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THE ROLE OF CENTROMERIC CHROMATIN AND KINETOCHORE-ASSOCIATED FACTORS IN CHROMOSOME SEGREGATION

THE ROLE OF CENTROMERIC CHROMATIN AND KINETOCHORE-ASSOCIATED FACTORS IN CHROMOSOME SEGREGATION

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Biology

By

Wesley Don Williamson
University of Arkansas
Bachelor of Science in Microbiology, 2005

December 2012
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ABSTRACT

Previous work in our lab has identified a point mutation in *HTA1*, one of the genes encoding histone H2A, which causes an increase-in-ploidy phenotype in *Saccharomyces cerevisiae.* This histone mutant strain was used to carry out a transposon insertion screen to identify suppressors of the increase-in-ploidy phenotype. This screen identified all three subunits of the Hda histone deacetylase complex, *HDA1, HDA2,* and *HDA3*. This study aims to elucidate the function of the Hda complex in chromosome segregation by exploring interactions among the members of the complex, as well as interactions between Hda complex and kinetochore components. We find that the Hda complex interacts with the chromosomal passenger complex (CPC), part of the tension-sensing machinery in the cell. Further experiments on the CPC revealed that a mutant allele of one of the components, *BIR1*, is synthetically lethal with our original histone H2A mutant. Our results led us to another component of the tension-sensing machinery, *SGO1*. Interestingly, *sgo1Δ* is also synthetically lethal with the histone H2A mutant. Our results indicate that the increase-in-ploidy phenotype of the histone H2A mutant is likely due to the inability to create or sense the adequate tension between kinetochores and microtubules that is necessary for faithful chromosome segregation.

This dissertation is approved for recommendation
to the Graduate Council.

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DEDICATION

This work is dedicated to my mother and father, Don and Leslie Williamson, my grandmothers, Emily Cupp and Frances Greene, and to the memory of my late grandfathers, Reeves Williamson and Wesley Cupp.

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Williamson, Wes D, and Ines Pinto. 2012. "Histones and Genome Integrity." *Frontiers in Bioscience:* 17: 984–995.

I. INTRODUCTION

All eukaryotes must carry out basic cellular processes, such as DNA replication, transcription, translation, and chromosome segregation, in order for survival. These basic cellular processes are well conserved from simple eukaryotes, such as the budding yeast *Saccharomyces cerevisiae*, to complex eukaryotes, such as metazoans. Using the simple eukaryote *S. cerevisiae* as a model organism, we aim to understand the role that chromatin plays in chromosome segregation.

Chapter II of this dissertation contains a published literature review in which we present the current body of knowledge regarding histones and genome integrity. This comprehensive review explores histones modifications, histone variants, histone gene dosage, and histone mutants that are relevant to chromatin dynamics, chromosome segregation, and genome integrity.

Chapter III focuses on the involvement of the Hda histone deacetylase complex with centromere function and chromosome segregation. The Hda complex has primarily been implicated in transcriptional regulation, but we identify a novel role for this complex in chromosome segregation. Additionally, we identify interactions between the Hda complex and kinetochore components.

In chapter IV, we expand upon the data shown in chapter III by presenting new finding of additional interactions between the Hda histone deacetylase complex and kinetochore components. This study reveals interesting interactions between chromatin and the tension sensing machinery of the cell. Chapters III and IV will be included in manuscripts which will be submitted for publication at a later date.

II. LITERATURE REVIEW

A. Abstract

Chromosomes undergo extensive structural rearrangements during the cell cycle, from the most open chromatin state required for DNA replication to the highest level of compaction and condensation essential for mitotic segregation of sister chromatids. It is now widely accepted that chromatin is a highly dynamic structure that participates in all DNA-related functions, including transcription, DNA replication, repair, and mitosis; hence, histones have emerged as key players in these cellular processes. We review here the studies that implicate histones in functions that affect the chromosome cycle, defined as the cellular processes involved in the maintenance, replication, and segregation of chromosomal DNA. Disruption of the chromosome cycle affects the integrity of the cellular genome, leading to aneuploidy, polyploidy or cell death. Histone stoichiometry, mutations that affect the structure of the nucleosome core particle, and mutations that affect the structure and/or modifications of the histone tails, all have a direct impact on the fidelity of chromosome transmission and the integrity of the genome.

B. Introduction

Eukaryotic chromosomal DNA is packaged in the cell nucleus as chromatin. The nucleosome is the fundamental repeat unit of chromatin, evolutionarily conserved and composed of histone proteins and DNA. Two molecules of histones H3 and H4 form a tetramer that is bound by two H2A-H2B dimers to form the histone octamer, to which 147 bp of DNA wrap around to form the nucleosome core particle [1-3]. The high-resolution structure of the nucleosome core has provided the framework for additional studies on histone-histone and histone-DNA interactions in eukaryotes. Histones are relatively small, basic proteins that consist of globular and tail domains. The globular domain is formed by the histone fold motif (helix-loop-helix-

loop-helix). The four core histones interact with each other and DNA through the histone fold domains to form the nucleosome core particle [1]. The flexible N-terminal tails protrude from the nucleosome and are important for inter-nucleosome interactions, which lead to higher order chromatin structure, in combination with linker histone H1 and a variety of non-histone proteins [2]. The N-terminal tails are also subjected to various covalent post-translational modifications, including phosphorylation, methylation, acetylation, ubiquitination, ADP ribosylation, and sumoylation. These modifications have been implicated in regulating several cellular processes such as DNA replication, transcription, and chromatin condensation, among others [4-8].

Extensive research over the last two decades has led to a better understanding of chromatin function, and replaced the original notion of histones being a structural component, the mere nuclear scaffolding for DNA compaction, with histones being a dynamic and interactive participant of cellular functions [9]. Although numerous *in vivo* and *in vitro* studies have demonstrated that histones affect all aspects of chromosome function, including transcription, replication, recombination and chromosome segregation, the particular roles in these processes are as yet poorly understood.

Here we provide an overview of the literature that implicates histone function in the maintenance of genome integrity. We focus on functions that affect the chromosome cycle, defined as the cellular processes involved in the maintenance, replication, and segregation of chromosomal DNA. Disruption of the chromosome cycle affects the integrity of the cellular genome, leading to aneuploidy, polyploidy or cell death.

C. Histone gene dosage

The earliest studies that linked histones with the maintenance of genome integrity were done by investigating the effects of differing the stoichiometry of individual histones within the

cell. The budding yeast *Saccharomyces cerevisiae* has proven to be an ideal model organism for these types of studies due to its relatively simple genomic organization of the histone genes. Cells subjected to overexpression of either the H2A/H2B or H3/H4 gene pairs show an increase in chromosome loss, which suggests that the ratio of H2A and H2B to H3 and H4 is important for proper chromosome segregation [10]. It was later shown that underexpression of the histone H3-H4 genes could also affect mitotic chromosome transmission [11]. Deletion of one of the gene pairs encoding H2A-H2B showed cell-cycle defects [12], and depletion of H2B and H4 by placing the genes under inducible promoters inhibits chromosome segregation and in turn causes cell cycle arrest [13, 14]. The connection between these genetic studies and altered chromatin structure was provided by micrococcal nuclease mapping of nucleosomes on isolated nuclei. Specific genetic loci showed disrupted nucleosome arrays in yeast cells lacking one of the two H2A-H2B coding gene pair [15]. One of the disrupted loci was the centromere of chromosome III. Additional chromatin mapping studies in cells repressed for expression of either H2B or H4 corroborated the sensitivity of centromeric chromatin structure to histone gene depletion [16]. Recent work in fission yeast has shown that the relative levels of histone H3, H4 and the centromere-specific histone H3 variant CENP-A influence the assembly of centromeric chromatin and recruitment of kinetochore proteins, affecting the fidelity of chromosome segregation [17]. In support of this finding, overexpression of H3 in budding yeast increases the rate of chromosome loss with a concomitant reduction in the levels of the centromere-specific histone H3 variant Cse4 [18]. In addition, partial depletion of H4 was shown to affect chromatin assembly during DNA replication that resulted in increased levels of homologous recombination, leading to genetic instability [19]. These studies clearly show that each of the four core histones

must be maintained in a proper stoichiometry for normal cell cycle progression and high-fidelity chromosome segregation.

D. Histone mutants

Mutational analysis of histones has allowed researchers to show that not only the balance of histones is important, but also that the histone proteins themselves can lead to phenotypes associated with defects in the chromosome cycle. Two independent mutants of H2A in *S. cerevisiae* cause increase in ploidy and increased frequency of chromosome loss. The mutations reside in evolutionarily conserved residues near the N-terminus of the structured globular domain (S19F and G29D) that make contact with DNA. These alleles show cell cycle defects, genetic interactions with kinetochore mutants, and altered centromeric chromatin structure, suggesting a role for H2A in microtubule attachment at the centromere-kinetochore [20]. Work in the fission yeast *Schizosaccharomyces pombe* has led to the finding of temperature sensitive mutations in the inner region of H2B that cause defects in centromeric chromatin and chromosome segregation [21]. These mutations affect DNA contact (G52D) as well as histone-histone interactions (P102L) in the core nucleosome particle, stressing the importance and stringency of the nucleosome architecture in chromatin function.

Early deletion studies in *S. cerevisiae* demonstrated that the highly conserved N-terminal tails of H3 and H4 are essential for cell cycle progression. Although H3 and H4 N-terminal tails can be individually deleted without losing cell viability, deletion of the H3 and H4 N-terminal tails in combination yields inviable cells with terminal phenotypes associated with cell division cycle defects [22]. In a more detailed study of H4 N-terminal tail mutations, Megee at al. [23] reported the requirement of the four most N-terminal lysine residues (domain A, positions 5, 8, 12 and 16) for normal nuclear division. The mutant cells activate the DNA damage checkpoint

and arrest at G2/M. Reintroduction of a lysine residue within domain A, without the requirement of polypeptide sequence specificity, restored cell-cycle progression, strongly supporting a role for the post-translational modifications of N-terminal lysines in cell division. In another study, a temperature-sensitive allele of H4, carrying two amino acid replacements (T82I and A89V) caused severe nuclear division and mitotic chromosome transmission defects [24]. The primary mutation at position 82 is located within one of the H4 surfaces that interacts with DNA, and the T82I mutation is lethal but rescued by the A89V mutation. Thus, similarly to the H2A mutants, H4 residues that lie in the path of DNA can have strong effects on cell cycle functions.

Recent analysis of a histone H3 mutant (G44S) that causes pleiotropic phenotypes related to cell cycle progression, including benomyl and hydroxyurea sensitivity, led to the discovery of a mitotic tension-sensing function [25]. Prior to anaphase, the bipolar attachment of sister chromatid kinetochores to the spindle microtubules generates tension that is monitored by the spindle assembly checkpoint. This tension-sensing checkpoint is essential to allow the cell to stall the cell cycle and correct erroneous or missing attachments that can result in aneuploidy. Yeast cells carrying the H3 G44S mutant fail to activate the spindle assembly checkpoint during tension-less situations, leading to the missegregation of chromosomes and aneuploidy. Interestingly, this impairment results from a defective interaction between H3 and Sgo1p (shugoshin), a protein required for tension sensing and present in pericentric chromatin.

Systematic histone substitution and deletion mutant collections have been created in *S. cerevisiae* to probe the contribution of each residue to chromosome function [26, 27]. These collections of alleles have been screened for phenotypes associated to DNA repair and sensitivity to DNA damaging agents, providing new insights into the contribution of each residue to the DNA damage response. These valuable resources will undoubtedly provide novel information as

the libraries are screened for additional phenotypes associated with the maintenance of genome integrity.

In many cases, mutational analyses of individual amino acids in each of the histones, particularly in their terminal "tails", have been carried out to study the effect of abolishing specific post-translational modifications. Those studies that link histone modifications to the chromosome cycle are discussed below.

E. Histone modifications

Covalent modifications of the N-terminal tails of histones have been implicated in the regulation of various cellular processes. The mechanisms by which many of these modifications carry out their effects in the cell are still largely unclear. Some may work by changing the charge of the histone, and in turn causing the DNA to associate more tightly or loosely with the nucleosome. Other modifications may serve as a "mark" to recruit chromatin remodeling complexes or other regulatory proteins. Lastly, there is an emerging "histone code" in which multiple modifications act in concert with each other and have a so-called "crosstalk" to regulate cellular functions [4, 5, 28]. Importantly, covalent modifications have been found in all organisms analyzed; however, the specific amino acids that are modified, the type of modification, and the associated function can vary among species, creating an enormous challenge in the efforts to decode the histone language.

1. Methylation

Histone methylation is the result of the covalent attachment of methyl groups from S-adenosyl-L-methionine onto the epsilon-amino group of lysine, arginine, and histidine residues catalyzed by specific histone methyltransferases [29, 30]. The reversible nature of this modification became clear many years later with the identification of histone demethylases [31]. Three forms of

methylated lysine –mono-, di- and tri-methylation- are found on histones, and each one can signal a different chromatin state. Methylation of histone H3 on Lys9 (H3K9me) has long been recognized as a determinant of silent chromatin and heterochromatin [32]. Fission yeast centromeres are marked by H3K9me heterochromatin that facilitates the assembly of the essential centromere-specific H3 variant CENP-A at the central domain [33, 34]. Mutations in the histone methyltransferase Clr4 distort the pericentric heterochromatin and disrupt chromosome segregation [35]. Dimethylation of histone H3 Lys9 (H3K9me2) and trimethylation of histone H3 Lys9 (H3K9me3) are also present in pericentric heterochromatin in Drosophila, mouse and human cells [36]. Similarly to fission yeast, loss of the suv39h histone methyltransferases disrupts mammalian heterochromatin and affects genome stability [37, 38]. The regulation of pericentric heterochromatin is carried out by the chromodomain proteins Swi6/HP1, which bind H3K9me2 and are essential for mitotic progression [39-42]. In mammalian cells, H3K9me3 methylation increases in late G2 phase and mitosis and rapidly decreases in G1. Loss of H3K9 methylation in G2 leads to centromere and kinetochore defects and chromosome misalignment [43, 44]. Histone H4K20 trimethylation has been described in fission yeast, Drosophila and mammalian cells [36]. In human cells, H4K20me3 is abundant in pericentric heterochromatin and cell-cycle regulated. H4K20me3 decreases in S phase and increases in late G2 and mitosis [45]. Interestingly, in murine cells H4K20 trimethylation is catalyzed by two histone methyltransferases, Suv4-20h1 and Suv4-20h2, which interact with HP1 and function in a suv39h dependent manner [46]. This finding led to the proposal of a sequential mechanism of H3K9me3 and H4K20me3 in the formation of pericentric heterochromatin. Although these results did not provide a direct connection between these histone modifications and mitosis, recent work has shown that the loss of methylation at H3K9 and H4K20 leads to less compact pericentric heterochromatin and loss of tension at the

centromere during mitosis [43]. In addition, the monomethyl to dimethyl transition of histone H4K20 has been associated with chromosome behavior during mitosis and cytokinesis. Subunits of the human factor HCF-1 associate with chromatin and regulate the expression of the H4K20 methyltransferase PR-Set7. Loss of HCF-1 during mitosis leads to increased PR-Set7 expression and dimethylation of H4K20, resulting in defective chromosome alignment and segregation [47].

