could potentially destabilize the protein folding properties of histone H2B as interaction between adjacent side chain(s) is altered. Preliminary screening of strain #150 revealed weak growth at 13°C, 37°C and canavanine sensitivity. Flow cytometry however verified the nuclei DNA content to be haploid. (Figure 12a) DAPI quantitation shows a total of 74% of strain #150 cells having large-budded morphology with 43% of cells having a single nucleus in one of the daughter cell, but not the other. (Figure 10) Additionally, fluorescence microscope images reveals chromosome segregation defects in strain #150. (Figure 11h)

Other than strain #150, plasmid isolated from strain SM3 is also found to have a mutation that changed lysine K82 to a leucine (K82L). The DNA mutation occurred at position 247 with an adenine to guanine shift, a transition. K82T mutation (Strain #150) is essentially a change from a polar positive residue to a polar neutral residue with an increased mass of about 27 Da whereas K82L represents a polar to a nonpolar (hydrophobic) residue. Again, K82 is located at the alpha helix 2 domain of the protein. Polyview 3D images (Figure 14) reveal K82 to be in the histone fold region with high proximity to the H2A protein. This may indicate K82 to be a significant residue with regards to histone - histone interaction. The phenotypes of SM3 include

weak growth at 13°C and canavanine sensitivity. DAPI quantitation shows total of 64% of cells of strain SM3 having large-budded morphology. (Figure 10)

Plasmid isolated from strain #154 shows DNA mutation at position 127 from the ATG start codon where a thymine is changed to a cytosine, a transition event. This DNA mutation translates to S42P in terms of protein expression. The S42 amino acid residue is found in the middle of the alpha helix 1 domain. The consequences of substituting a serine on the middle of an alpha helix with a proline will mean that the alpha helix structure will be completely disrupted. Amino acid replacements with proline can function as disruptors of secondary structures such as alpha helices. In effect, the S42P mutation may have caused the histone fold region of H2B to wrap in a functionally different way to form an lettered histone octamer in nucleosomes. This in turn could cause chromatin instability which may lead to chromatin segregation defects. Preliminary screening of strain #154 indicates the strain to have weak growth at 13°C, 37°C and canavanine sensitivity. Flow cytometry data suggests absence of ploidy defect, as the DNA content is haploid. (Figure 12a) Nevertheless, fluorescence microscope images do indicate segregation defects. (Figure 11i) DAPI quantitation shows total of 60% of strain #154 cells having large-budded morphology. (Figure 10)

Plasmid isolated from strain SM2A reveals a mutation at position 268 on the ORF of the *HTB1* gene. This mutation is adenine to guanine transition which is responsible for K89E amino acid mutation. This is a lysine to glutamic acid residue change which means the side group has changed from a positive polar residue to a negative polar residue. K89 is situated at alpha helix 3 region of H2B protein. Polyview 3D image (Figure 14) reveals K89 to be adjacent to the DNA which wraps around the histone octamer. Since alpha helix 3 region is part of the histone fold, there is also a chance of histone-histone interaction between this section of H2B and H2A.

Glutamic acid residue, which is a negatively charged residue, could undo various interactions such as DNA interactions with the original lysine, a positive residue. The negatively charged phosphate group of the DNA backbone, when placed in close proximity to a glutamic acid residue could repel each other, and lead way to destabilizing the nucleosomes. This repulsion, repeated in a global scale could possibly destabilize the chromatin. Chromatin destabilization is known to have effects on chromosome segregation processes. As an example, the attachment of sister chromatids to spindle fiber during chromosome segregation could be affected negatively when the chromatin is destabilized. The screening of phenotypes of strain SM2A indicates the strain to have weak growth at 13°C and canavanine sensitivity. DAPI quantitation shows total of 76% of strain SM2A cells having large-budded morphology of which 46% of large-budded cells have single nucleus in one cell and no nucleus in the daughter cell. (Figure 10)

Plasmid isolated from strain SM7 is confirmed to carry a mutation at position 266 in the ORF of the *HTB1* gene. The mutation at this position changed an adenine to a guanine, which in turn changed the gene expression at amino acid residue K88. The lysine at this position was mutated to an arginine residue (K88R). K88 is the last residue on loop 2 region right before the alpha helix 3 secondary structure which starts off with K89. When mutated, also reveals chromosome segregation defects. K88 residue, just like the K89 residue, seems to be adjacent to the DNA. The chromosome segregation defects exhibited by K88R may be explained by the possibility that K88 is a post-translationally modifiable residue. There is also the possibility of steric effects associated with the volume occupied by all the atoms of each residue. The functional group of lysine is a single amino group that occupies a smaller space compared to the functional group of arginine, the guanido group, which takes up a bigger space in terms of spatial volume. Therefore, when atoms are brought closer together in space, the overlapping electron

clouds could cause repulsion between molecules, which can lead way to an altered conformation of the H2B protein. Changes in protein conformation can definitely give rise to changes in the functional properties of the protein. Preliminary screening of strain SM7 indicates the strain to have weak growth at 13°C. DAPI quantitation shows total of 76% of cells in strain SM7 having large-budded morphology. (Figure 10)

The final plasmid isolated, which is from strain SM14 had a mutation on nucleotide position 361, changing a guanine to an adenine (transition). The functional change from this DNA mutation is A120T. Alanine 120 is located at the C –terminal tail region of the H2B protein. This mutation represents a change from a nonpolar hydrophobic residue to a neutral polar residue, which is threonine. Change in polarity may result in a secondary structure change as amino acid side group interaction at this A120 residue accommodates a polar residue at a position previously occupied by a hydrophobic residue. Phenotypic analysis of strain SM14 indicates the strain to have weak growth at 13°C and canavanine sensitivity. DAPI quantitation shows total of 76% of cells in strain SM14 having large-budded morphology. (Figure 10)

Overall, this study provides a strong indication that histone H2B is required for normal chromosome segregation by playing an essential role in centromere function. This conclusion is based on several observations. Firstly, at least three *htb1* mutants are unable to maintain a normal haploid state, becoming diploids or polyploids after replacing their wild type H2B with the mutant protein. Secondly, nuclear staining (DAPI) reveal some *htb1* mutants having a large proportion of cells with a large-bud and undivided nucleus, characteristic of cells blocked at G_2-M (Figure 16), and missegregation of chromosomes (Figures 11a to 11i). Thirdly, most *htb1* mutants have slow growth and sensitivity to microtubule depolymerizing drugs, suggesting a defect in cell cycle and chromosome segregation.

In conclusion, we have provided strong molecular data that supports the view that histone H2B is essential to proper chromosome segregation and ploidy stability. The mutations identified from these studies may result in defective microtubule attachment or play a role in the disruption in the tension-sensing pathway involved in the chromosome segregation process. Such defects in these processes have been known to lead to increase-in-ploidy and chromosome segregation defects.

Further genetic and biochemical studies will be required to clarify the exact functions of histone H2B in chromosome segregation. For instance, one type of genetic study that could be performed on these *htb1* mutants is a double mutation study with centromere-binding proteins. The current evidence suggests that a complex array of interactions occur between histones and non-histone proteins such as the centromere-binding proteins. These interactions seem necessary for the formation of a functional centromere-kinetochore complex. (Yen et al., 1991; Pinto and Winston, 2000)

Other potential studies include: (1) Mapping of chromatin structure to see if centromere chromatin is altered in the *htb1* mutants; (2) immunoblotting studies using antibodies that recognize specific histone posttranslational modifications to see if there are specific defects on the mutant positions; (3) biophysical studies on individual *htb1* mutants within the nucleosome context to find out if there are specific defects in DNA-histone interactions or histone-histone interactions. Ultimately, the understanding of the role of evolutionarily conserved histone H2B in chromosome segregation in yeast will do much to contribute to understanding of the same process in higher eukaryotes.

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Table I

Saccharomyces cerevisiae Strains

IPY 698	*MATa his3-11,15 trp1-1 leu2-3,112 ura3-1 can1-100*
IPY 811	pIP123/pAFs 135#1 GFP-CEN from IPY 698
IPY 983	*MATa hta2-htb2*Δ *:: CloNAT* in IPY 811 (from CloNAT plate)
IPY 984	*MATa hta2-htb2*Δ *:: CloNAT* in IPY 811 with pSAB6 (from *SC-Ura*)
IPY 1001	Derivative of IPY 984; *(hta1-htb1)* Δ*:: kanMx (hta2-htb2)*Δ *:: CloNAT* pSAB6 GFP-CEN colony 1
IPY 1002	Derivative of IPY 984; *(hta1-htb1)* Δ*:: kanMx (hta2-htb2)*Δ *:: CloNAT* pSAB6 GFP-CEN colony 2
WX46-3A	*Matα his3-11,15 :: lacI-GFP-HIS3 CAN*$^{+}$ *trp1-1 :: lacOTRP1 leu2 ura3 dam1-1*^{ts}
IPY 1009	*Mata (hta1-htb1)* Δ*:: kanMx (hta2-htb2)*Δ*:: CloNAT pSAB6 – URA3 CAN*$^{+}$ *DAM1*$^{+}$ *leu2 ura3* [cross between WX46-3A and IPY 1002]

Table II

Escherichia coli Plasmids

pJH 55	A *HIS3* plasmid containing the wild type copy-I histone H2A and H2B *(HTA1-HTB1)*
pIP164	*LEU2* plasmid containing the wild type copy-I histone H2A and H2B *(HTA1-HTB1)*