Methylation of H3K79 and H4K20 are the main modifications involved in DNA repair, hence, essential to the integrity of the cell's genome. Although methylated H3K79 and H4K20 are present throughout the genome, they become evident at DNA repair foci after DNA damage [48]. Dimethylated H4K20 at these foci is specifically recognized and bound by the checkpoint protein Crb2/53BP1, which triggers a G2/M arrest to allow DNA repair to take place [49, 50]. Consistent with these data, depletion of the methyltransferases Suv4-20h1 and Suv4-20h2 decreases the number of DNA repair foci containing 53BP1 [51]. Surprisingly, Crb2/53BP1 only recognizes H4K20me and H4K20me2, but not H4K20me3 [50, 52], therefore, it appears that different functions are associated with different proteins that recognize distinct levels of methylation at the same histone residue. Methylation of H3K79 is the main signal for DNA repair in budding yeast. Dot1 is the evolutionarily conserved methyltransferase capable of adding mono-, di-, and trimethyl groups to H3K79. Originally identified by mutations that disrupted telomeric silencing, cells lacking Dot1 are also defective in the checkpoint response to DNA damage and DNA repair pathways [53, 54]. Supporting the critical function of H3K79 methylation in the chromosome cycle, mouse ES cells lacking Dot1L, the murine Dot1 homologue, show reduced levels of the heterochromatic marks H3K9me2 and H4K20me3 at centromeres and telomeres, along with the general depletion of H3K79me. These histone changes are likely the cause of the aneuploidy and telomere elongation defects observed in these

cells [55].

Histone methylation can also influence cell cycle progression in a more indirect fashion. H3K4 methylation has been shown to increase in mitosis and is thought to be a mark for the activation of certain mitotic-specific genes, such as cyclin B1 [56]. This finding provides a connection between the transcriptional regulation of factors driving the cell cycle and histone methyltransferases.

2. Acetylation

Histones are reversibly acetylated on lysine residues primarily in the N-terminal tails. The transfer of the acetyl group from acetyl-coenzyme A is catalyzed by histone acetyltransferases (HATs). Histone acetylation has been mainly implicated in transcriptional regulation, with histones in transcriptionally active regions being acetylated. The reversal of acetylation has been associated with transcriptional repression and chromatin compaction [57, 58]. Underacetylated histones H3 and H4 are abundant in centromeric heterochromatin of metaphase chromosomes [59], and an overall reduction of histone H3 and H4 acetylation occurs in the transition from interphase to mitosis [60]. However, histones H3 and H4 remain acetylated in loci that are still transcriptionally active during mitosis or need to be reactivated quickly following mitosis [56]. Deletion of the H3 acetyltransferases *SAS3* and *GCN5* in *S. cerevisiae* leads to G2/M mitotic arrest [61], perhaps as a result of transcriptional defects.

Histone deacetylase activity is essential for mitotic progression. Inhibition of deacetylation has been associated with delayed G2/M transition [62] and mitotic arrest [63]. Treatment of cells with histone deacetylase inhibitors affects the formation of pericentric heterochromatin, resulting in kinetochore assembly defects [64], chromosomal instability and defective checkpoint activation [65]. Depletion of the mammalian histone deacetylase HDAC3

also affects chromosome condensation, sister chromatid cohesion, and kinetochore-microtubule attachment, leading to defective chromosome segregation [66-68]. Depletion of the human histone deacetylase HDAC3 causes premature dissociation of sister chromatids and acetylation of centromeric H3K4, which correlates with the loss of dimethylation at the same position, illustrating the complexity of the "histone code" in the regulation of mitotic events [67]. It has also been suggested that deacetylation of H4K16 by the SirT2 deacetylase during the G2/M transition is required for chromatin condensation [69].

Additionally, deletion of a histone deacetylase complex (Hda1) in *S. cerevisiae* suppresses a histone H2A mutant that causes increase in ploidy and increased frequency of chromosome loss, providing a genetic link between histone deacetylation and mitotic function [70]. These histone H2A mutants alter the nucleosome architecture and pericentric chromatin structure in a significant way, leading to the hypothesis that pericentric chromatin contributes to kinetochore formation and microtubule attachment in budding yeast [20]. The increased acetylation observed in the suppressors may compensate directly for a defective histone post-translational modification in the H2A mutant-containing nucleosomes, restoring an epigenetic mark specific for pericentric chromatin. Alternatively, indirect suppression may occur by bypassing the chromatin structural defect, creating an epigenetic environment favorable for the formation of a functional centromere-kinetochore complex and microtubule attachment. Further studies will be necessary to decipher the factors that interact with pericentric chromatin and contribute to the bipolar kinetochore-microtubule attachment and proper chromosome segregation in *S. cerevisiae*.

Acetylation of H3K56 deviates from the well-characterized modifications of the histone

tails, but this modification has been shown to play an important role in DNA replication and repair. Lysine 56 resides in the H3 core and is acetylated in yeast cells by the Rtt109 acetyltransferase as a mark of newly synthesized chromatin during S phase. Although this modification was originally described in yeast, it has recently been identified in mammalian cells [71]. In the absence of DNA damage H3K56 acetylation is removed during the G2/M phase of the cell cycle. In contrast, cells with DNA lesions maintain high levels of acetylated H3K56, modification that is crucial for the DNA damage response [72, 73]. Consistently, *rtt109* mutants display hypersensitivity to DNA damaging agents and elevated levels of spontaneous chromosome breaks [74, 75]. Moreover, H3K56R mutants are also sensitive to DNA-damaging agents and unable to reassemble chromatin after DNA repair [76, 77].

3. Phosphorylation

Phosphorylation of histones, mainly at serine residues, has long been recognized as an important modification involved in chromosome dynamics during mitosis and DNA repair processes. Phosphorylation of histone H3 at serine10 (H3S10pho) has been found in all organisms analyzed so far, and shown to be required for chromatin compaction and condensation in mammals and most eukaryotes [78, 79]. During mitosis, levels of H3S10pho are high through the activity of the evolutionarily conserved Aurora B kinase [80]. Mutants of H3S10 in *Tetrahymena* that are unable to be phosphorylated display problems in chromosome segregation caused by lack of chromosome condensation [81]. In a converse study, increased mitotic levels of H3S10pho induced by overexpression of the mammalian AIM-1 (Aurora B) kinase led to lagging chromosomes and aneuploidy [82]. Thus, regulated levels of H3S10pho are required for proper mitotic progression. One of the proposed mechanisms by which H3S10pho may function is a binary switch responsible for the association /dissociation of the chromodomain protein HP1

from mitotic chromosomes. Phosphorylation of H3S10 in mitosis induces the dissociation of HP1 bound to H3K9me, the latter required for heterochromatin maintenance, while the levels of H3K9me remain unchanged [83, 84]. Another study has suggested that phospho-acetylation of H3 (SP10-K14Ac) is required for eviction of HP1 from chromatin [85]. Recent work has shown that H3S10pho also regulates the binding of two human SR protein splicing factors, SRp20 and ASF/SF2, with chromatin. These SR proteins associate with interphase and late post-mitotic chromatin, but are dissociated from mitotic chromatin following H3S10 phosphorylation. They also interact with HP1, which fails to dissociate from chromatin when the SR proteins are absent [86]. Much like H3S10, H3S28 is also phosphorylated and is closely correlated with chromatin condensation [87]. An additional phosphorylation event at threonine 3 (H3T3), catalyzed by the Haspin kinase, has been shown to be required for metaphase chromosome alignment [88]. The functional connection between the H3T3 and H3S10 phosphorylation events remains unknown. Phosphorylation of H3 at Thr 45 has been recently reported in budding yeast and linked to DNA replication [89]. Although the specific functions of H3 phosphorylation remain to be elucidated, the emerging information points to a dynamic interaction between H3 kinases, phosphatases, and chromatin associated factors required for the formation of the proper chromatin conformation of the mitotic chromosome.

Two other phosphorylations, H2A-S1 and H4S1, are also associated with mitotic chromatin condensation. [90]. While most histone modifications are at the N-terminal tails, they can be modified elsewhere as well. Phosphorylation of H2A-T119 takes place at the C-terminus and happens specifically during mitosis [91], where it is enriched at centromere regions in Drosophila [92]. Recent work has provided a functional link to this modification. In fission yeast, H2A-S121 (equivalent to Drosophila H2A-T119) is phosphorylated by the mitotic kinase Bub1

and recruits shugoshin/Sgo1 to centromeres, which secures proper chromosome partitioning. [93]. These data establish an essential function for H2A phosphorylation in maintaining mitotic chromosome stability.

4. Ubiquitination

Ubiquitination is the covalent conjugation of ubiquitin to lysine residues. Histones are usually monoubiquitinated, a modification that does not lead to protein degradation. Monoubiquitination of H2BK123 in *S. cerevisiae* is mediated by the Rad6/Ubc2 ubiquitin conjugating enzyme and the Bre1 ubiquitin ligase. Mutants that are unable to be ubiquitinated at H2BK123 show mitotic and meiotic defects [94]. Recently, it has also been shown that ubiquitination of H2BK123 is required for trimethylation of H3K4 and H3K79 [95], a cross talk that has been mainly implicated in the regulation of gene expression. Histone ubiquitination has also been linked to DNA repair. DNA lesions caused by UV-irradiation induce monoubiquitination of histone H2A by the Ring2 ubiquitin ligase [96], as well as ubiquitination of H3 and H4 by the CUL4-DDB-Roc1 ubiquitin ligase complex [97]. It is likely that these modifications alter the chromatin structure and facilitate the recruitment of repair proteins to the damage loci.

In mammalian cells, deubiquitination of H2A is required for normal mitosis and cell cycle progression. It is also apparent that deubiquitination of H2A is required for H3S10 phosphorylation [98]. Thus, histone ubiquitination has emerged as an important signal for various cellular processes. Further research is needed to determine the specific involvement of this modification in cell cycle progression.

F. Histone variants

Histone variants are specialized histones that replace core histones in a DNA-replication independent manner, generating an altered chromatin structure with distinct cellular functions [99].

1. CenH3

All eukaryotes, from yeast to humans, have a histone H3 variant (called CenH3, in general) that takes the place of the canonical H3 in centromeric nucleosomes. CenH3 is called Cse4 in *S. cerevisiae,* Cnp1 in *S. pombe*, CID in *Drosophila,* and CENP-A in mammals. CenH3 is only 50% identical to the canonical H3, compared with most other histone variants that are more conserved with respect to the canonical histone. CenH3 in *Saccharomyces cerevisiae*, called Cse4, occurs only in one nucleosome per chromosome directly at the centromere because the budding yeast centromeres are only 125bp long [100]. On the other hand, higher eukaryotes have regional centromeres that can be up to 1 megabase long; blocks of CenH3-containing nucleosomes are interspersed with blocks of H3-containing nucleosomes [101].

The composition of the centromeric nucleosome in *S. cerevisiae* has been a topic of recent debate. It was reported that a nonhistone protein, Scm3, could assemble with Cse4 and histone H4 to form a centromeric nucleosome hexamer that lacked H2A-H2B [102]. A later study showed that Cse4 forms an octameric nucleosome with H2A, H2B, and H4 [103]. The latter study suggests that Scm3 is perhaps intimately associated with Cse4-H4 tetramers as an intermediate complex before nucleosome formation, but is not included in the resultant histone octamer.

CenH3-containing nucleosomes are assembled into centromeric chromatin, which becomes the scaffolding on which the kinetochore is formed during mitosis. CenH3 is essential for the formation of a stable kinetochore. Mutational analysis in *S. cerevisiae* has demonstrated that loss of CenH3 leads to mitotic arrest and missegregation of chromosomes [104]. Lastly, CenH3

is an important epigenetic mark in organisms with regional centromeres, as the highly variable centromeric DNA of higher eukaryotes is not sufficient for kinetochore formation. Specification of kinetochore location is directed by the epigenetic mark of CenH3 dilution to daughter DNA strands following S phase, allowing the centromeric chromatin to be heritable [105].

2. H3.3 and H3.1

In addition to CenH3, there are two other histone H3 variants in higher eukaryotes called H3.1 and H3.3. While not as well characterized as the other histone variants with respect to genome integrity, they do appear to play significant roles in the chromosome cycle. In metazoans, H3.3 is a replication-independent H3 variant that has mainly been implicated as an epigenetic mark for active chromatin [106]. Interestingly, *Drosophila* that are deficient for H3.3 display widespread transcriptional defects, sterility, and semi-lethality [107]. H3.1 and H3.3 have nearly identical sequences to the canonical H3, with only a stretch of 4 amino acids contributing to the difference in function and selective deposition at specific genetic loci [108]. H3.1 is a replication-dependent H3 variant found in mammals, the function of which remains unknown.

3. H2A.Z

Another histone variant that has been implicated in genome integrity is the H2A variant H2A.Z. Studies in *D. melanogaster* have shown that the loss of H2A.Z leads to depletion of HP1alpha from chromosome arms, thus affecting the integrity of heterochromatin. This defect in forming higher order chromatin structures is likely the cause of the chromosome segregation errors [109]. H2A.Z has also been shown to affect chromosome segregation and centromere silencing in the fission yeast *S. pombe.* It was determined that H2A.Z is required for the expression of Cnp3, the *S. pombe* homolog of CENP-C, which is a centromere protein that is

essential for maintenance of centromere silencing [110]. H2A.Z is not an essential protein in the budding yeast *S. cerevisiae*, but phenotypic and genetic studies have implicated it in genome stability [111]. Unlike CenH3, which has a direct effect on chromosome segregation at centromeric regions, H2A.Z has a more indirect effect on genome integrity by affecting heterochromatin at chromosome arms as well as transcription of certain centromeric proteins.

4. H2AX

Histone modifications provide a critical signal during the DNA damage response, by marking the sites of DNA lesions and making them accessible to the repair machinery [48]. In mammalian cells, the histone variant H2AX becomes rapidly phosphorylated in response to double-strand breaks (DSB) [112]. The phosphatidylinositol-3-OH kinase-like family of protein kinases, which include ataxia telangiectasia mutated (ATM), ataxia telangiectasia-related (Rad-3 related or ATR) and DNA-dependent protein kinase (DNA-PK), catalyzes the phosphorylation of Ser 139 in the highly conserved carboxy terminal Ser-Gln-Glu (SQE) motif, generating gamma-H2AX [113, 114]. In *S. cerevisiae* and *D. melanogaster*, which lack H2AX, a conserved SQ motif is found at the C-terminus of the canonical H2A and the H2Av variant, respectively. Phosphorylation of Ser 129 of H2A in yeast signals DSB repair via non-homologous end-joining [115]. The presence of gamma-H2AX on the chromatin surrounding the DNA lesion triggers a signal cascade for the recruitment and retention of the DNA repair proteins to the damaged site, along with chromatin remodeling complexes and mitotic checkpoint factors [116]. Recent work has provided evidence that additional post-translational modifications, including acetylation and ubiquitination of gamma-H2AX and other chromatin components, are necessary for the repair

process, either through the non-homologous end-joining or homologous recombination pathways [116, 117].

5. Macro H2A

This is the most atypical histone variant. MacroH2A (mH2A) is a vertebrate specific variant, consisting of an N-terminal domain homologous to the canonical H2A and a large C-terminal region referred to as the macro domain, connected by a basic hinge region [118]. This non-histone like region accounts for two thirds of the molecular mass of mH2A. There are two closely related variants, macroH2A1 and macroH2A2, which preferentially associate with the inactive X chromosome (Xi), suggesting a role in transcriptionally repressed chromatin. However, they are also found in autosomes, where they appear to exert a function in gene repression and heterochromatization [119]. *In vitro* studies have shown that nucleosomes containing mH2A1 can interfere with chromatin remodeling and transcription initiation [120, 121]. Interestingly, the macro domain can bind and maintain in an inactive form poly(ADP-ribose) polymerase 1 (PARP-1), contributing to X chromosome inactivation and gene silencing. Release of mH2A from promoters activates PARP-1, which in turn activates transcription through ADP ribosylation [122]. Recently, extensive analysis of hundreds of mH2A targets reveled that they are enriched in genes controlling developmental processes and cell fate decisions [123]

Like other histones, mH2A variants are also subjected to post-translational modifications [124]. Phosphorylation of S137 in mH2A1, which resides in the hinge region of mH2A, was shown to be present in male and female cells during mitosis, but excluded from the X chromosome [125]. This finding suggests a role for mH2A in chromatin function throughout the cell cycle, outside Xi, and regulated by its own post-translational modifications.

G. Conclusions

In order to maintain the integrity of the genome the cell requires precise temporal and spatial chromatin dynamics during the chromosome cycle. Ample evidence indicates that the proper balance of histones is essential for maintaining nucleosome assembly and chromatin structure. The centromeric and pericentric regions of the chromosome are particularly sensitive to histone balance, since the incorporation of the cenH3 variant to centromeric regions and the structure of pericentric chromatin are perturbed when histone stoichiometry is altered. Aneuploidy is a common consequence of altered histone balance, likely the result of defective centromere-kinetochore structures, although not necessarily the only cause of it. More studies are needed to understand genomic instabilities associated with defective chromatin assembly during DNA replication as a consequence of histone imbalance.

Histone mutations can be generally divided into two groups, those that affect the globular domain of histones in the core nucleosome particle, and those that affect the flexible histone tails. The first group of mutations usually leads to distortions in the nucleosome architecture, which correlate with phenotypes that can be associated with specific cellular functions. It is becoming apparent that there are domains within the nucleosome particle that may be recognized by specific proteins devoted to distinct cellular functions. This possibility raises questions of specificity, recognition, and targeting that will require extensive research to be elucidated. Most mutations within the histone tails have been induced to study the effect of their post-translational modifications. It is clear that all four histones undergo modifications that are crucial for the chromosome cycle; what is not so clear yet is the specific role of each modification, although some correlations are evident. Examples of such associations are the methylation of H3 lysines and heterochromatin formation, histone deacetylation and mitotic progression, and H3

phosphorylation with chromosome condensation. Most of what we have learned so far comes from studies that abolish individual modifications, either by mutations in the modified amino acid, or by mutations in the modifier enzyme (methyltransferase, acetyltransferase, etc.). The use of modification-specific antibodies has provided major advances in connecting specific histone modifications to cellular functions. Undoubtedly, histones provide key signals in the dynamic behavior of chromatin throughout the chromosome cycle. The difficult task ahead lies in the identification of the proteins that recognize and bind nucleosomal histones in their specific modified state, and to link them to their respective cellular pathway.

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III. INVOLVEMENT OF THE HDA HISTONE DEACEYLASE COMPLEX WITH CHROMOSOME SEGREGATION

A. Introduction

DNA is packaged into eukaryotic cells in the form of chromatin, a dynamic structure consisting of nucleic acid, histone proteins, and a variety of non-histone proteins. Histones are highly evolutionarily conserved from yeast to humans. Two each of the core histones H2A, H2B, H3, and H4 come together to form the histone octamer, and approximately 147 bp of DNA wraps around each octamer twice to form the fundamental unit of chromatin, the nucleosome (Luger et al. 1997; van Holde 1988; White, Suto, and Luger 2001). The nucleosomes are packaged into higher order structure to form chromosomes.

An important feature of histone proteins is the N-terminal tails that protrude from the nucleosome. These tails can be post-translationally modified by acetylation, methylation, ubiquitylation, and phosphorylation (reviewed in Williamson and Pinto 2012). Histone modifications affect a very diverse array of cellular processes including DNA damage repair, transcription, silencing, and chromosome segregation. Modifications can work in parallel or antagonistically, and much remains unclear about the complex “histone-code” that promises to unlock many secrets of epigenetics.

For chromosome segregation to proceed properly, the chromosomes must satisfy all requirements of the spindle assembly checkpoint (SAC), including accurate biorientation of kinetochores on sister chromatids and attachment of microtubules to kinetochores. Kinetochores assemble on the centromeric (*CEN)* regions of DNA, and facilitate the binding of microtubules during mitosis. Budding yeast *Saccharomyces cerevisiae* have “point centromeres” consisting of only 125bp of DNA (Furuyama and Biggins 2007). This is in contrast to the much larger regional centromeres of higher eukaryotes that can be as large as 100 megabases long (Cleveland, Mao, and Sullivan 2003).

Although *S. cerevisiae* lacks the centromeric heterochromatin that is seen in higher eukaryotes, there is a nuclease-resistant core flanked by phased nucleosomes (Bloom and Carbon 1982). The centromeric phasing of nucleosomes has been shown to be affected by mutations in genes encoding the histone proteins (Pinto and Winston 2000). These studies clearly link histones to chromosome segregation, although their particular roles are not clearly understood.

Previously, we used a transposon insertion screen to identify suppressors of an increase-in-ploidy phenotype of two mutants of *HTA1*, one of the two genes encoding histone H2A (Kanta et al. 2006). From this screen we identified *HDA1, HDA2, AND HDA3*, the three subunits of the Hda histone deacetylase complex (Wu, Carmen, et al. 2001). Histone deacetylation has been shown to be essential for normal mitotic progression, and loss of histone deacetylation activity gives rise to G2/M delay (Mikhailov, Shinohara, and Rieder 2004), mitotic arrest (Sandor et al. 2000), and abnormal pericentric heterochromatin (Shin et al. 2003).

The Hda complex has primarily been implicated in transcriptional regulation (Wu, Suka, et al. 2001), but here we propose a role for this complex in chromosome segregation. Here we show that the original truncated tn-alleles identified from the screen behave as null alleles, indicating that loss of deacetylase activity is necessary for suppression of the *hta1* mutants. Previously, we showed by chromatin immunoprecipitation that the Hda complex is present at centromeric regions of DNA, and able to deacetylate centromeric histones. Here we show that the Hda complex binds to DNA up to 20kb away from the centromere, indicating that pericentric chromatin may also be an important target of deacetylation, and required for kinetochore function. We also report genetic and physical interactions between the Hda complex and kinetochore components. Lastly, we demonstrate that the suppression of an *ndc10* kinetochore

mutant by loss of Hda1 deacetylation restores the defective chromosome segregation of the mutant further implicating the importance of histone deacetylation in chromosome segregation.

B. Materials and Methods

Yeast strains, genetic methods, growth, and media: The yeast strains used are listed in Table 1. Unless indicated, strains are isogenic to FY2, originally derived from S288C (Winston, Dollard, and Ricupero-Hovasse 1995). Strain construction and other genetic manipulations were carried out by standard methods (Guthrie and Fink 1991; Rose, Winston, and Hieter 1990). All yeast media, including YPD, synthetic minimal, omission media (SC), and media containing 5-fluoroorotic acid (5-FOA) were made as described previously (Rose, Winston, and Hieter 1990). Benomyl plates were made by adding benomyl (Sigma, St. Louis) to hot YPD to a final concentration of 10 μg/ml. Canavanine plates contain 60 μg/ml of canavanine sulfate (Sigma).

Genes were tagged with 13x*MYC* at the 3' end by PCR using plasmid GHB160 as template, with 3x*FLAG* at the 3' end by PCR using plasmid GHB342 as template, and with 3x*HA* at the 3' end by PCR using GHB159 as template as previously published. (Longtine et al. 1998; Schneider et al. 1995)

Synchronization of cells in G1 was carried out by adding a final concentration of 0.9mM α-factor to exponentially growing cells. Cultures were incubated at 30° for 2.5 hours, washed twice in α-factor-free medium, and resuspended in fresh YPD. Synchronization of cells in S-phase was carried out by adding a final concentration of 200mM hydroxyurea (USBiological) to exponentially growing cells. Cultures were incubated at 30°C for 2.5 hours, washed twice in hydroxyurea-free medium, and resuspended in fresh YPD. Synchronization of cells in G2/M was carried out by adding a final concentration of 15 μg/ml nocodazole (USBiological) to exponentially growing cells. Cultures were incubated at 30°C for 2.5 hours, washed twice in nocodazole-free medium, and resuspended in fresh YPD.

For the recovery from nocodazole assay, nocodazole was added to a liquid culture of exponentially growing cells in a final concentration of 50 μg/ml and incubated for 6 hours at 30°C. Approximately 200 cells were plated on YPD, allowed to grow for 2 days at 30°C, and assessed for viability by counting CFU's.

Bacterial strains and plasmids: Plasmids were amplified and isolated from Escherichia coli strain DH5α, according to standard procedures (Ausubel et al. 1988).

Flow cytometry: DNA content of yeast cells was determined as described, using a Becton Dickinson (San Jose, CA) FACSCalibur instrument (Pinto and Winston 2000).

Canavanine assay of ploidy: The ploidy status of yeast cells was assessed by monitoring the function of the *CAN1* gene (Schild, Ananthaswamy, and Mortimer 1981). Recessive *can1* mutations confer resistance to canavanine (Can), therefore the frequency of Can^r mutants is greater in haploid cells than in diploid cells, or among strains with two copies of chromosome V, the location of *CAN1*. Patches of yeast cells grown on YPD were replicated onto SC-Arg plates with or without canavanine and mutagenized by UV irradiation (300 ergs/mm^2) with a UV source (Stratalinker UV crosslikker, Stratagene, Agilene Technologies). Plates were incubated at 30°C for 4 days. Haploid cells that became Can^r would form papillae on plates containing canavanine after exposure to UV irradiation. Diploid cells would remain Can^s.

Yeast Two-hybrid analysis: The yeast strain EGY48 was transformed with the bait plasmid pEG202 and the prey plasmid pJG4-5, both containing the genes of interest. Strains were plated

on SC-Leu+X-Gal media containing either glucose or galactose as the carbon source and incubated at 30°C for 2 days.

β-galactosidase assay: β-galactosidase activity was determined from liquid cultures using ONPG as substrate as previously described (Clontech Laboratories, Inc.).

Immunoprecipitation: Protein extracts were prepared by resuspending 50ml of exponentially growing cultures in 500μl of RIPA buffer (10mM Tris-HCl pH 8, 250mM LiCl, 0.5% NP-40, 0.5% DOC, 1mM EDTA, 1X Roche EDTA-free protease inhibitor cocktail). Primary antibody was added and the slurry was incubated at 4°C for 2 hours. 15μl of Protein A Dynabeads (Dynal, Great Neck, NY) was added to the reaction and incubated for 2 hours at 4°C. Beads were washed 5 times with 1ml RIPA buffer, resusupended in 20μl of 2x SDS loading buffer (100mM Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 0.2% bromophenol blue, 200mM DTT), and incubated in boiling water bath for 5 minutes. 10μl of the immunoprecipitate was loaded on 4-20% gradient polyacrylamide iGels (NuSep) for SDS-PAGE, transferred to PVDF membrane, and analyzed by Western blot.

Western blot: Extracts were prepared by resuspending 1.5ml of an exponentially growing culture in 200μl of Rapid Protein Extract Sample Buffer (60mM Tris-HCl pH 6.8, 6M urea, 2% SDS, 5% β-mercaptoethanol, 0.0025% bromophenol blue) and incubating in a boiling water bath for 5 minutes. Following centrifugation, 10ml of the supernatants were loaded onto a 4-20% gradient polyacrylamide iGel (Nusep) for SDS-PAGE. Proteins were transferred to PVDF membrane, and the membrane was blocked with 5% nonfat dry milk in TBST (150mM NaCl,

100mM Tris-HCl pH 8.0, 0.5% Tween-20). Primary and secondary antibodies were diluted in 5% nonfat dry milk in TBST, and blots were developed with chemiluminescent substrate (Millipore). Blots were imaged using a FluorChem 8900 (Alpha Innotech).