Table III

Oligonucleotide primers

Name	Sequence	Purpose
oIP276	(5' – 3') CCT TGC CGC ATA AGC GCA TT	5' confirmation of *(hta1-htb1)* Δ; 5' PCR Mutagenesis; 5' DNA Sequencing.
oIP287	(5' – 3') GCA ACA GTG CCC AAT GAA CC	3' confirmation of *(hta1-htb1)* Δ; 3' confirmation of *(hta1-htb1)* Δ *(hta2-htb2)* Δ
oIP280	(5' – 3') AAC GTC AAA GCT GTA GTA CG	5' confirmation of (hta2-htb2) Δ
oIP281	(5' – 3') GAA TGC TCG TGT AGT GAA CC	3' confirmation of (hta2-htb2) Δ
oIP343	(5' – 3') AAC CAA AGG AAG TGA TTT CAT TAT GCT TGA GTA GAG GAA G-CGG ATC CCC GGG TTA ATT AA	5' for deletion of *HTA1-HTB1*
oIP344	(5' – 3') GTT TAG TTC CTT CCG CCT TCT TTA AAA TAC CAG AAC CGA T-GAA TTC GAG CTC GTT TAA AC	3' for deletion of *HTA1-HTB1*
oIP345	(5' - 3') GAC ATA CCA GTC TTC CTC ATA TGA CCT ACT TTA AAA CCC C- TCA GGG GCA TGA TGT GAC T	5' for deletion of *HTA2-HTB2*
oIP346	(5' – 3') CCT TCC AAT CAT ATC TGG ACA AGT AAC AGA ACC CTA ATG TTA - AGC TCG TTT TCG ACA CTG GAT	3' for deletion of *HTA2-HTB2*
oIP349	(5' – 3') TGA TTT TGA TGA CGA GCG TAA T	5' confirmation of *(hta1-htb1)* Δ *(hta2-htb2)* Δ
oIP350	(5' – 3') TGA TCA TCT CAG ATG GTC AG	3' PCR Mutagenesis
oIP382	(5' – 3') CTG GTC TTG TTG AAC CAT CA	5' Re-integration confirmation
oIP383	(5' – 3') CTC GTT TCT GAT AAA CCA GG	3' Re-integration confirmation

Table IV

***htb1* mutation summary**

Colony no.	Preliminary screening	Flow Cytometry	Nucleotide change (Mutation)	H2B Amino acid residue change
11	Weak at 13C, 37C, Benomyl, Canavanine	Haploid	G223A	E74K
14	Weak at Benomyl	Diploid	A263C	N87T
21	Weak at 13C, 37C, Canavanine	Haploid	T197C & T278C	L65S & I92T
78	Weak at 13C, 37C, Benomyl, Canavanine	Haploid	A68G	K22R
87	Weak at Benomyl, Canavanine	Diploid & Beyond	A335G	K111R
143	Weak at Benomyl, Canavanine	Diploid	T250A	L83M
150	Weak at 13C, 37C, Canavanine	Haploid	A166G & A248C	T55A & K82T
154	Weak at 13C, 37C, Canavanine	Haploid	T127C	S42P
SM1	Weak at 13C, 37C, Benomyl, Canavanine	Haploid	A263T	N87I
SM2A	Weak at 13C, Canavanine	Haploid	A268G	K89E
SM3	Weak at 13C, Canavanine	Haploid	A247G	K82L
SM7	Weak at 13C	Haploid	A266G	K88R
SM13A	Weak at 13C, 37C, Canavanine	Haploid	A334G & A49G	K111E & K16E
SM14	Weak at 13C, Canavanine	Haploid	G361A	A120T
12	Weak at 13C, 37C, Benomyl	Haploid	WT	WT
13	Weak at 13C, 37C, Benomyl	Haploid	WT	WT
23	Weak at 13C, 37C, Benomyl	Haploid	WT	WT
19	Weak at 37C, Benomyl, Canavanine	Diploid & Beyond	WT	WT
31	Weak at 37C, Benomyl, Canavanine	Diploid & Beyond	WT	WT
35	Weak at 37C, Benomyl, Canavanine	Diploid & Beyond	WT	WT
37	Weak at 37C, Benomyl, Canavanine	Diploid & Beyond	WT	WT
82	Weak at 13C, 37C, Benomyl, Canavanine	Haploid	WT	WT
89	Weak at 13C, 37C, Benomyl, Canavanine	Haploid	WT	WT
119	Weak at Benomyl, Canavanine	Diploid	WT	WT
134	Weak at 37C, Benomyl, Canavanine	Diploid & Beyond	WT	WT
141	Weak at 37C, Benomyl,	Diploid	WT	WT

	Canavanine			
144	Weak at 13C, 37C, Benomyl, Canavanine	Diploid	WT	WT
149	Weak at 13C, 37C, Benomyl, Canavanine	Diploid	WT	WT

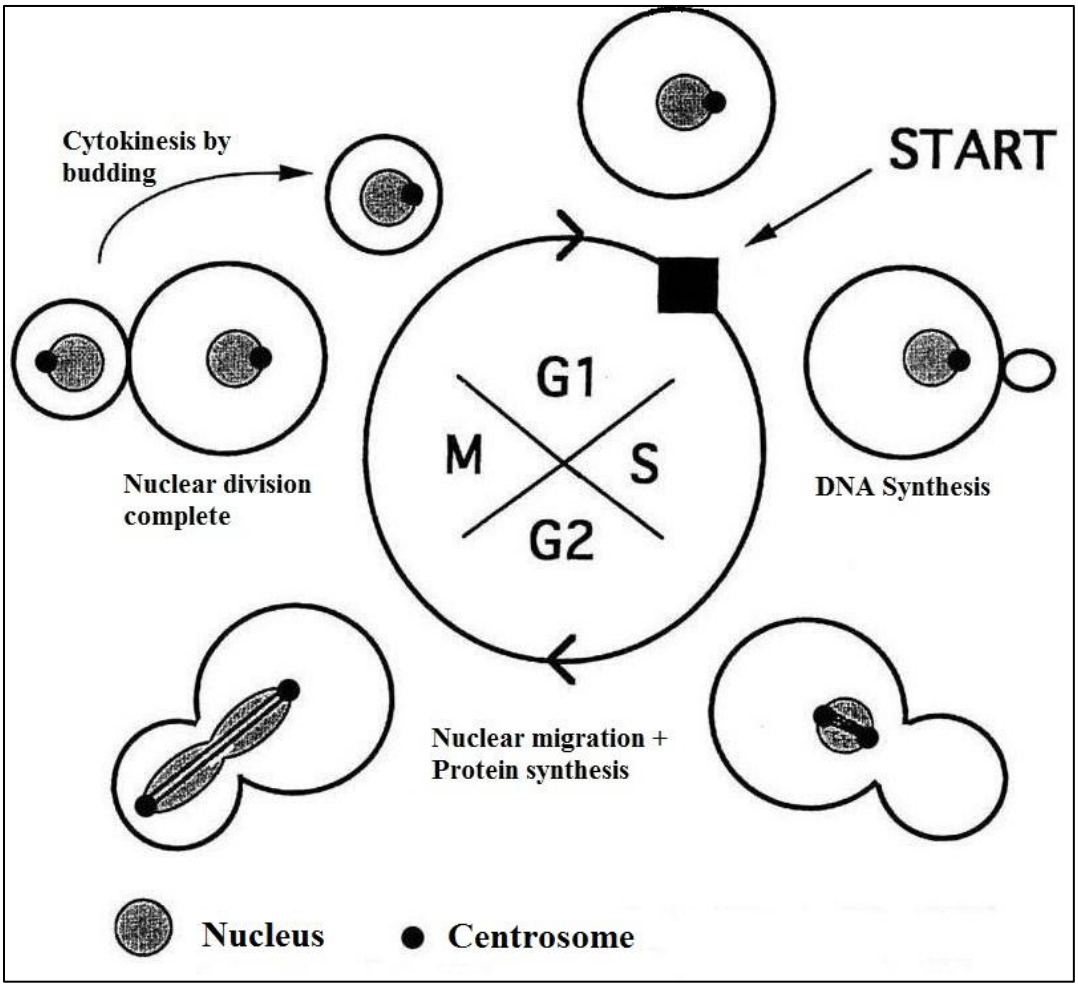


FIGURE 1. *Saccharomyces cerevisiae* haploid cell cycle. The DNA is replicated by the end of S-phase. All required protein is synthesized by the end of G2 phase. Complete nuclear and cell division occcurs at the end of mitosis. Daughter cell pinches off at cytokinesis before reaching the same size of the parent cell.

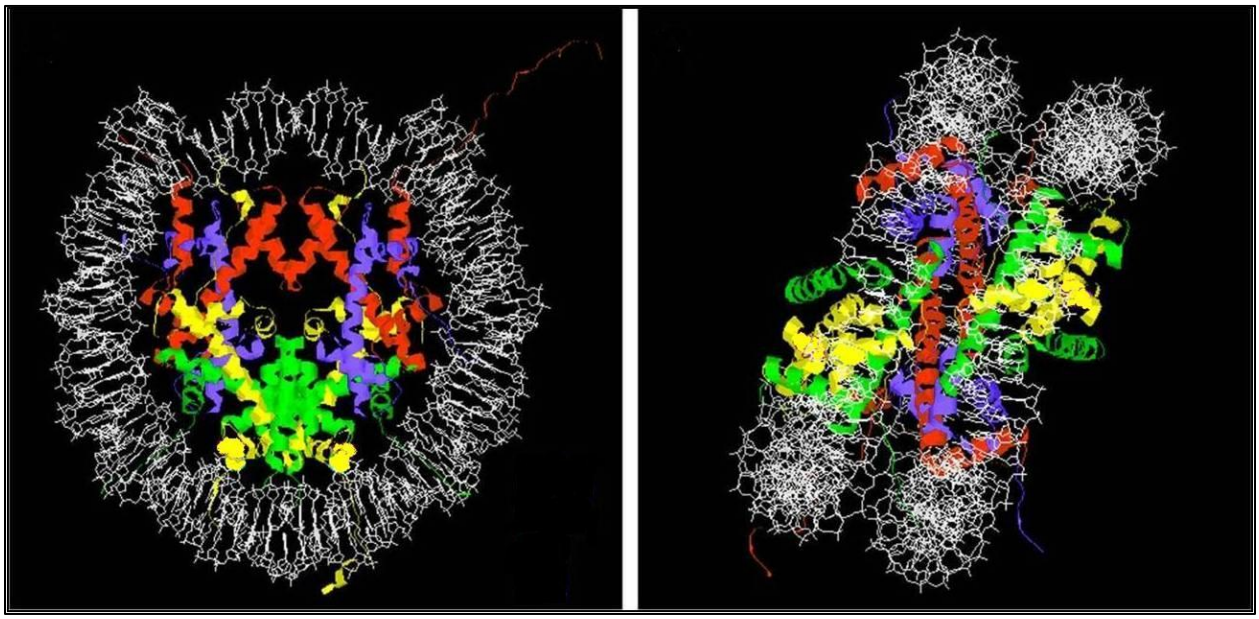


FIGURE 2. Model of a single unit of the nucleosome based on X-ray Crystallography data. Left is the front top-down view and right image is the side view. Images were acquired using Polyview3D software. H2A shown in yellow, H2B in green, H3 in red and H4 in purple/blue. The DNA is shown in white.