Chromatin Immunoprecipitation: ChIP was carried out as previously described (Kanta et al. 2006). ChIP results were quantified by resolving the PCR products on 1.5% agarose gels stained with ethidium bromide. Gels were imaged using a FluorChem 8900 (Alpha Innotech), and relative band intensity was determined using AlphaEase FC software. The following primers were used for PCR (Table 2): *CEN3* (oIP142, oIP143), *CEN1* (oIP140, oIP141), *CEN4* (oIP144, oIP145), *ENA1* (oIP193, oIP194), *TEL-VIR* (oIP150, oIP151), *PGK1* (oIP92, oIP93), and *HO* (oIP234, oIP235). The following primers were used for PCR walking away from *CEN3*: 5kb L (oIP204, oIP205), 2kb L (oIP206, oIP207), 1kb L (oIP208, oIP209), 0.5kb L (oIP210, oIP211), 0.25kb L (oIP212, oIP213), 0.25kb R (oIP214, oIP215), 0.5kb R (oIP216, oIP217), 1kb R (oIP218, oIP219), 2kb R (oIP220, oIP221), and 5kb R (oIP222, oIP223).

Fluorescent microscopy: Cells carrying GFP-tagged centromeres were imaged using an Axio Imager M1 (Zeiss). Ten focal slices of each image was obtained, and compiled into one image using ImageJ (NIH).

Table 1. List of yeast strains used in this study.

EGY48	*MATa his3 trp1 ura3 LexA*$_{op(x6)}$*-LEU2*
FY1333	*MATα leu2Δ0 ura3Δ0*
IPY247	*MATa/α leu2Δ1/leu2Δ1 ura3-52/ura3-52 his3Δ200/his3Δ200 TRP1/trp1Δ63 lys2Δ202/LYS2*
IPY466	*MATa leu2Δ0 ura3Δ0 BIR1-3xHA*
IPY461	*MATa leu2Δ0 ura3Δ0 HDA1-13xMYC::KanMX*
IPY475	*MATα leu2Δ0 ura3Δ0 trp1Δ63 HDA1-13xMYC::KanMX HDA2-3xFlag::KanMX HDA3-3xHA*
IPY476	*MATα leu2Δ0 ura3Δ0 HDA1-13xMYC::KanMX hda2-546-3xFlag::KanMX HDA3-3xHA*
IPY478	*MATa ura3Δ0 HDA1-13xMYC::KanMX HDA2-3xFlag::KanMX hda3-548::3xHA*
IPY481	*MATa leu2Δ0 ura3Δ0 hda1-646-13xMYC::KanMX HDA2-3xFlag::KanMX HDA3-3xHA*
IPY491	*MATα leu2Δ0 ura3Δ0 hda1Δ::URA3 HDA2-3xFlag::KanMX HDA3-3xHA*
IPY541	*MATa leu2Δ1 ura3-52 his3Δ200 trp1Δ63 (hta2-htb2)Δ::TRP1*
IPY543	*MATα leu2Δ1 ura3-52 his3Δ200 trp1Δ63 (hta2-htb2)Δ::TRP1 hda1Δ::HIS3*
IPY549	*MATa leu2Δ1 ura3-52 his3Δ200 trp1Δ63 (hta2-htb2)Δ::TRP1 hda1Δ::HIS3 hta1-300*
IPY550	*MATα leu2Δ1 ura3-52 his3Δ200 trp1Δ63 (hta2-htb2)Δ::TRP1 hta1-300*
IPY811	*MATa his3-11,15::lacI-GFP-HIS3 trp1-1::lacO*$_{256}$*–TRP1 leu2-3,112 ura3-1*
IPY979	*MATα leu2Δ0 or leu2-3,112, ura3Δ0 or ura3-1 trp1::lacO*$_{256}$*-TRP1 his3-11,15::lacI-GFP-HIS3 hda1Δ::KanMX*
IPY980	*MATα leu2Δ0 or leu2-3,112, ura3Δ0 or ura3-1 trp1::lacO*$_{256}$*-TRP1 his3-11,15::lacI-GFP-HIS3 ndc10-1*
IPY981	*MATα leu2Δ0 or leu2-3,112, ura3Δ0 or ura3-1 trp1::lacO*$_{256}$*-TRP1 his3-11,15::lacI-GFP-HIS3 hda1Δ::KanMX ndc10-1*
IPY631	*MATα leu2Δ0 ura3Δ0 DAM1-13xMYC::KanMX*
IPY1000	*MATa his3Δ200 lys2-801 CSE4-3xHA::URA3 HDA1-13xMYC::KanMX*
IPY943	*MATa ura3Δ0 HDA1-3xFLAG::KanMX DAM1-13xMYC::KanMX*
IPY1010	*MATa ura3Δ0 or ura3-52 leu2Δ1 or leu2-3,112 his3Δ200 dam1-1*
IPY1011	*MATa ura3Δ0 or ura3-52 leu2Δ1 or leu2-3,112 his3Δ200 dam1-1 hda1Δ::HIS3*

Table 2. Primers used in this study.

oIP-92	5’-CACACTCTTTTCTTCTAACCA-3’
oIP-93	5’-CTTCAAGTCCAAATCTTGGACAGAC-3’
oIP-140	5’-CTCGATTTGCATAAGTGTGCC-3’
oIP-141	5’-GTGCTTAAGAGTTCTGTACCAC-3’
oIP-142	5’-GATCAGCGCCAAACAATATGG-3’
oIP-143	5’-AACTTCCACCAGTAAACGTTTC-3’
oIP-144	5’-GCGCAAGCTTGCAAAAGGTCACATG-3’
oIP-145	5’-CGAATTCATTTTGGCCGCTCCTAGGTA-3’
oIP-150	5’-GCGTAACAAAGCCATAATGCCTCC-3’
oIP-151	5’-CTCGTTAGGATCACGTTCGAATCC-3’
oIP-193	5’-CACCTGACAGAAGAAAAAACAAGG-3’
oIP-194	5’-CACTTGATGAAGATATCTGCTT-3’
oIP-204	5’-GCGAACCCTTCTCCATTTGGCAAT-3’
oIP-205	5’-CCTCGAAGGCCATCAAGTAGAAAA-3’
oIP-206	5’-CCGAAGGCTGGTATGTGATTTGTT-3’
oIP-207	5’-GATGGGCCAAAATACTGGAATATCG-3’
oIP-208	5’-ACTGCTATTAAGCGCCACTT-3’
oIP-209	5’-TTCTAACCACTGTGTCATCCGT-3’
oIP-210	5’-CCGTATCATGGACGATTTCCTT-3’
oIP-211	5’-TTGTCAAGTTGCTCACTGTGATTT-3’
oIP-212	5’-CCATCCAATACCTTGATGAACTTTTC-3’
oIP-213	5’-CGCCATGCCATGTTTATGAA-3’
oIP-214	5’-CGTTTACTGGTGGAAGTTTTGCTC-3’
oIP-215	5’-GGGGCGGAAATTCATTTGAA-3’
oIP-216	5’-CAAATGAATTTCCGCCCCAT-3’
oIP-217	5’-CCAGTAGGTTTGTACTATAATGTGGGTG-3’
oIP-218	5’-ACGTGCATTAAATCTCACTGTCAC-3’
oIP-219	5’-TGCAGGTGCTATTTGACGACT-3’
oIP-220	5’-CGTCCAAACATGAAAGTGCTCCTT-3’
oIP-221	5’-CTGGCCTTCTTATCATACGTTGTC-3’
oIP-222	5’-GGAAAACGCATACCGCTAAAGAAG-3’
oIP-223	5’-CCGCTCCTTGTATTCTACCATTG-3’
oIP-226	5’-GAAGCCTATCAATAAGTGGA-3’
oIP-227	5’-CTTACTGTCCTACTACACCT-3’
oIP-234	5’-CATGATGAAGCGTTCTAAACGCAC-3’
oIP-235	5’-TAGCCGTGACGTTTGCGATGTCTT-3’

C. Results

Suppressor alleles of Hda complex are deficient in complex formation

Prior work in our lab led to the identification of truncated alleles of all three members of the Hda complex as suppressors of the increase-in-ploidy phenotype of *hta1-300* (Figure 1). To better understand the mechanism of this suppression, we carried out yeast two-hybrid experiments to study the binding among Hda complex members using both full-length and truncated proteins (Figure 2). The yeast two-hybrid strain EGY48 was transformed with a plasmid containing either *HDA2* or $hda2_{546}$ fused to the LexA DNA binding domain (bait, pEG202), and a second plasmid containing *HDA3*, $hda3_{548}$, *HDA1, or* $hda1_{646}$ fused to the B42 activation domain under the control of a *GAL4* promoter (prey, pJG4-5). A third plasmid (pSH18-34) was also present in these strains that contains the LexA operator sequence with β-galactosidase as a reporter gene. A positive interaction between the bait and the prey yields a complete transcription factor that can bind to the LexA operator sequence and drive transcription of β-galactosidase, which can easily be observed on media containing X-Gal. From this experiment, we observed that the truncated alleles have much lower binding activity, indicating that less or no Hda complex is being formed. One exception is $Hda3_{548}$, which shows strong binding to Hda2 and $Hda2_{546}$. This is likely due to the fact that Hda3 can activate the system on it's own without the need for an activation domain (data not shown). Therefore, endogenous Hda3 that is binding to the Hda2 or $Hda2_{546}$ bait could activate transcription of β-galactosidase and give a false positive.

These results were confirmed by β-galactosidase assays (Figure 3 and 4). Protein extracts were obtained from the two-hybrid strains and incubated with the chromogenic substrate ortho-nitrophenyl-β-galactoside (ONPG) to determine the levels of enzymatic activity. The two-hybrid

results were also confirmed by co-immunoprecipitation (Figure 5). Alleles corresponding to the original transposon insertion mutants obtained from the suppressor screen of the increase in ploidy phenotype of the histone *hta1-300* mutation were generated in their chromosomal locations and epitope-tagged with different epitopes to allow us to use commercially available antibodies for their identification. The truncated alleles, $hda1_{646}$, $hda2_{546}$, and $hda3_{548}$ were then used for pairwise combinations of immunoprecipitation and immunoblotting (Western blot) with antibodies that recognized their individual epitopes. . All strains were immunoprecipitated with anti-Flag antibodies, which recognized the wild-type or truncated form of Hda2, and blotted with the corresponding antibody against the wild type or truncated forms of Hda2 (Flag), Hda3 (HA) or Hda1 (Myc). Lane 2 shows that all three members of the complex; Hda1, Hda2, and Hda3, were pulled down by Hda2, as predicted by previous work (Wu, Carmen, et al. 2001) However, when the complex contained any of the truncated forms of the Hda subunits there was a significant decrease in the amount of protein associated with the complex (lanes 3-5), with minimal amounts of Hda3. In addition, $Hda1_{646}$ appears particularly unstable, and also critical for the stability of Hda3 (lane 5). This finding was surprising, since the deacetylase activity is considered to be associated with the N-terminus of Hda1 (Lee, Maskos, and Huber 2009), and the *hda1-646* allele only cleaves less than 9% of the Hda1 C-terminus. Since $Hda1_{646}$ appeared to be essential for the stability of the complex, we confirmed its behavior by comparing it with a strain that carries a deletion of *HDA1*. Lane 6 shows that in the absence of Hda1, Hda2 and Hda3 no longer form the complex. Therefore, we conclude that the truncated alleles of the Hda complex are deficient in complex formation.

Deletion of *HDA1* suppresses the increase-in-ploidy phenotype of histone H2A mutants.

Based on our finding that the *hda1*, *hda2* and *hda3* suppressors appear to be loss of function alleles deficient in forming the Hda complex, we created a double mutant strain carrying the histone *hta1-300* and *hda1Δ* alleles to test whether *hda1Δ* would suppress the increase-in-ploidy defect caused by *hta1-300*. Segregants from a cross containing the individual mutations as well as the double mutant were tested for ploidy by the canavanine test. As shown in Figure 6, the double mutant shows papillation consistent with a haploid strain, and similar to the suppression seen with the original *hda1* suppressor (Kanta et al. 2006). We confirmed these results by determining the DNA content of the strains using flow cytometry (Figure 7). The results show that the *hta1-300 hda1Δ* strain remains haploid, in contrast to the *hta1-300* strain that has become completely diploid.

***CEN* localization of the Hda complex is not affected by the cell cycle**

It has been shown that many kinetochore and centromeric proteins only bind to the *CEN* regions in G2/M phase (Dorn and Maddox 2012). Previous work from our lab has shown by chromatin immunoprecipitation that the Hda complex localizes to centromeric regions (Kanta et al. 2006), and that is active in deacetylating lysine 14 of histone H3 and lysine 16 of histone H2B in centromeric chromatin (Almutairi, Williamson and Pinto, unpublished). Here we build upon these studies by assaying whether the Hda complex has a cell-cycle specific *CEN* binding similar to kinetochore proteins by arresting cells in different phases of the cell cycle, and then used chromatin immunoprecipitation to assay Hda1 binding at *CEN3*. Cells were arrested in G1 phase using α-factor, in S phase using hydroxyurea, and in G2/M phase using nocodazole. We show that the Hda complex is present at the centromere in all phases of the cell cycle (Figure 8 and 9).

One possible explanation is that the Hda complex is always present at *CEN* regions in order establish a particular "chromatin environment" that is beneficial for genome integrity.

Pericentric localization of the Hda complex

It is widely accepted that *S. cerevisiae* has a "point centromere" consisting of only 125 bp of DNA that are sufficient for proper centromere function and kinetochore formation. Additionally, the pericentric chromatin on either side of the centromere forms an elastic loop consisting of roughly 15kb of DNA (reviewed in Bouck, Joglekar, and Bloom 2008). Using chromatin immunoprecipitation and PCR primers walking away from the centromere, we found that the Hda complex is present on every chromosomal locus that we assayed, spanning up to 5 Kb away on either side of the centromere (Figure 10 and 11). Further studies confirmed that the Hda complex is present at up to 20 Kb away from the centromere (data not shown). This is in clear contrast to the outer kinetochore protein Dam1, which is localized to the centromere only and not to the pericentric regions (Fig 12 and 13).

Interactions with kinetochore components

Because we have shown that the Hda complex is present and active at centromeric regions, and that deletion of this complex suppresses a chromosome segregation defect caused by mutations in histone H2A, we hypothesized that the Hda complex could be interacting with other components of the chromosome segregation machinery, namely the kinetochore. To test this hypothesis, we explored genetic interactions between the Hda complex and kinetochore components. We created strains carrying double mutations between *hda1Δ* and temperature sensitive alleles of *NDC10* and *DAM1*. Similar to other mutants defective in kinetochore

function, these alleles are also sensitive to the microtubule-depolymerizing drug benomyl. *NDC10* encodes the p110 subunit of the CBF3 complex, which binds to the CDEIII region of the centromere and is essential for kinetochore assembly (Goh and Kilmartin 1993; Lechner and Carbon 1991). Dam1 is an essential component of the DASH complex and is involved in kinetochore-microtubule attachments (Hofmann et al. 1998; Westermann et al. 2006). We tested growth of the single and double mutants at 26°C (permissive temperature for all strains) in YPD and YPD containing benomyl (Figure 14). At 26°C in YPD, the *hda1Δ ndc10-1* and *hda1Δ dam1-1* double mutants showed no synthetic interactions. In YPD containing 10μg/ml of benomyl, the *ndc10-1* and *dam1-1* single mutants showed decreased viability that was partially suppressed by combining the mutations with *hda1Δ*. Additionally, *hda1Δ* was also able to suppress the temperature sensitivity of *ndc10-1* and *dam1-1,* as seen by the *hda1Δ ndc10-1* double mutant strain at 29.5^{o}C and the *hda1Δ dam1-1* double mutant at 34°C.