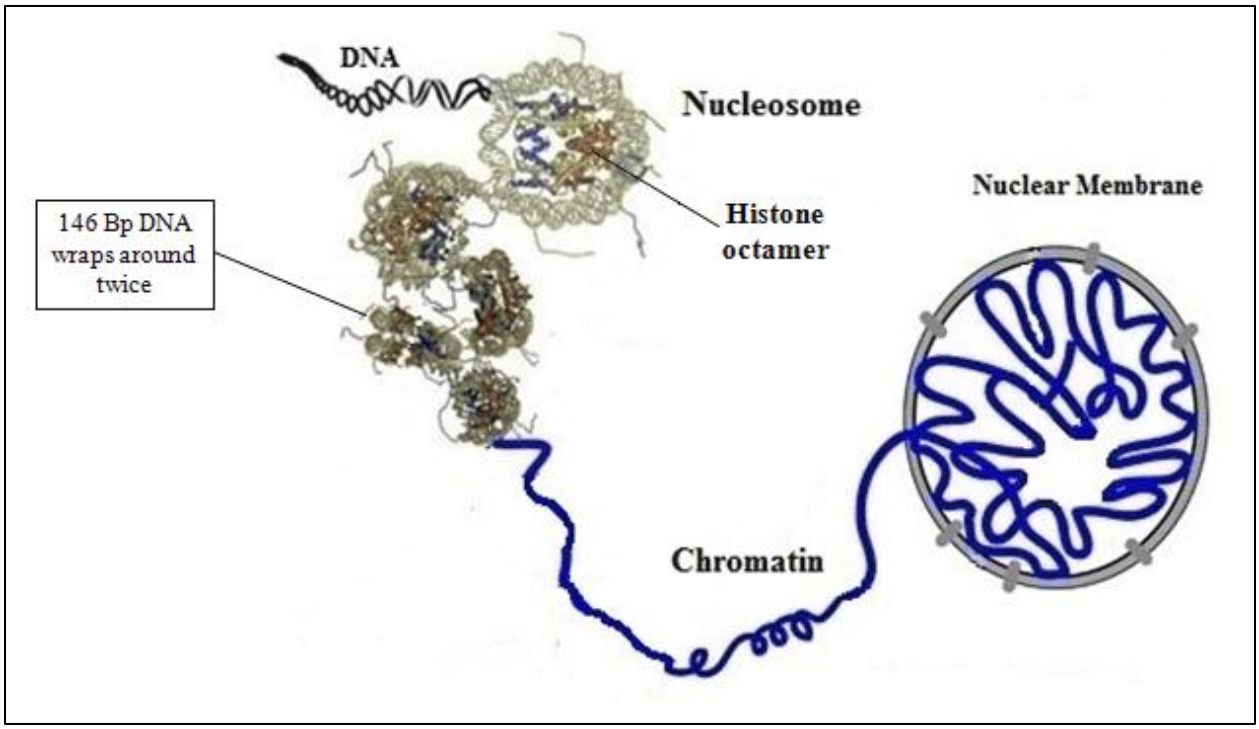


FIGURE 3. Hierarchy of chromatin assembly. Minimum unit of chromatin is the nucleosome. 146 bp of DNA is wrapped around twice around histone octamer to make up a mononucleosome.

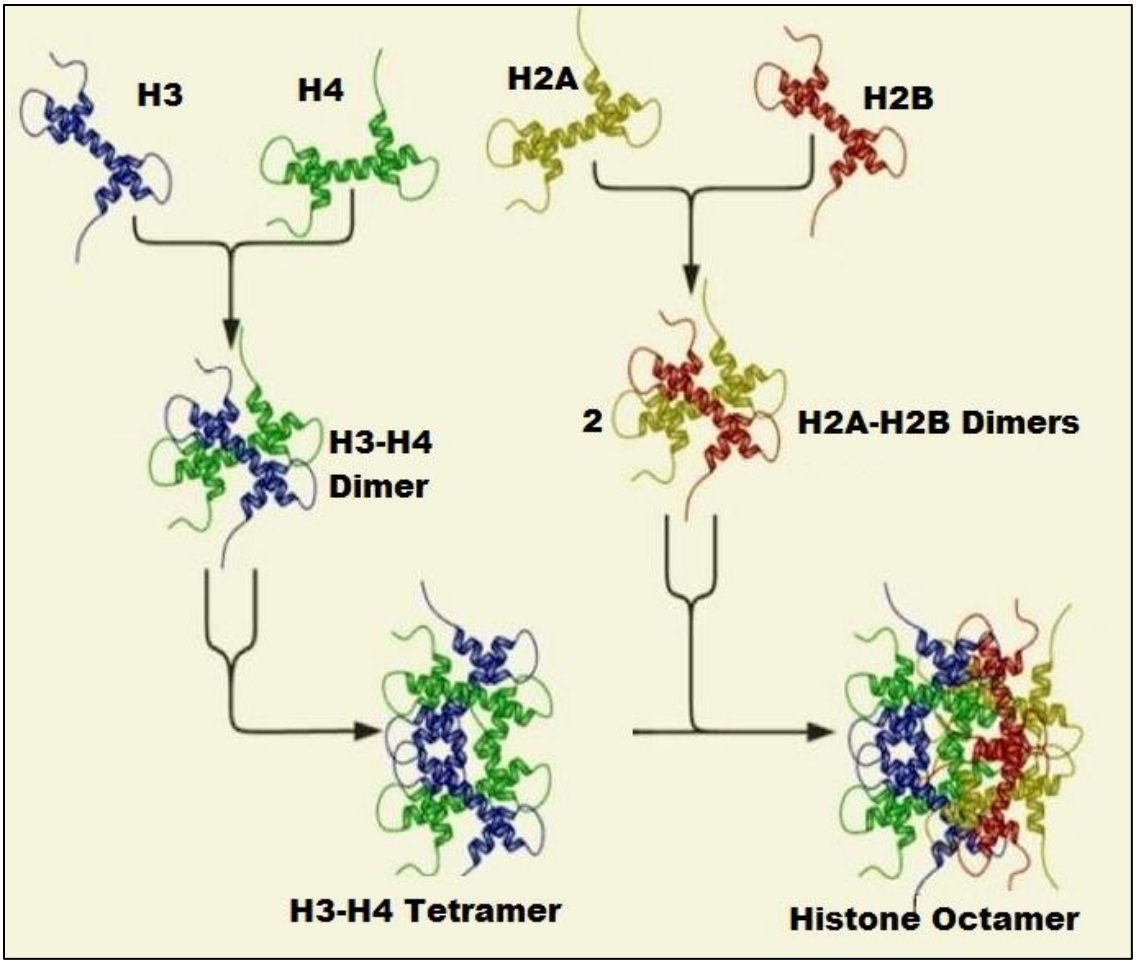


FIGURE 4. Histone octamer core assembly. One copy of H3 and H4 form a H3-H4 dimer. Two H3-H4 dimers associates and forms a H3-H4 tetramer. Next, two copies of H2A-H2B dimers interact with the surface of the H3-H4 tetramer and form the histone octamer. 146 bp of DNA wraps around twice on the histone octamer, constituting a nucleosome.

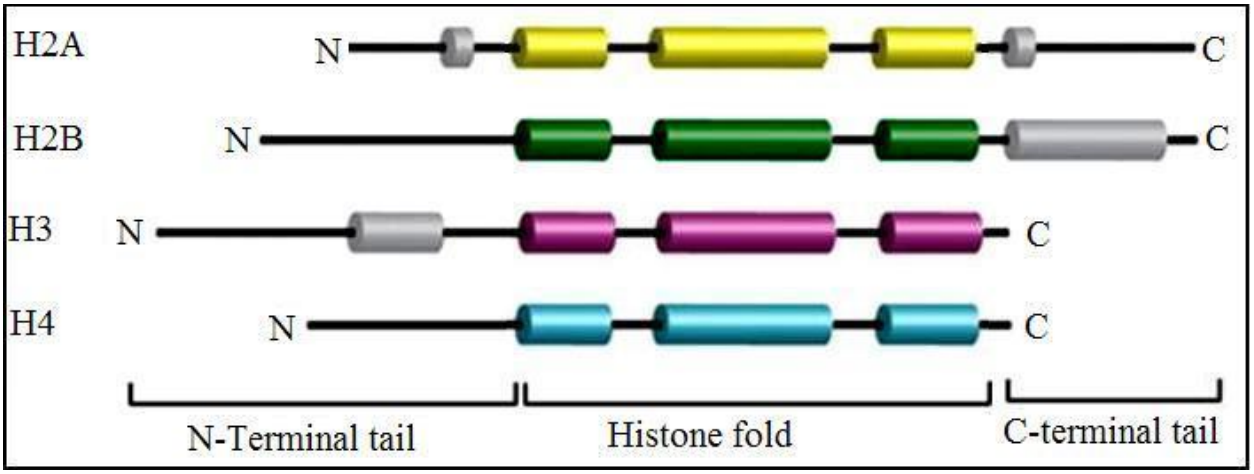


FIGURE 5. Protein motif of four core histones; H2A, H2B, H3 and H4. All four histone motifs are similar; made up of an N-terminal tail, histone fold region which mediates histone-histone interaction and a C-terminal tail. In the histone fold region, the secondary structures are the same; alpha helix 1-loop 1- alpha helix 2- loop 2- alpha helix 3. Histones H2A and H2B have alpha helix secondary structures in the C-terminal tail. Histones H2A and H3 have alpha helix in their N-terminal tail.