Upon discovering the genetic interactions between the Hda complex and components of the kinetochore, we asked if there was also physical interactions. Strains were created carrying epitope-tagged *HDA1* in combination with either epitope-tagged *NDC10* or *DAM1*. We show by co-immunoprecipitation that Hda1 interacts with Dam1 (Figure 15). However, we did not detect a significant interaction with Ndc10 (data not shown). Together with the evidence from genetic interactions, this data strongly indicates that Hda1 has a role in kinetochore function.

***hda1Δ* suppresses *ndc10-1* mutant by restoring normal chromosome segregation**

To address the mechanism of how *hda1Δ* suppresses the benomyl sensitivity phenotype of the *ndc10-1* mutant, we used fluorescence microscopy with strains carrying GFP-tagged centromeres. These strains carry an array of 256 *lac* operators integrated at the *TRP1* locus,

which is approximately 12kb away from *CEN4*. Additionally, *lacI-GFP* is expressed from the *HIS3* locus (Straight et al. 1996). This results in a GFP signal very close to the centromere of chromosome IV and allows viewing centromere movement through the cell cycle. We created single mutants of *hda1Δ* and *ndc10-1* as well as the double mutant *hda1Δ ndc10-1* in the GFP-tagged centromere background. These strains were grown in liquid YPD cultures with a low concentration (5µg/ml) of nocodazole, which is a microtubule-depolymerizing drug similar to benomyl. Following incubation with the nocodazole for 2 hours, the cells were viewed under fluorescence microscopy to calculate the percentage of cells that had missegregated chromosomes. Due to the low dose of nocodazole, some cells continued dividing (cycling) normally. We considered missegregated chromosomes to be those that showed two distinct GFP foci that were misaligned. Cells that showed one distinct GFP focus with a large budded daughter cell were considered to be the normal response to the drug (Figure 16). Under these conditions, we found that the

ndc10-1 mutant had a 12% missegregation rate, while the *hda1Δ ndc10-1* double mutant had a 6% missegregation rate which is comparable to wild type and the *hda1Δ* single mutant (Figure 17).

Next we addressed the issue of viability concerning these mutants in response to nocodazole. Strains carrying single mutations of *hda1Δ* and *ndc10-1* as well as the double mutant *hda1Δ ndc10-1* were grown to saturation overnight in YPD, and then diluted in YPD containing 50µg/ml of nocodazole and incubated for 6 hours. Following exposure to the drug, cells were washed and approximately 200 cells were plated on YPD. After incubation, colony-forming units (CFU's) were counted for viability. Our results show that the *ndc10-1* mutant has an 81% survival rate, and the double mutant *hda1Δ ndc10-1* has a 100% survival rate which is

comparable to wild type and the single mutant *hda1Δ* (Figure 18). Together, these results indicate that deletion of the Hda complex suppresses the chromosome segregation defects of *ndc10-1,* and this suppression restores normal viability. Therefore, we conclude that deacetylation by the Hda complex affects centromere-kinetochore function.

D. Discussion

Our previous work has shown that deletion of the Hda histone deacetylase complex can suppress a chromosome segregation defect caused by mutations in histone H2A. We have also showed by chromatin immunoprecipitation that the Hda complex is localized to centromeric regions of DNA (Kanta et al. 2006), and the complex is active in deacetylating lysine 14 of histone H3 and lysine 16 of histone H2B (Almutairi, Williamson and Pinto, unpublished). In this study, we used yeast two-hybrid and co-immunoprecipitation assays to investigate the interactions between subunits of the Hda complex. We found that the original suppressor alleles, which encode truncated Hda subunits, are defective in complex formation, and these alleles behave the same as the null mutants of the Hda complex with respect to their suppressor function of the increase-in-ploidy defect caused by the H2A mutations. These findings also imply that the C-teminus of each subunit, Hda1, Hda2, and Hda3, is essential for the formation of the complex.

Next, we wanted to know if the centromeric localization of the Hda complex is cell-cycle dependent. Due to the fact that we are implicating the Hda complex in chromosome segregation, we hypothesized that the Hda complex would be enriched at the centromere in G2/M. Here we show that the Hda complex is present at the centromere in all phases of the cell cycle in relatively equal amounts. One possible explanation is that the Hda complex is always present at *CEN* regions in order establish a particular "chromatin environment" that is beneficial for genome integrity. Looking further away from the centromere, the data shows that the Hda complex is present up to 20kb away from the centromere in either direction. This is in contrast to the outer kinetochore protein, Dam1, which is found only very close to the centromere, suggesting that the Hda complex is not behaving as a true kinetochore component. This extensive association of the Hda complex with pericentric chromatin might relate to the

specialized chromatin loop structures proposed to take place during mitosis when bioriented kinetochores are pulled toward the spindle poles (Yeh et al. 2008).

We also explored genetic interactions between the Hda complex and kinetochore components, finding that *hda1Δ* can suppress the temperature and benomyl sensitivities of both *ndc10-1* and *dam1-1*. This information is supported by co-immunoprecipitation data that shows an interaction between Hda1 and Dam1. We did not, however, find interactions by co-immunoprecipitation between Hda1 and Ndc10 or the centromeric H3 variant, Cse4 (data not shown). Dam1 has been shown to be phosphorylated by the Aurora kinase Ipl1 (Cheeseman et al. 2002) and methylated by Set1 (Zhang et al. 2005), and we speculate that it is possible that Dam1 could also be regulated by acetylation. In this context, acetylation by the Hda complex would modulate the activity of Dam1, or other potential kinetochore substrates, in addition to its role on histones. Even though Hda1 does not appear to behave as a true kinetochore protein, its localization to *CEN* regions is dependent on Ndc10. Since a functional kinetochore does not assemble in the absence of Ndc10, we can infer that a functional kinetochore is necessary for Hda1 activity at centromeric and pericentric regions.

To better understand the mechanism of how *hda1Δ* suppresses the benomyl sensitivity phenotype of *ndc10-1,* we used strains carrying a GFP-tagged centromere to follow chromosome segregation by fluorescence microscopy in these cells. We find that the *ndc10-1* single mutants have double the rate of missegregated chromosomes as the wild-type, and the double mutant *hda1Δ ndc10-1* has a rate of missegregation comparable to wild-type. We infer that *hda1Δ* is suppressing *ndc10-1* by restoring normal chromosome segregation, further strengthening our proposed role for the Hda complex in centromere-kinetochore function. Our current model proposes a role for histone deacetylation by the Hda complex at centromeric and pericentromeric

regions, as well as a function through deacetylation of potential kinetochore substrates (Figure 19).

A recent report has shown that hypoacetylation of H4K16 at centromeric regions is important for proper kinetochore function and accurate chromosome segregation (Choy et al. 2011). H4K16 is deacetylated by Sir2 and is not a known target of the Hda complex. Inhibiting HDAC's in human tumor cell lines blocks mitotic progression by altering pericentric chromatin and in turn interfering with kinetochore assembly (Robbins et al. 2005). In addition, deacetylation and methylation of H3K9 is involved in chromosome condensation during cell cycle progression in a variety of human tumor cell lines (Park et al. 2011). In human tumor cell lines, HDAC3 interacts with AKAP95 and HA95, which are Aurora kinase B-anchoring proteins. This interaction is required for deacetylation of H3 in mitosis and allows for optimal H3S10 phosphorylation by Aurora B. H3S10 phosphorylation leads to dissociation of HP1 from H3K9 and allows normal mitotic progression (Li et al. 2006). Work in *S. pombe* has shown that inhibiting deacetylation with the HDAC inhibitor trichostatin A or by mutations affects chromosome segregation by interfering with the anaphase promoting complex cyclosome (APC/C) (Kimata et al. 2008). These examples underscore the connection between deacetylation and chromosome segregation. They also emphasize the conservation of function among species. It is unclear at this point if the main centromeric role of the Hda complex in *Saccharomyces* is deacetylating histones or non-histone proteins with centromere function. Certainly these two functions of the Hda complex are not mutually exclusive. Our future studies will aim to clarify the function of deacetylation at the kinetochore.

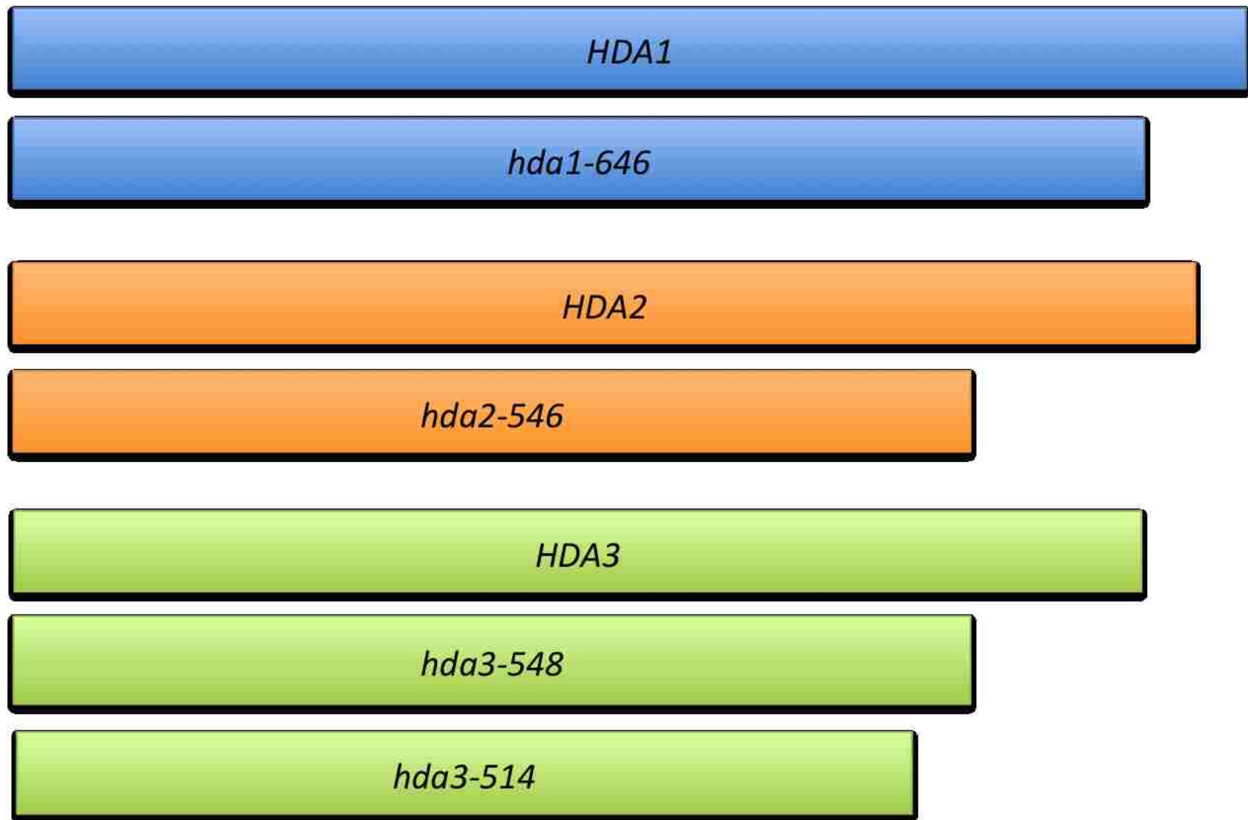

Figure 1. Structure of the *hda1*, *hda2* and *hda3* alleles isolated as suppressor of the *hta1-300* increase-in-ploidy phenotype. The isolated *alleles had1-646, hda2-546, hda3-548* and *hda1-514* carry transposon insertions followed immediately by stop codons, creating proteins with a C-terminal truncation. The bars representing the length of the alleles are shown to scale.

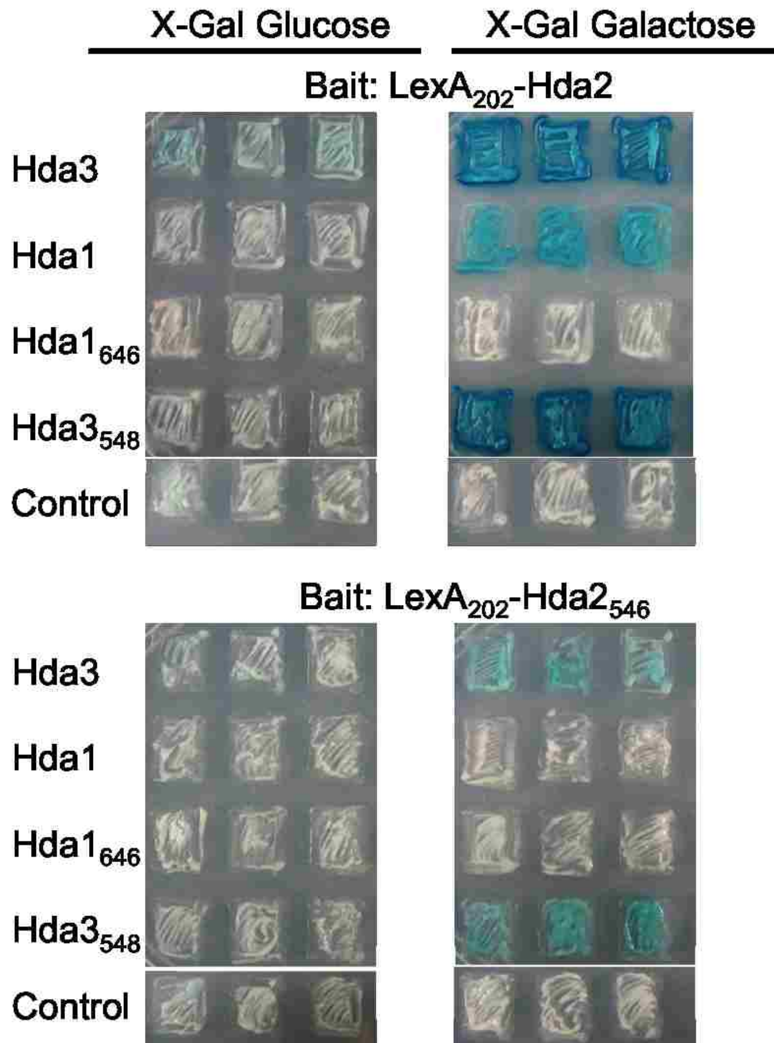

Figure 2. The yeast two-hybrid strain EGY48 was transformed with a plasmid containing either *HDA2* or *hda2*$_{546}$ fused to the LexA DNA binding domain (bait, pEG202), and a second plasmid containing *HDA3, hda3*$_{548}$*, HDA1, or hda1*$_{646}$ fused to the B42 activation domain under the control of a *GAL4* promoter (prey, pJG4-5). A third plasmid (pSH18-34) was also present in these strains that contains the LexA operator sequence with β-galactosidase as a reporter gene. Strains were grown on X-Gal media containing either glucose or galactose and incubated at 30°C for 2 days.

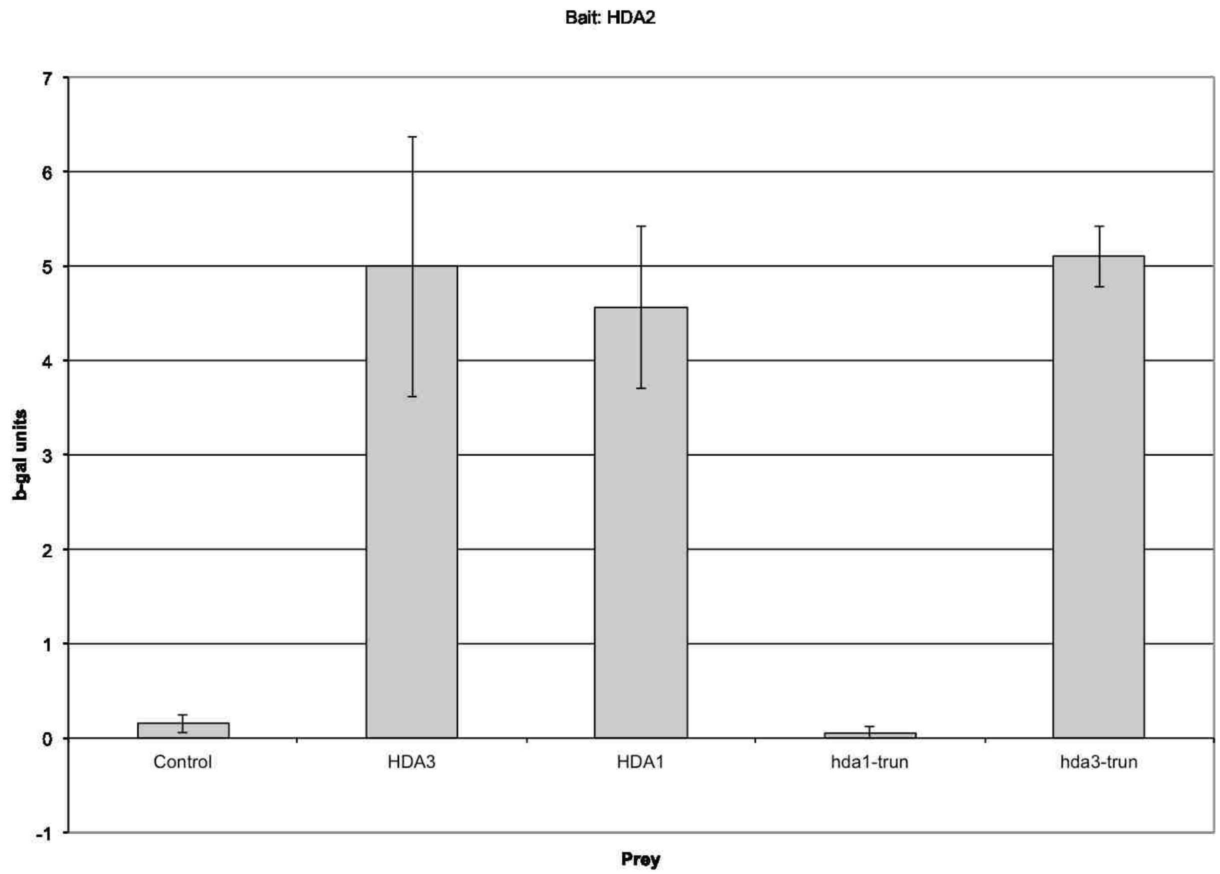

Figure 3. β-galactosidase assay in correspondence with Figure 2.

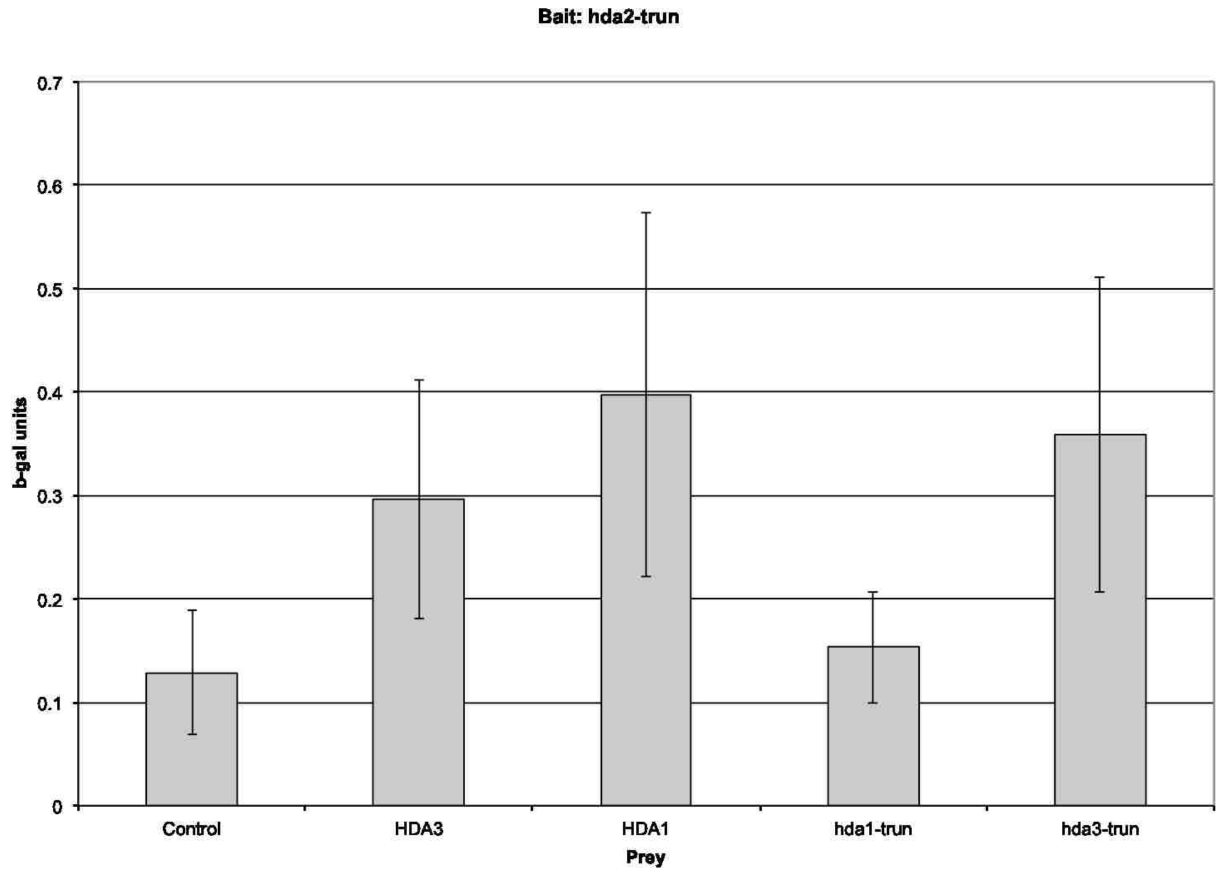

Figure 4. β-galactosidase assay in correspondence with Figure 2.

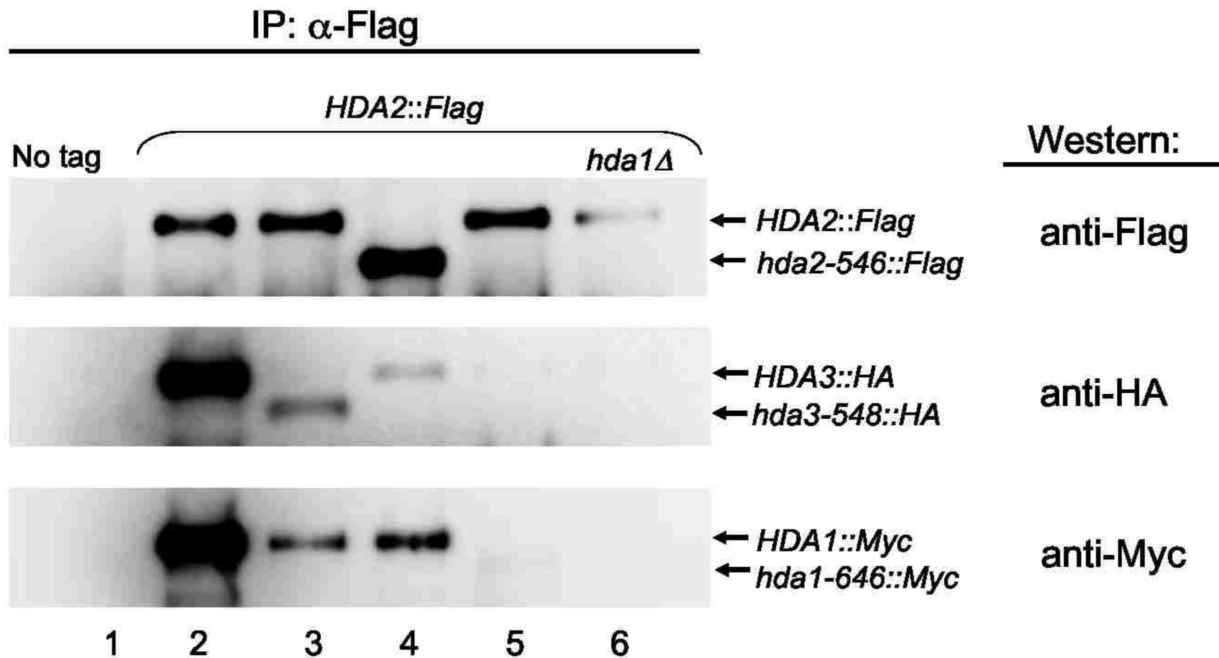

Figure 5. Interactions among the Hda complex subunits in strains carrying the *hda1-646*, *hda2-546*, and *hda3-548* suppressor alleles. Co-immunoprecipitation experiments were carried out with yeast extracts from untagged (lane1, FY1333) or epitope-tagged strains (lanes 2-6, IPY475, IPY478, IPY476, IPY481, IPY491). Relevant genotypes are indicated. Lane 1, untagged. Lane 2, wild *type HDA1-Myc HDA2-Flag HDA3-HA*. Lane 3, *HDA1-Myc HDA2-Flag* $hda3_{548}$*-HA*. Lane 4, *HDA1-Myc* $hda2_{546}$*-Flag HDA3-HA*. Lane 5, $hda1_{646}$*-Myc HDA2-Flag HDA3-HA*. Lane 6, *hda1Δ HDA2-Flag HDA3-HA.*

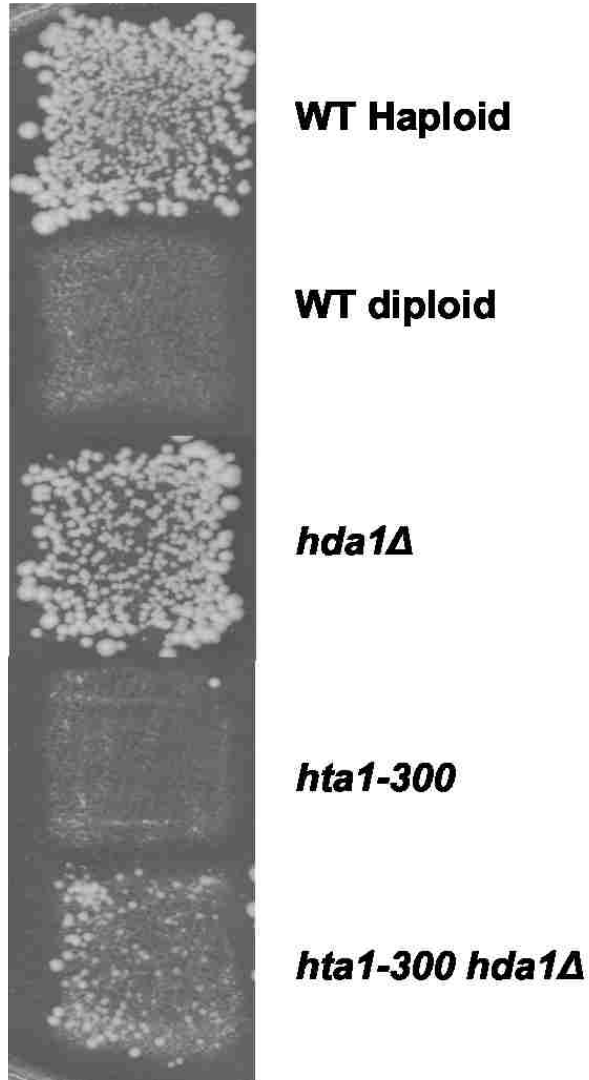

Figure 6. Canavanine assay for monitoring cell ploidy. Strains were patched on YPD and replica plated on SC-arg plus canavanine plates, exposed to UV irradiation and incubated at 30dC. Haploid *can1* cells that become Can^r forms colonies (papillae), while cell that are diploids for the *CAN1* locus remain Can^s. Strains are wild type haploid (IPY541), wild type diploid (IPY247), *hda1Δ* (IPY 543), *hta1-300* (IPY550), and *hta1-300 hda1Δ* (IPY549).