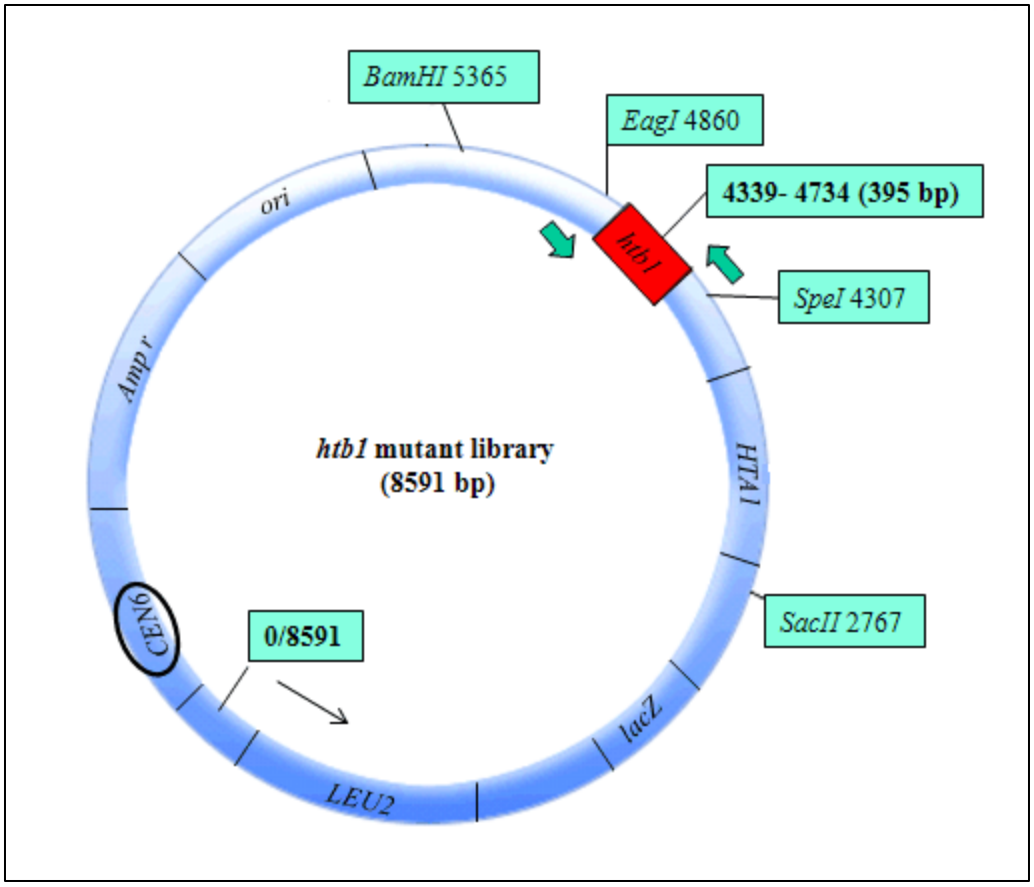


FIGURE 6. *htb1* mutant library generated using PCR mutagenesis. The goal was to generate a single mutation in the 395 bp of the *HTB1* gene, using an error-prone, low fidelity PCR approach. The amplified *htb1* gene was ligated into a *LEU2-CEN* plasmid at the *SpeI* and *EagI* restriction sites. Ligated plasmids were propagated in *E.coli*.

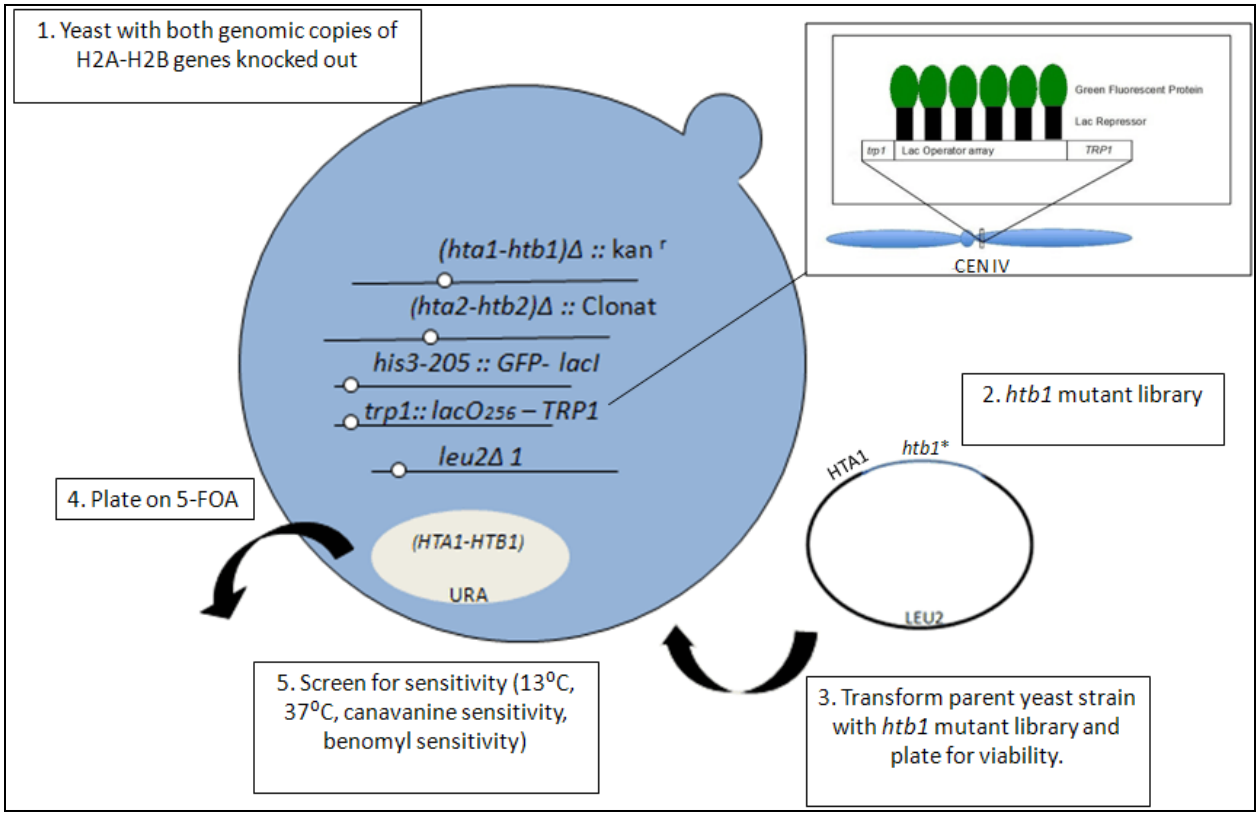


FIGURE 7. Model for the plasmid shuffle used for screening the *htb1* library. PCR mediated disruption was used to delete both genomic copies of genes *HTA1-HTB1* and *HTA2-HTB2*. Using DNA primer flanking regions of *HTA1-HTB1* and *HTA2-HTB2*, we amplified by PCR the genes that provide resistance to Clonat and Kanamycin, transformed the original yeast strain and selected media containing antibiotic. The parent strain was kept alive by a *URA3* rescue plasmid which carries the *HTA1-HTB1* gene. *htb1* mutant library plasmids (*LEU* plasmid) were then introduced into the parent strain and the rescue plasmid was shuffled out by plating transformants-strains on 5-FOA. The surviving strains were screened for various phenotypes.

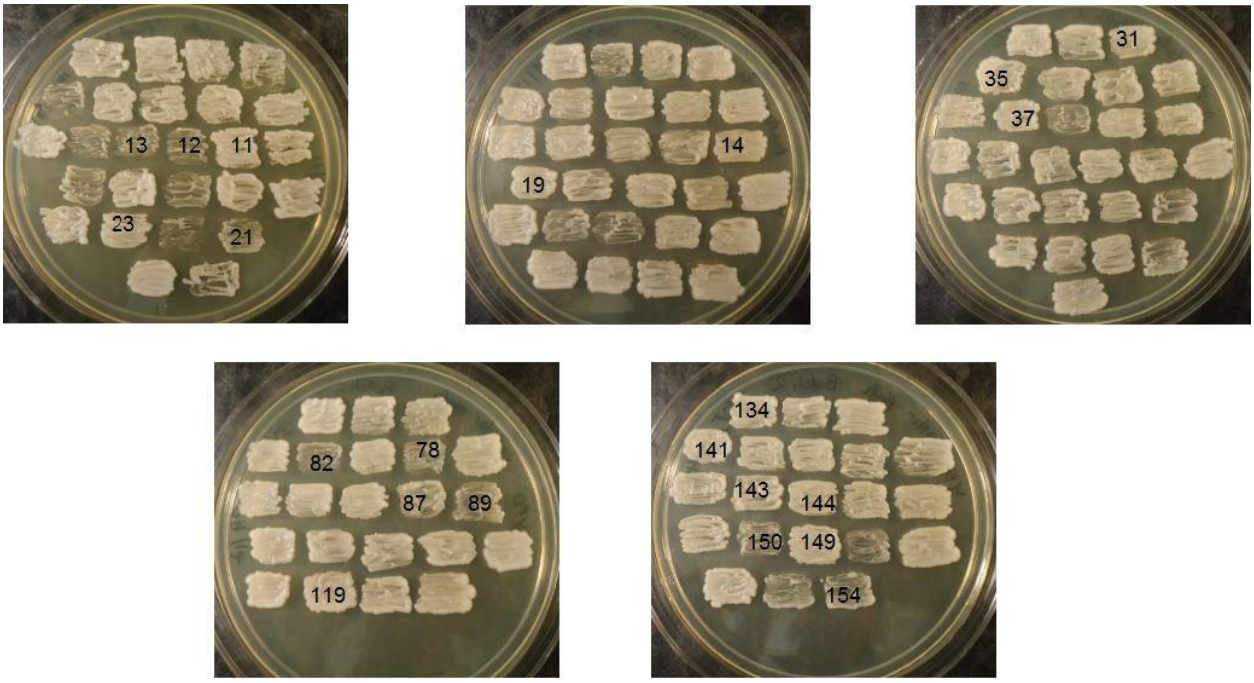


FIGURE 8. 126 strains which showed weak growth at13°C, 37°C, benomyl and canavanine media streaked as patches on YPD. Plates were incubated for 2 days at 30°C. 22 strains with most clear phenotypes were chosen for further study is shown with specific numbers. SM strains are not shown.