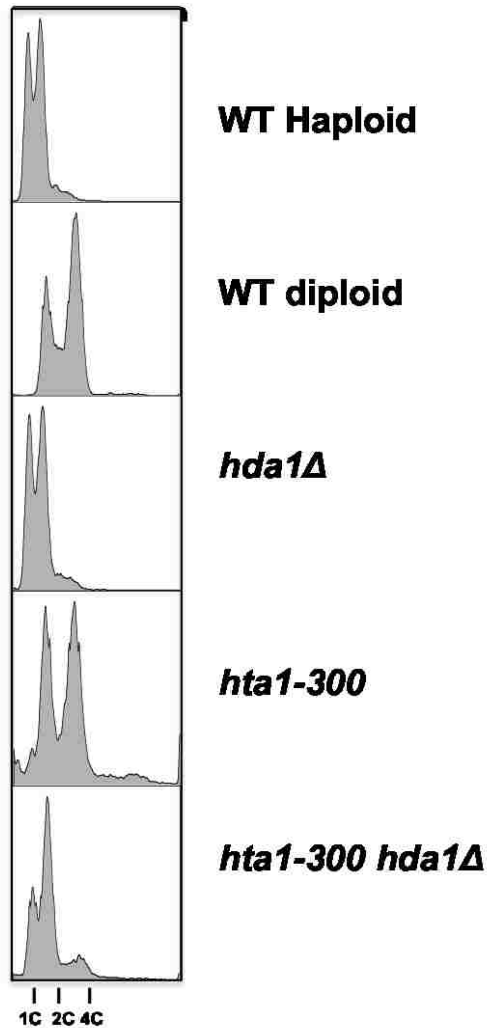

Figure 7. Ploidy determination by flow cytometry. DNA content represented by relative fluorescent of wild type haploid (IPY541), wild type diploid (IPY247), *hda1Δ* (IPY 543), *hta1-300* (IPY550), and *hta1-300 hda1Δ* (IPY549).

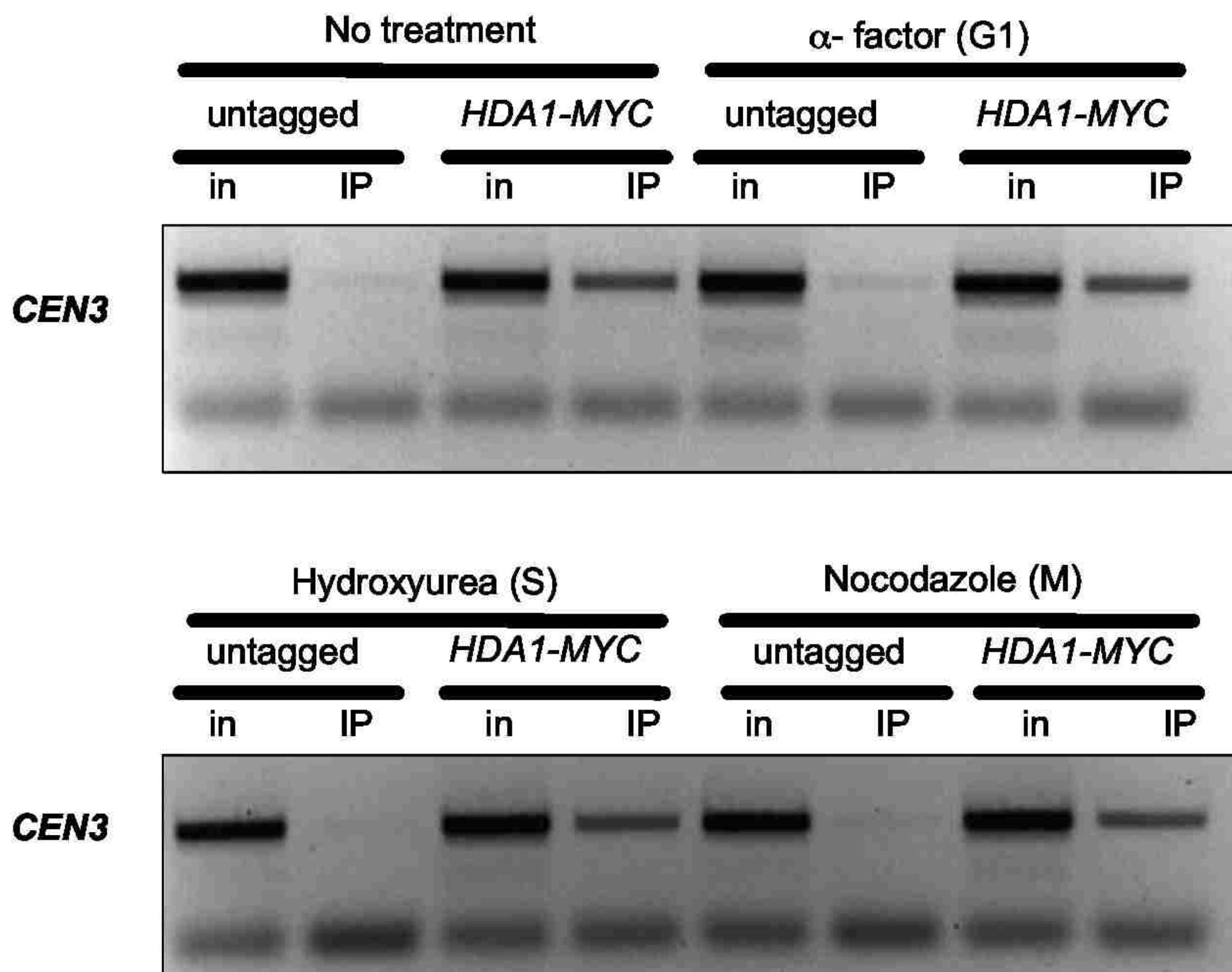

Figure 8. Hda1 associates with centromeric chromatin in all phases of the cell cycle. Wild-type strains containing untagged (IPY466) or Myc-epitope tagged (IPY466) Hda1 were arrested in G1 using alpha-factor, in S-phase with hydroxyurea, or in G2/M with nocodazole. Cells that were not arrested (no treatment) represent unsynchronized, exponentially growing cultures. Formaldehyde cross-linked chromatin was prepared, and extracts were immunoprecipitated with anti-Myc antibodies. PCR was performed on total input DNA (in) and immunoprecipitated DNA (IP) to visualize the core centromeric region of *CEN3*.

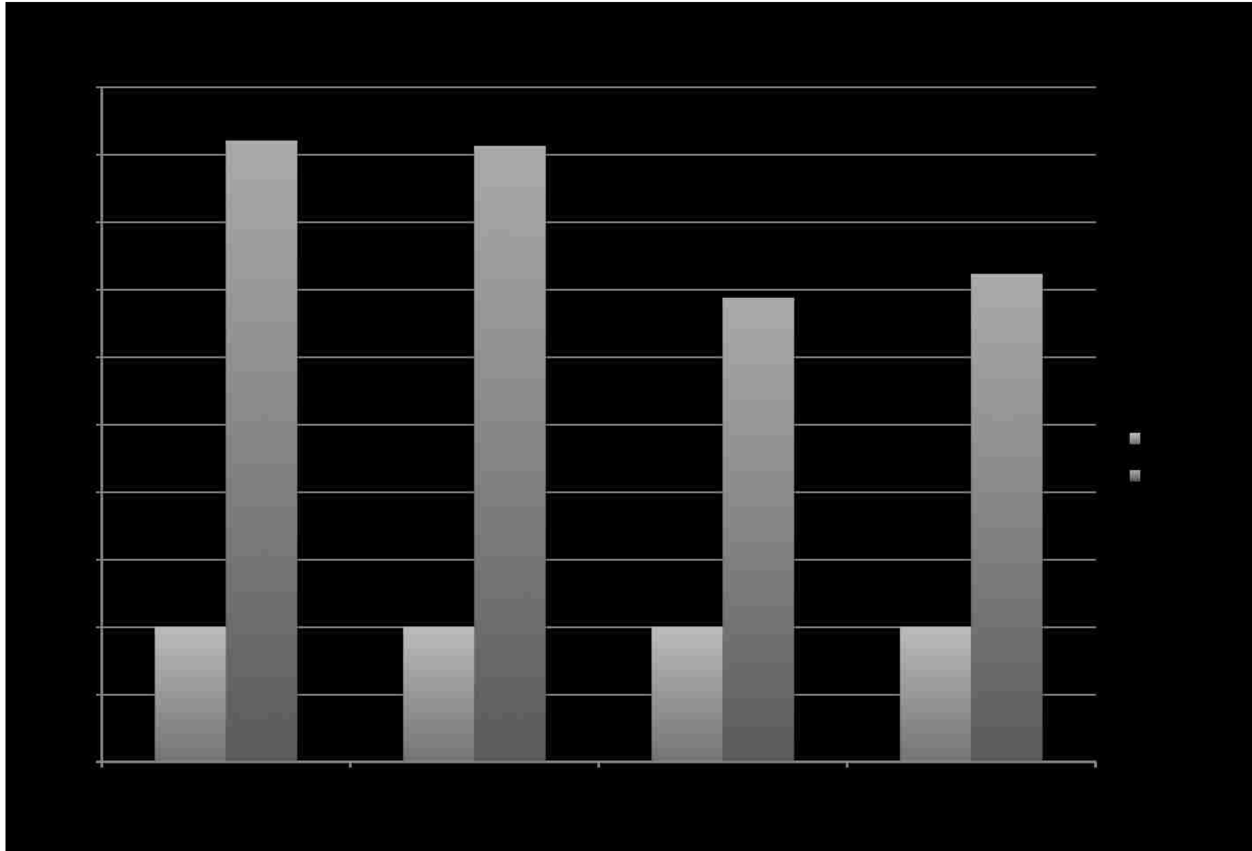

Figure 9. Graph showing relative chromatin immunoprecipitation of Hda1 at *CEN3* in different phases of the cell cycle.

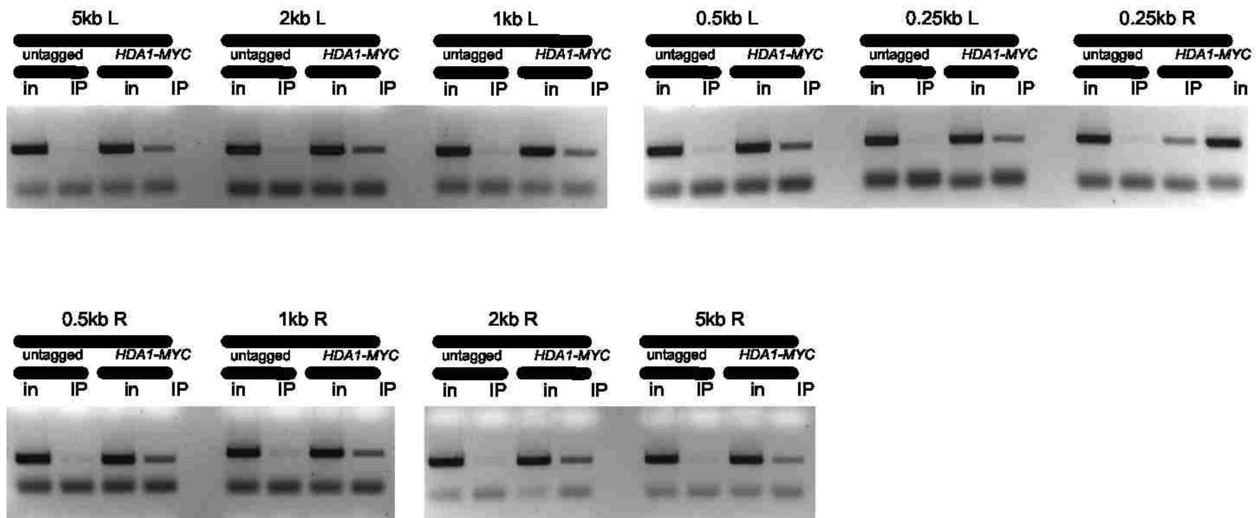

Figure 10. Pericentric localization of Hda1. Formaldehyde cross-linked chromatin was prepared from wild type strains that were untagged (IPY466) or Myc-epitope tagged Hda1 (IPY461). Extracts were immunopreciptated with anti-Myc antibodies. PCR was performed on total input DNA (in) and immunoprecipitated DNA (IP) using primer walking up to 5kb right (R) or left (L) of *CEN3*.

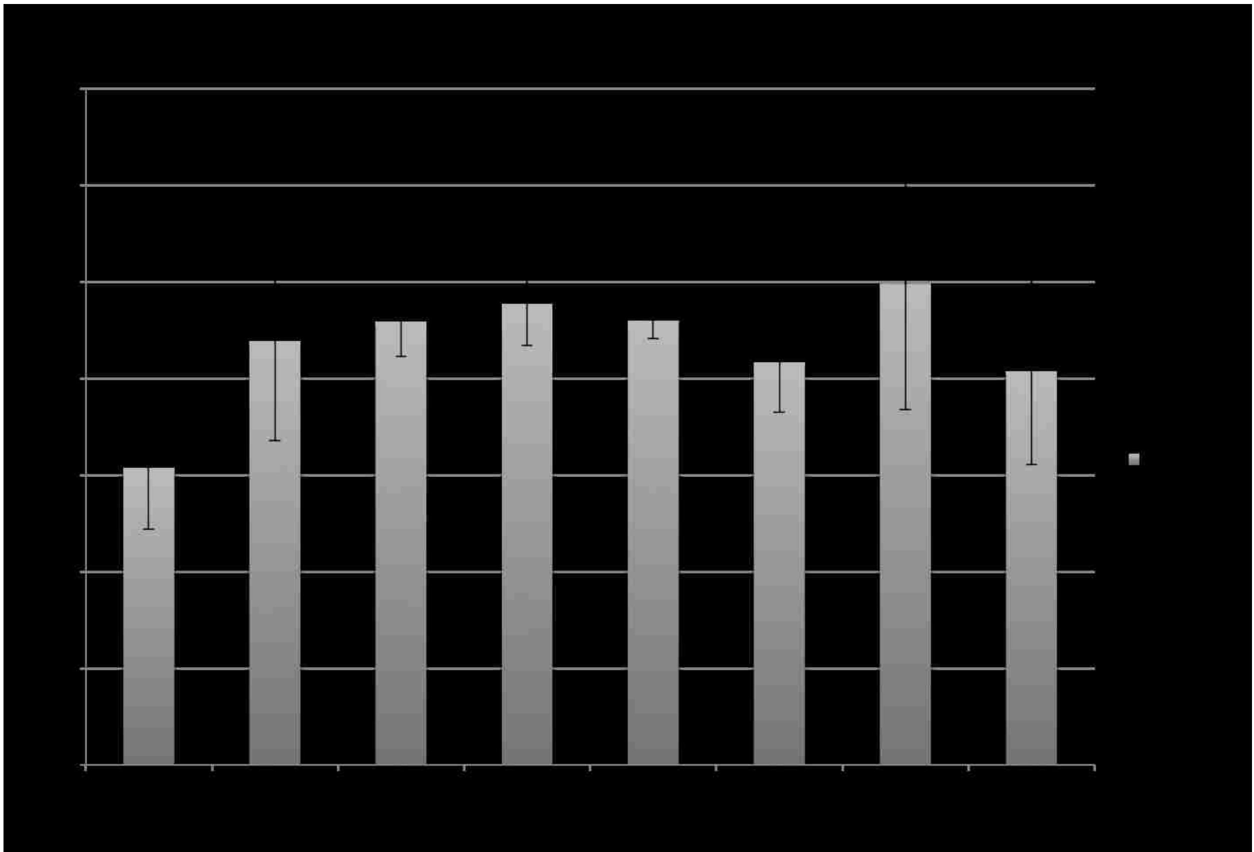

Figure 11. Graph showing relative chromatin immunoprecipitation of Hda1 at 5kb left and right of *CEN3*.