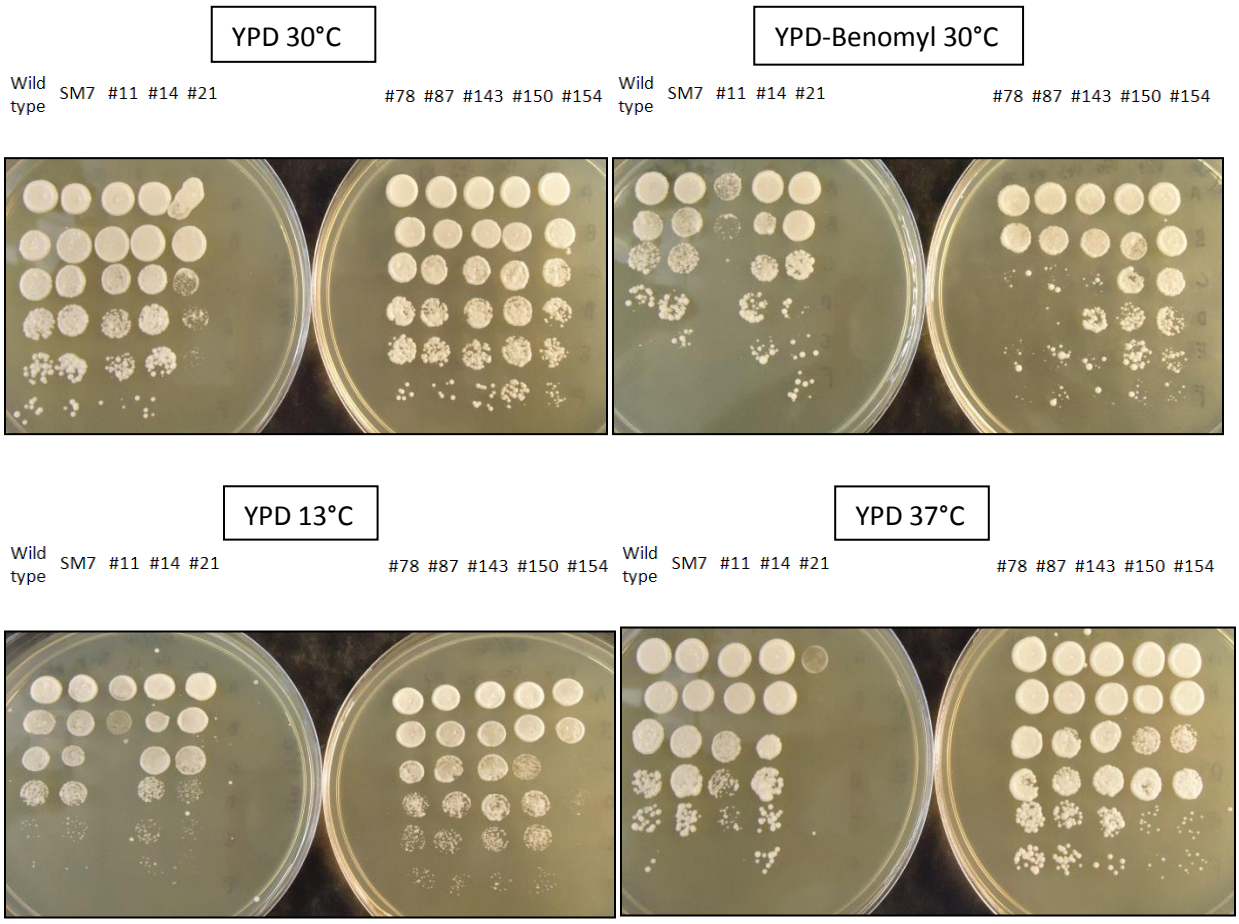


FIGURE 9. Mutant strains SM7, #11, #14, #21, #78, #87, #143, #150 and #154 at various conditions. Serial dilutions (6.0×10^7 to 1.4×10^8 cells/ml) were spotted onto YPD or benomyl plates and incubated at 30°C (2 days), 37°C (2 days), 13°C (4 days) and benomyl 30°C (3 days). Each dilution spotting has a decrease in number of cells by a factor of 10.

	UB		SB	LB				DB
Strain								
WT	22	0	65	2	1	10	0	0
#11	7	2	8	47	3	22	10	1
#14	25	0	6	34	3	30	2	0
#21	25	1	8	29	1	34	1	1
#78	29	0	10	23	2	36	0	0
#87	33	0	9	20	3	33	1	1
#143	50	0	7	18	0	22	1	2
#150	15	0	9	43	2	29	1	1
#154	24	3	12	21	1	38	0	1
SM1	26	0	10	33	1	29	1	0
SM2A	15	1	6	46	1	27	2	2
SM3	19	1	14	31	0	34	0	1
SM7	19	0	5	35	1	40	0	0
SM13A	14	0	14	45	0	27	0	0
SM14	17	0	7	36	0	40	0	0

FIGURE 10. *htb1* mutants arrest at G_2-M. The nuclear and bud morphology were quantified using DAPI staining (in %). UB, unbudded; SB, small budded; LB, large budded; DB, double budded. Number of cells counted is between 201 and 215 for each strain.

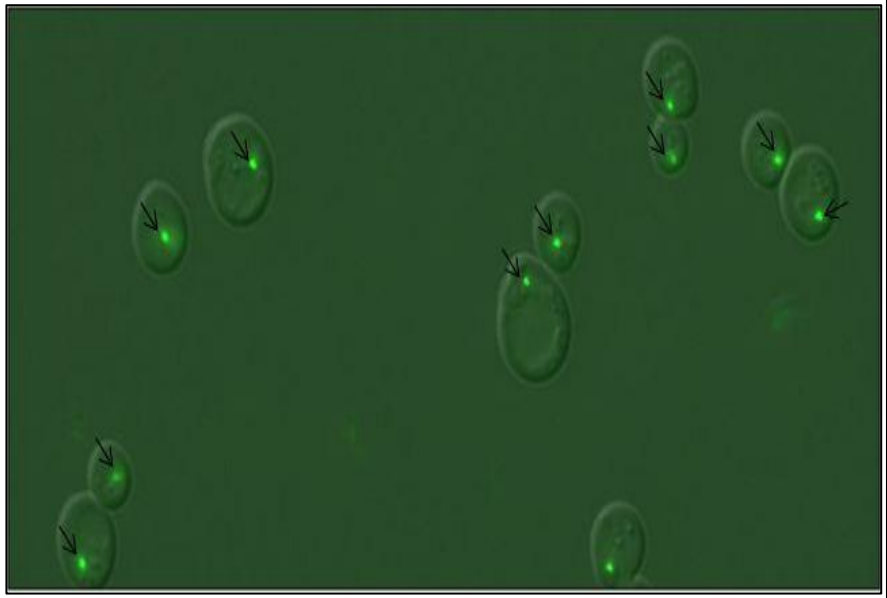


FIGURE 11a. Wild type haploid showing bipolar segregation with each dividing cell containing one copy of chromosome (GFP green dot). Undivided cells contain single copy of chromosome. Arrows indicate green dots.

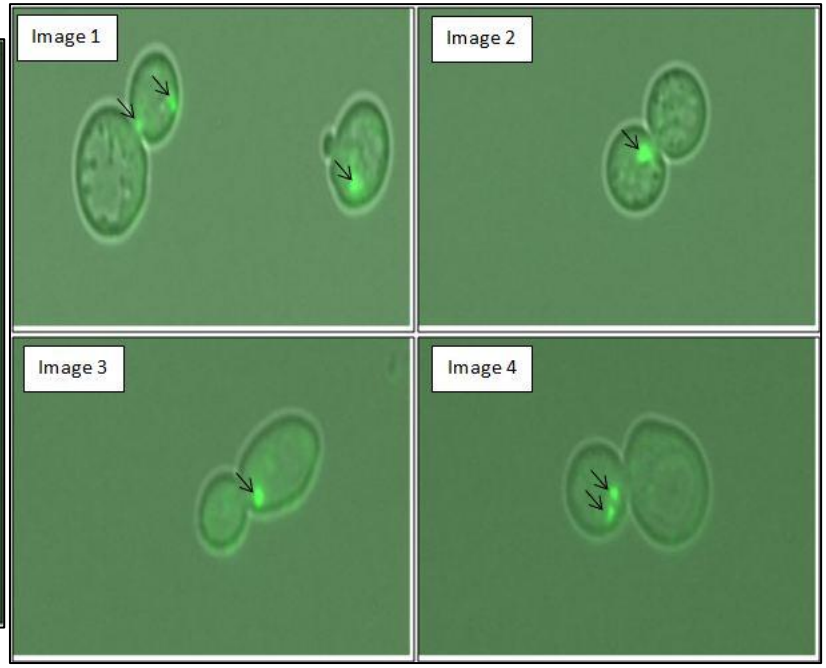


FIGURE 11b. Strain #11. Images 1 & 4 show bipolar segregation with both duplicated chromosome going into a single cell. Images 2 & 3 show mitotic arrest with yeast cells having dumbbell shape.

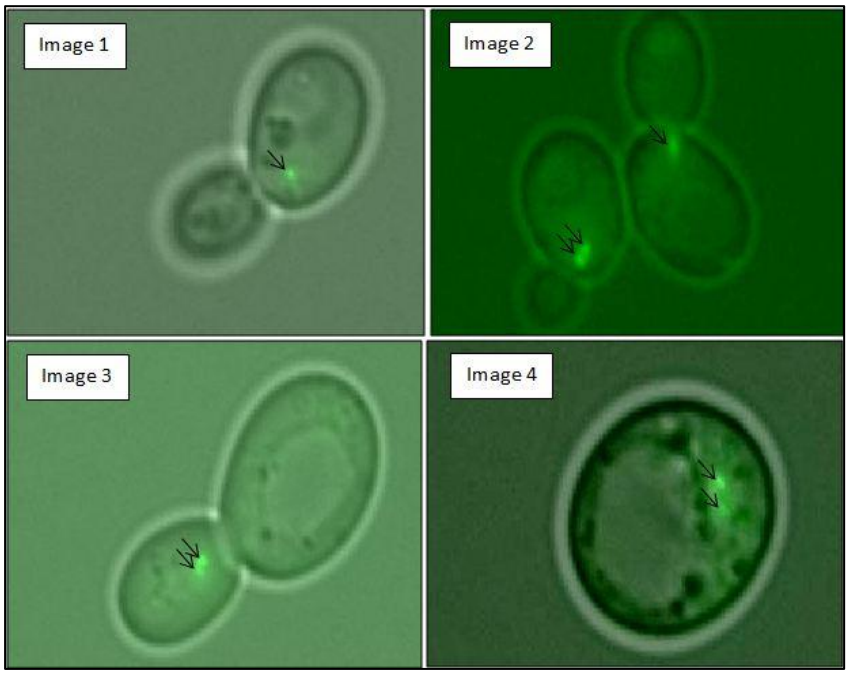


FIGURE 11c. Strain #14. Yeast cells in images 1 & image 2 (top right) show mitotic arrest with yeast cells. Cells in images 2 (bottom left), 3 and 4 show two copies of chromosomes in a single cell.

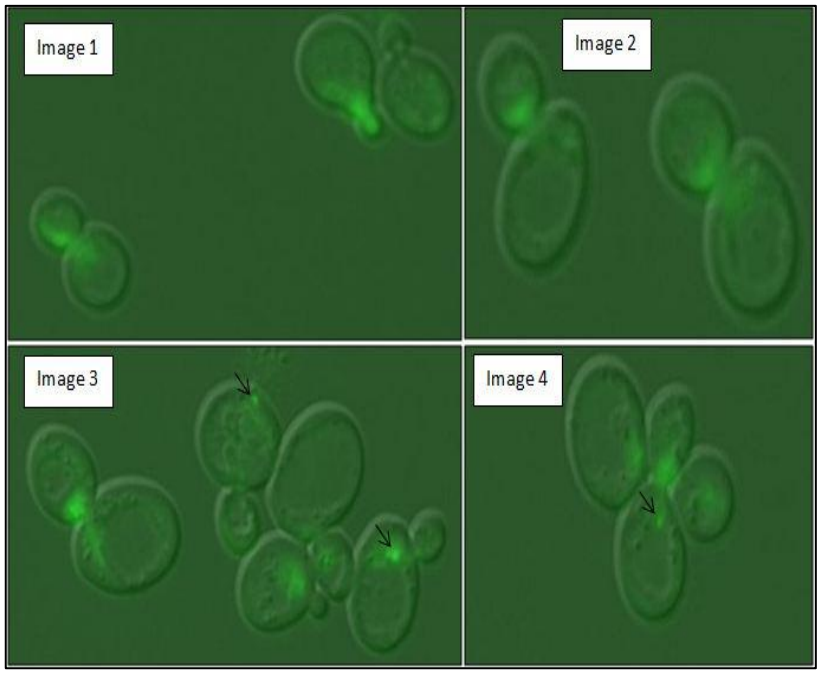


FIGURE 11d. Strain #21. The strain seems to have diffused lac operator arrays to which the GFP binds. Nevertheless, there are still a few GFP dots observable in images 3 & 4.

FIGURE 11e. Strain #78. Yeast cells in all 3 images show mitotic arrest. They also seem to exhibit a strange bulging.

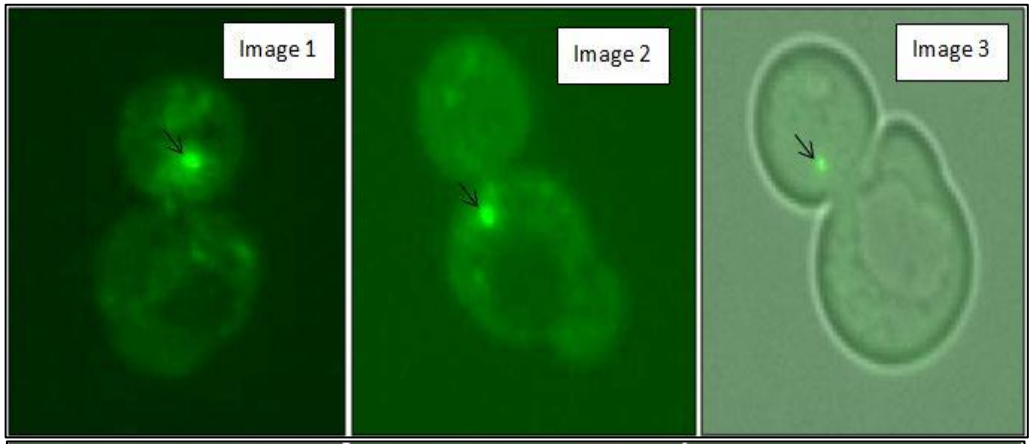
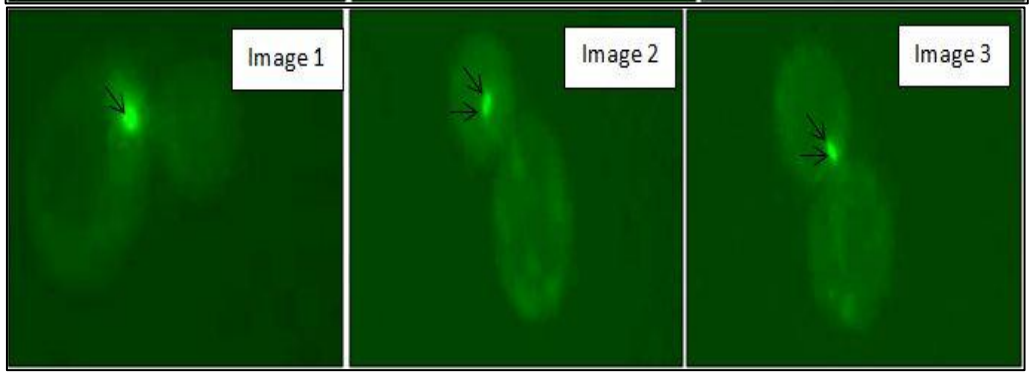


FIGURE 11f. Strain #87. The yeast cell in image 1 show mitotic arrest. Cells in images 2 & 3 show unipolar segregation with one dividing daughter cell having both the duplicated copies of chromosomes.



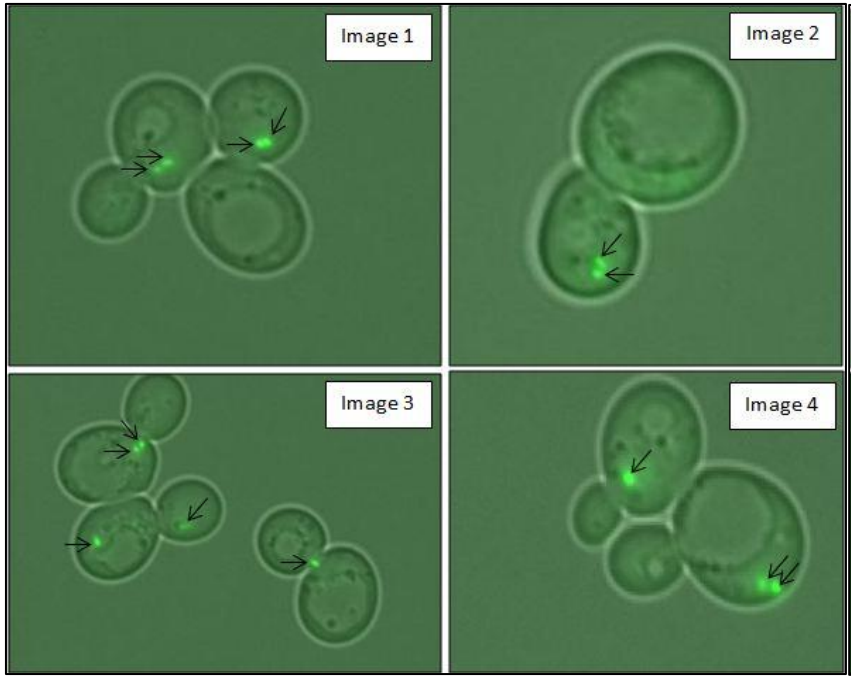


FIGURE 11g. Strain #143. Yeast cells in all images 1, 2, 3 (top left) & 4 (bottom right) show unipolar segregation. Yeast cells in images 3 (bottom right) & 4 (top center) show mitotic arrest.

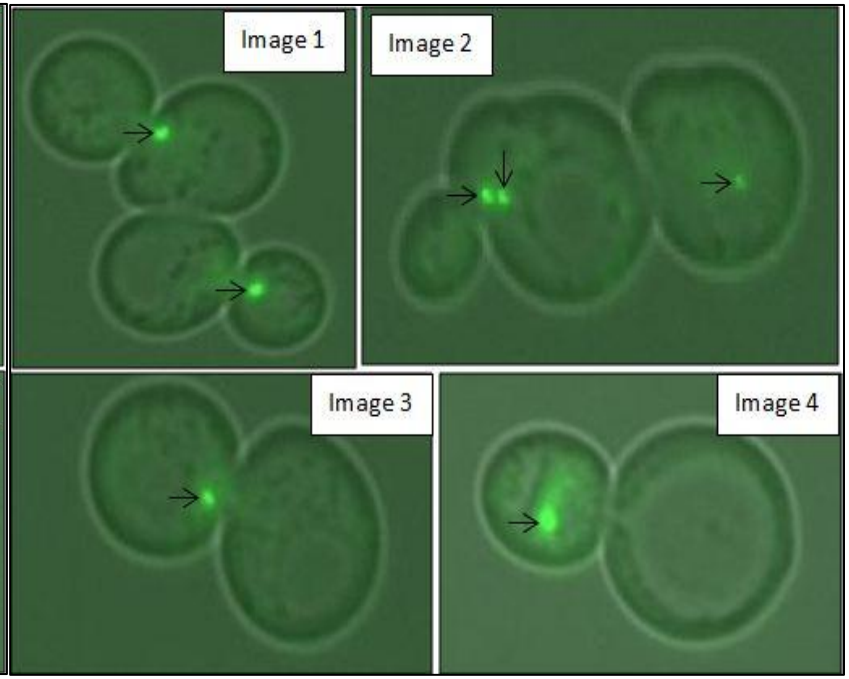


FIGURE 11h. Strain #150. Yeast cells in all images 1, 3 & 4 all show mitotic arrest. The yeast cell in image 2 (left) is showing unipolar segregation.

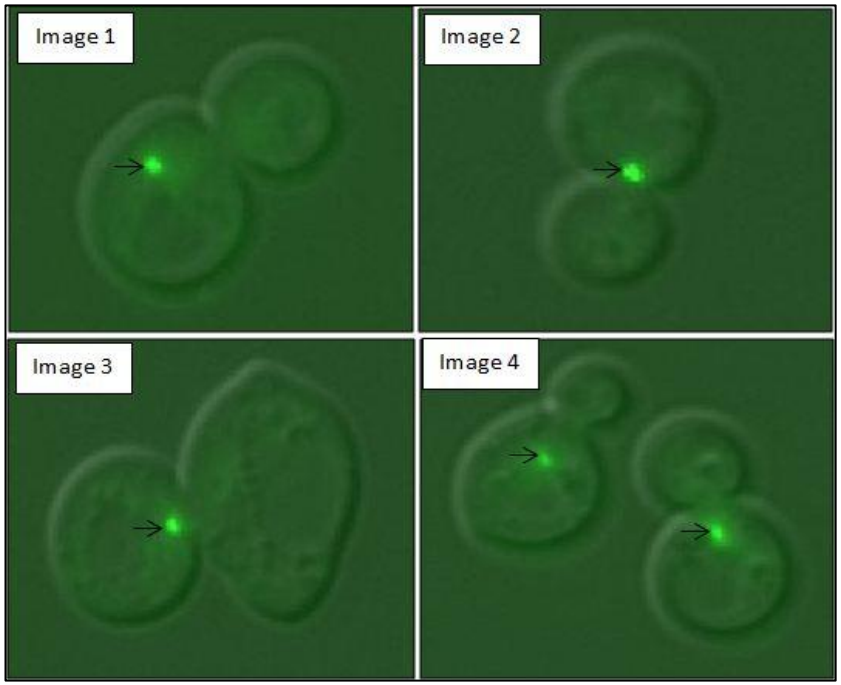


FIGURE 11i. Strain #154. Yeast cells in all 4 images show mitotic arrest.

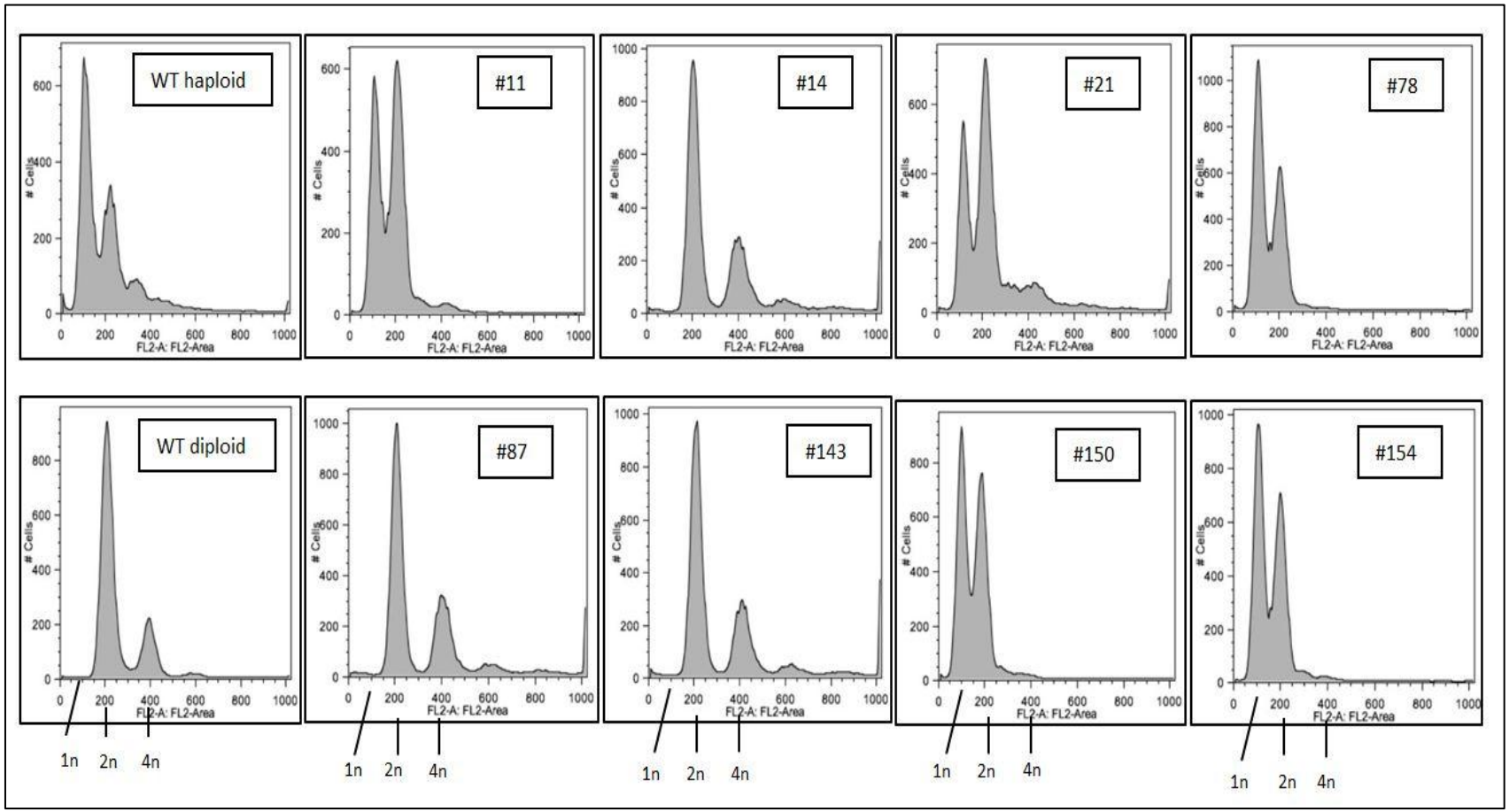


FIGURE 12a. *htb1* mutant strains #14, #87 and #143 show clear increase-in-ploidy phenotype. Strain #11 shows a very slight increase at 4n peak. Strain #21 has a shifted n peak slightly to the right. All the other strains (#78, #150 and #154) show haploid DNA content.

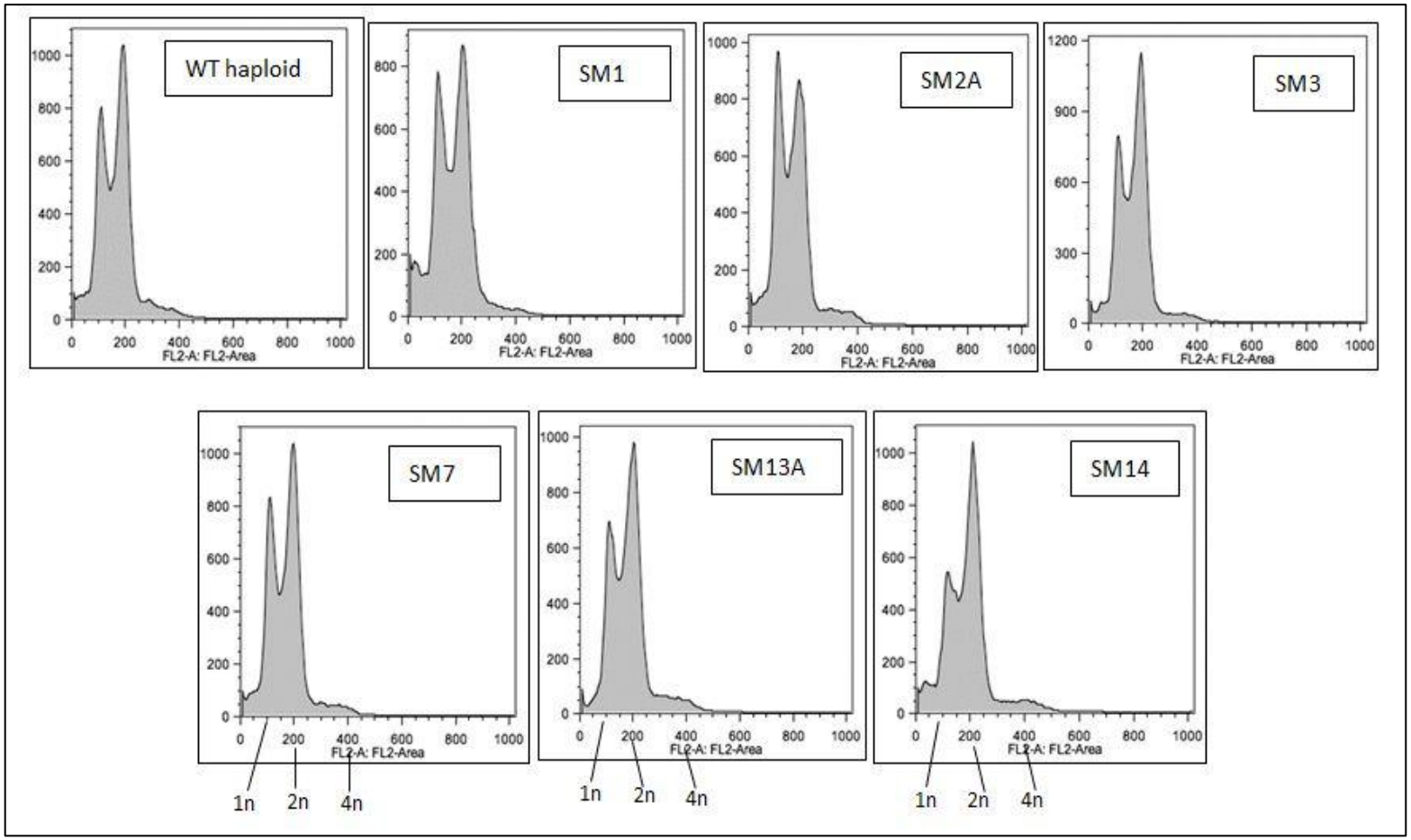


FIGURE 12b. *htb1* mutant strains SM1, SM2A, SM3, SM7, SM13A and SM14. All the strains show haploid DNA content.

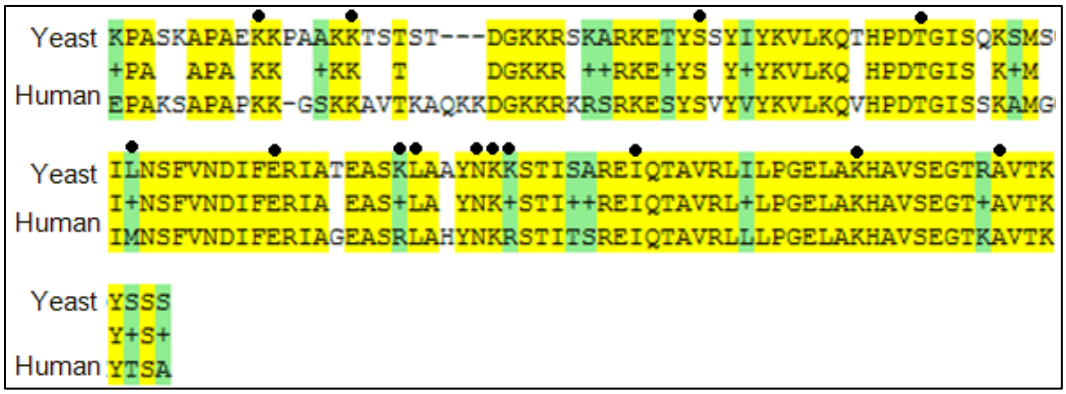


FIGURE 13. Histone H2B alignment between *S.cerevisiae* and human histone H2B type 1-K. Amino acid substitution occurred at 14 different positions (black dots on the protein sequence). 11 out of 14 mutations are in residues evolutionarily identical between the yeast and human proteins. The remaining 3 mutations are changes in conserved residues (positively charged amino acids). Alignment was created using ClustalW2 Multiple Sequence Alignment tool by European Bioinformatics Institute.

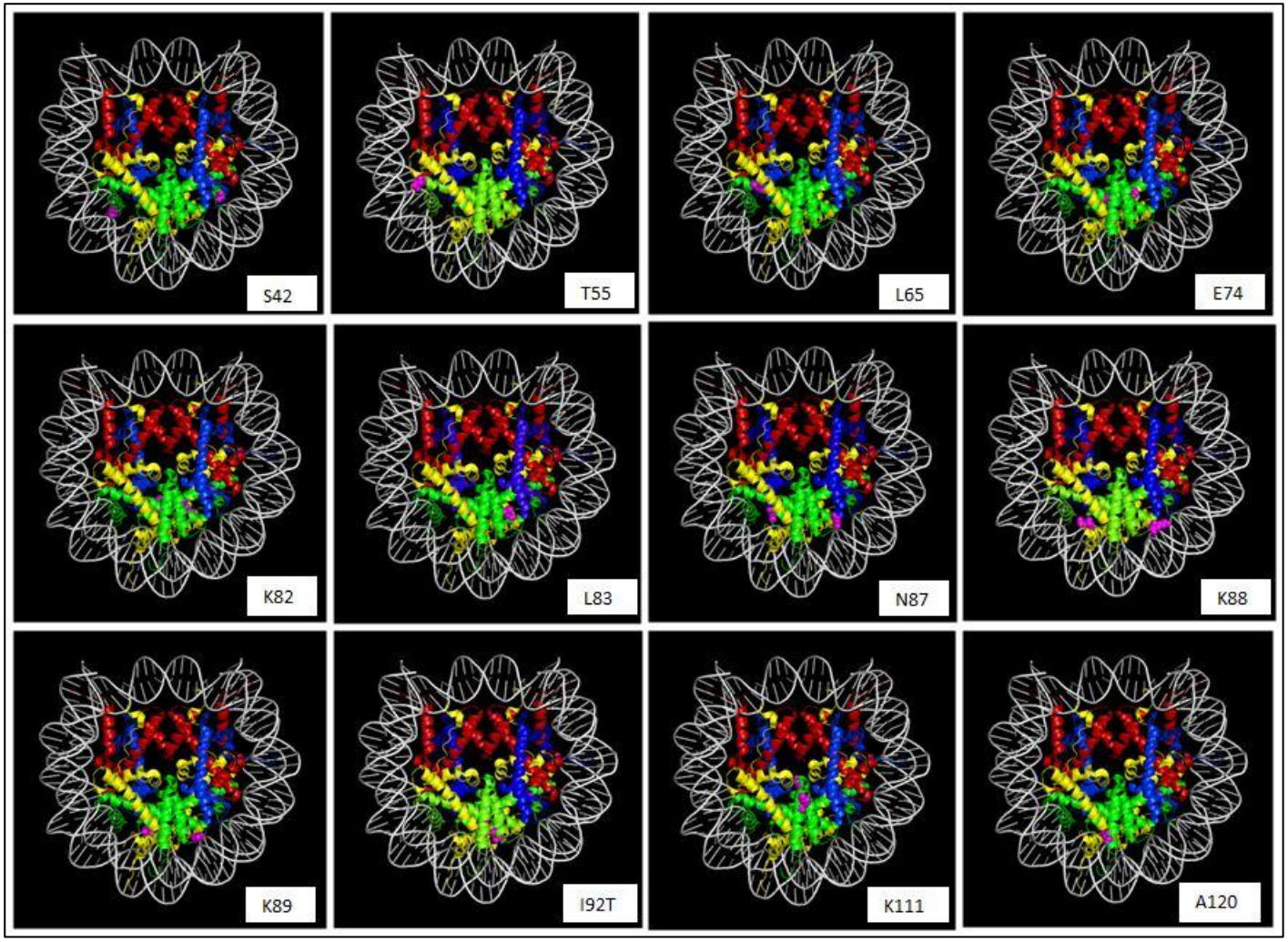


FIGURE 14. Amino acid residue changes in *htb1* mutant strains at positions S42, T55, L65, E74, K82, L83, N87, K88, K89, I92, K111 and A120. (Positions K16 and K22 are not shown). All mutant amino acid residues are colored in magenta. H2B (green), H2A (yellow), H3 (red), H4 (blue) and DNA (white). Images constructed in POLYVIEW 3D.

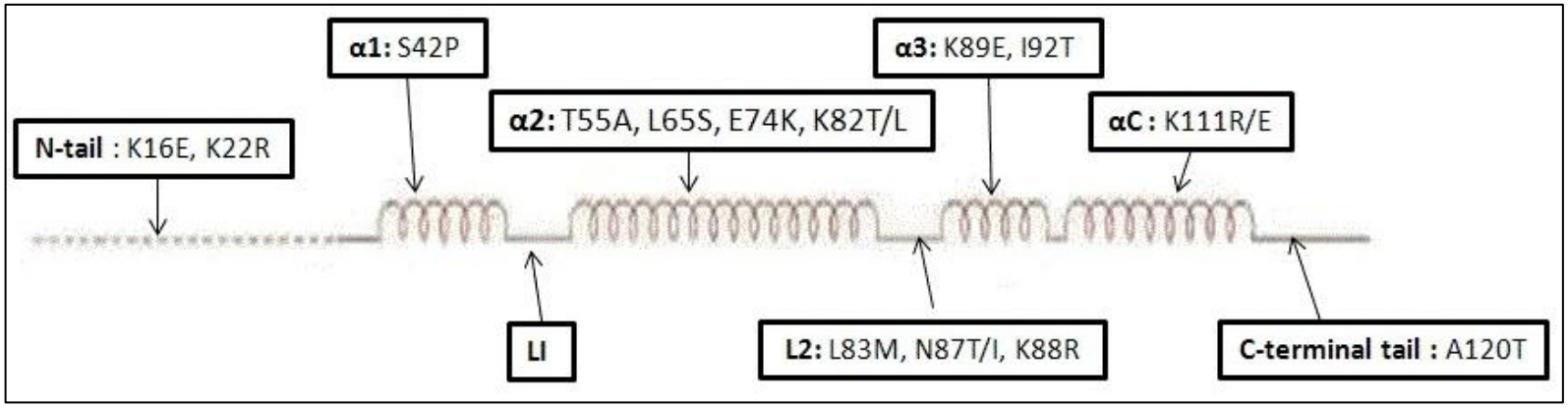


FIGURE 15. *htb1* mutant profile. Mutations were essentially scattered through the entire length of the H2B histone protein except Loop 1 (L1). Amino acid residues K82, N87 and K111 were each found to be mutated in two different strains.

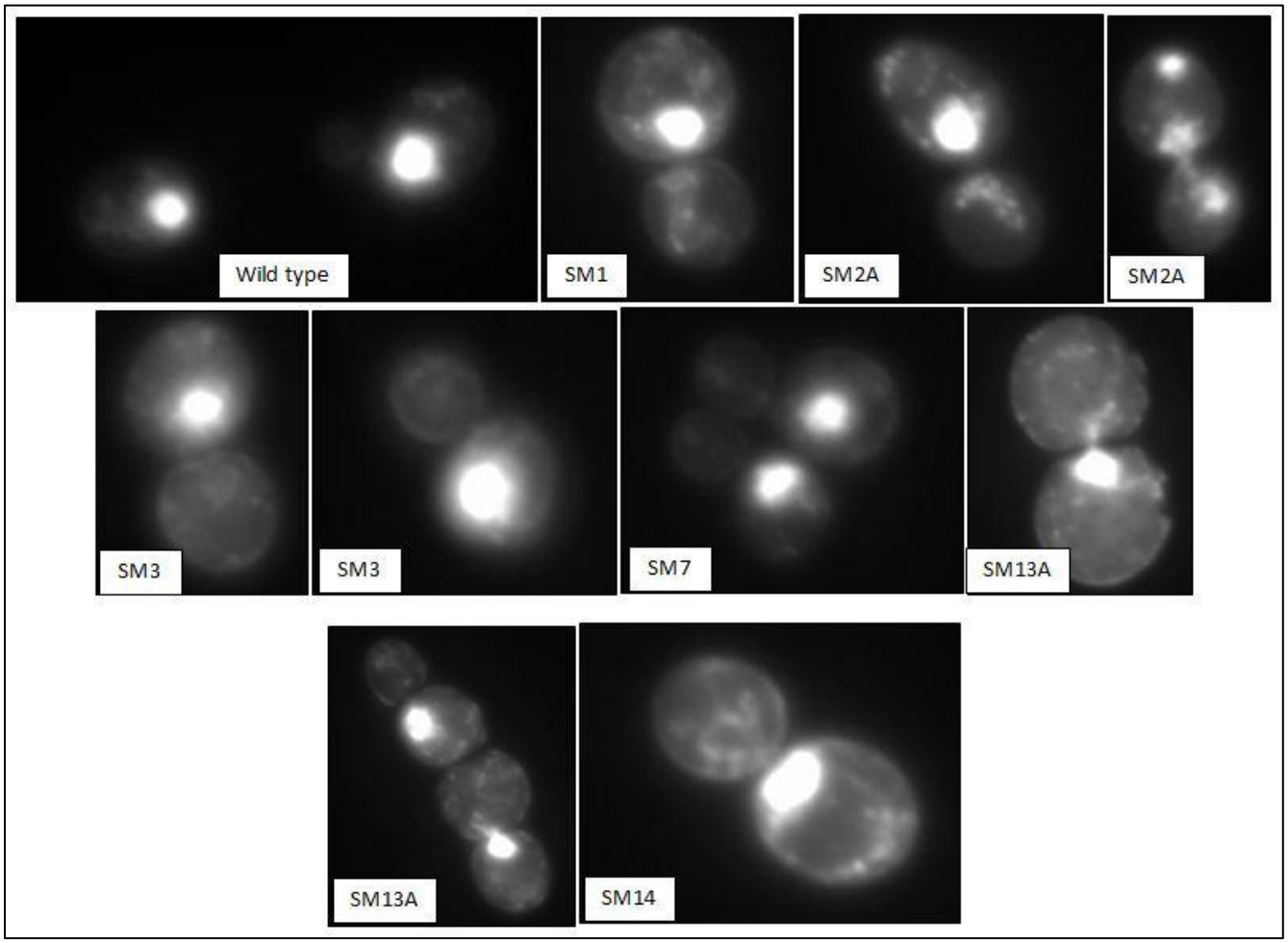


FIGURE 16. DAPI staining of wild type (WT) and all six SM *htb1* mutant strains. WT strain shows unbudded cell on the left and small budded cell on the right. SM1, SM2A (left), SM3, SM7, SM13A and SM14 all show large budded cell with a single nucleus in one daughter cell and none in the other. SM2A (right) shows large budded cell with two or potentially three nuclei between two daughter cells.