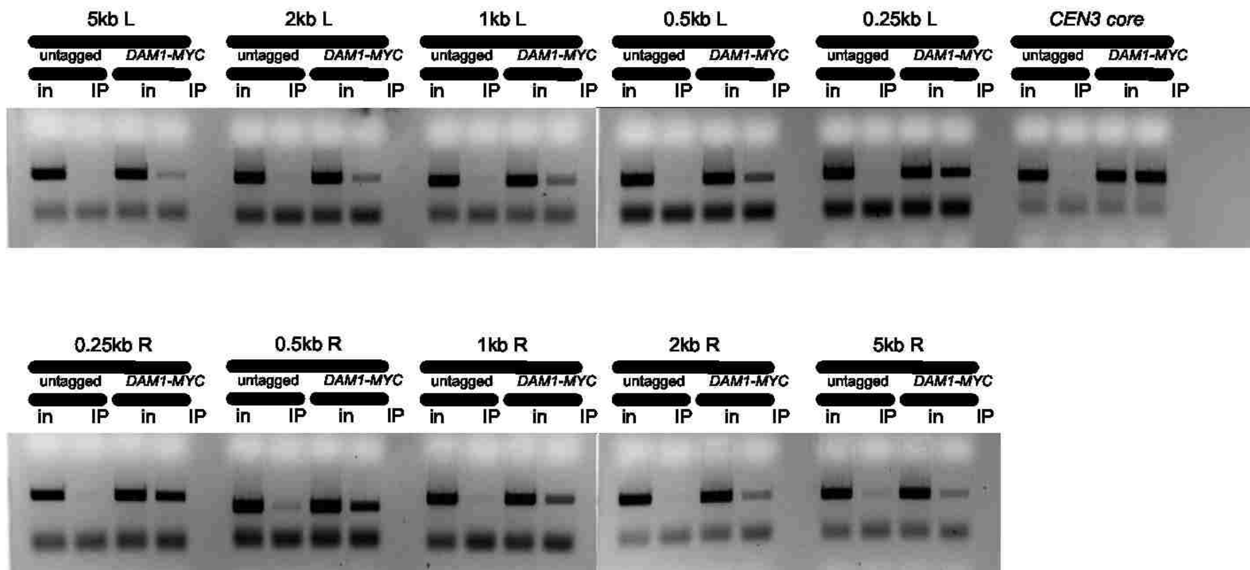

Figure 12. Pericentric localization of Dam1. Formaldehyde cross-linked chromatin was prepared from wild type strains that were untagged (FY1333) or Myc-epitope tagged Dam1 (IPY631). Extracts were immunopreciptated with anti-Myc antibodies. PCR was performed on total input DNA (in) and immunoprecipitated DNA (IP) using primer walking up to 5kb right (R) or left (L) of *CEN3*.

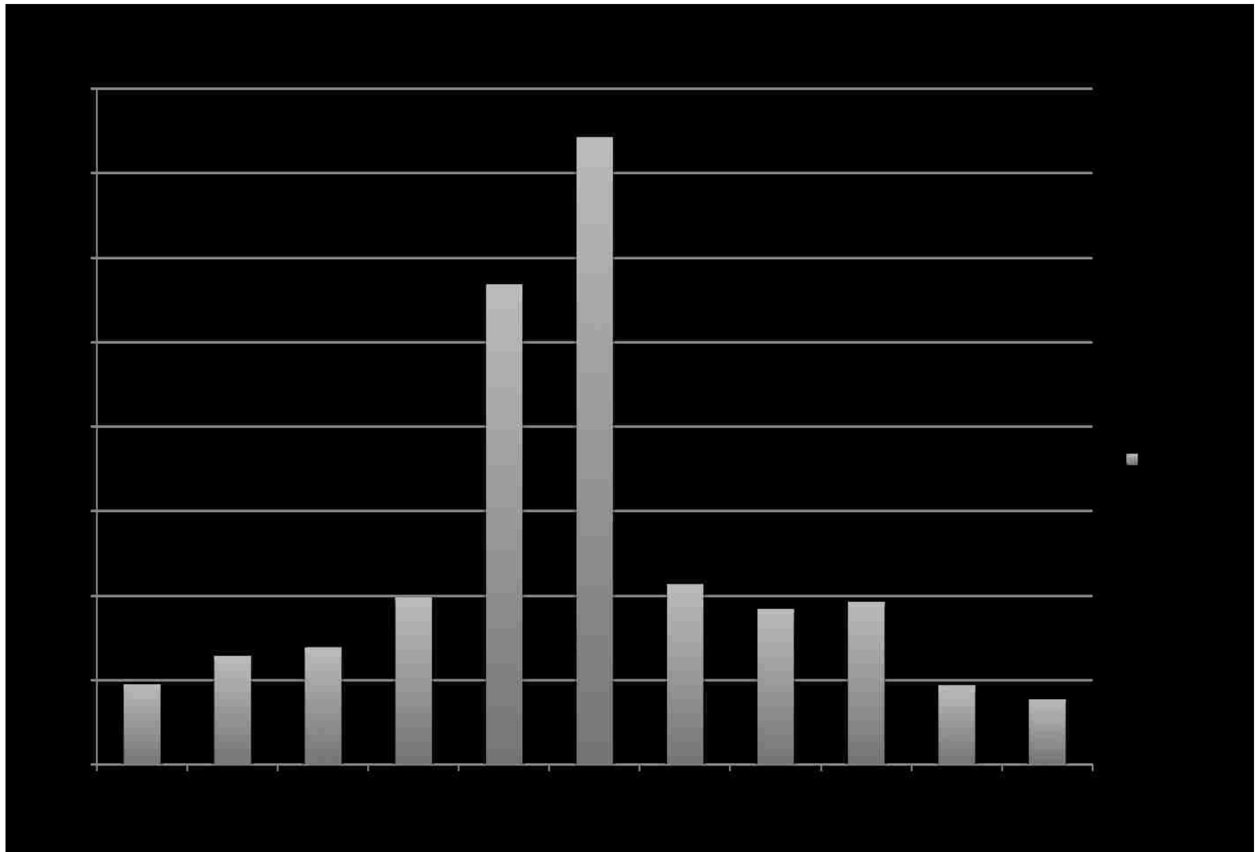

Figure 13. Graph showing relative chromatin immunoprecipitation of Dam1 at 5kb left and right of *CEN3*.

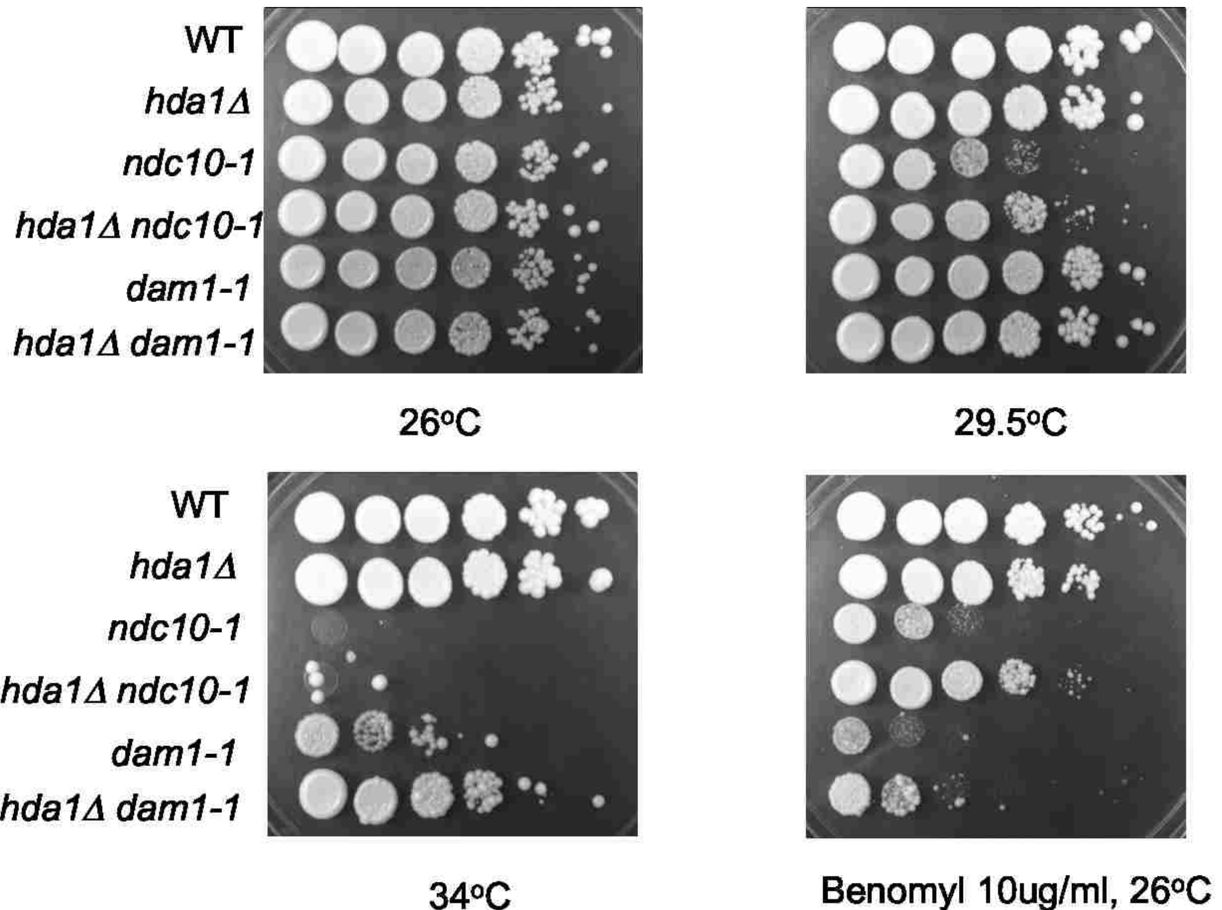

Figure 14. Interactions between Hda1 and kinetochore components. Suppression of *ndc10-1* and *dam1-1* by *hda1Δ*. Double mutants were generated by crosses between *hda1Δ* strains and kinetochore mutants. Serial dilutions (108–10^3cells/ml) were spotted (4μl) onto YPD or benomyl plates and incubated at the indicated temperatures for 2 days. The genotypes correspond to the following strains: wild-type (IPY811), *hda1Δ* (IP979), *ndc10-1* (IPY980), *hda1Δ ndc10-1* (IPY981), *dam1-1* (IPY1010), and *hda1Δ dam1-1* (IPY1011).

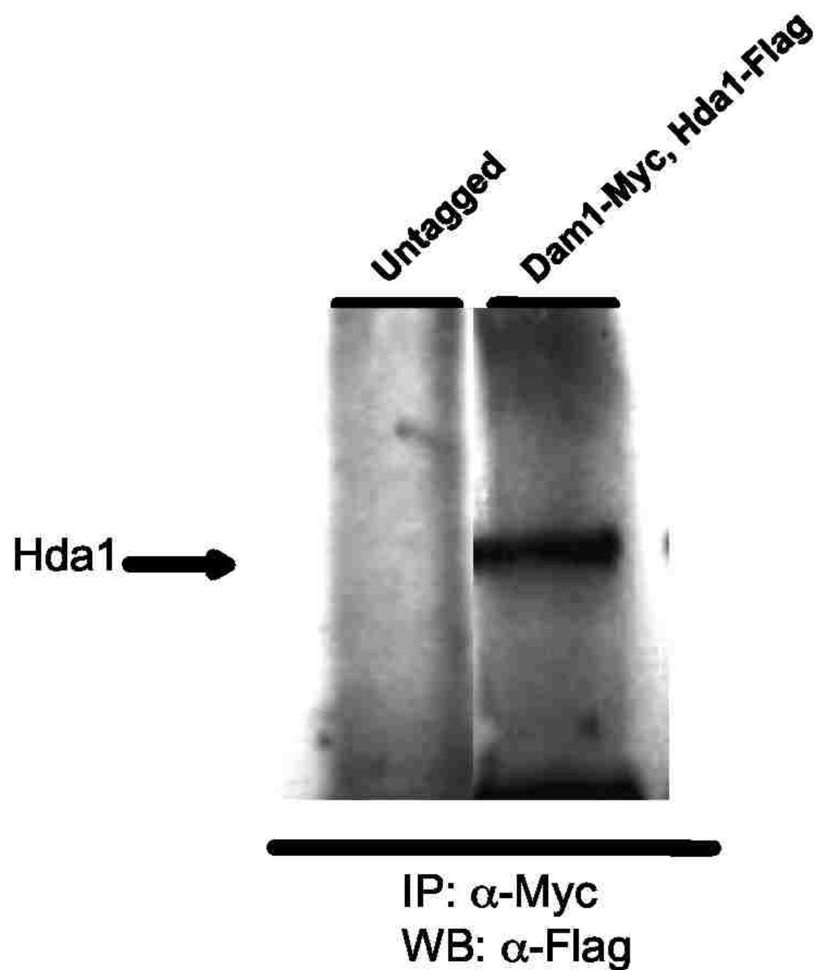

Figure 15. Co-immunoprecipitation interaction between Hda1 and Dam1. Protein extracts from wild-type strains untagged (FY1333) or double tagged *DAM1-MYC HDA1-FLAG* (IPY943) were immunoprecipitated with anti-Myc antibodies, separated by SDS-PAGE, followed by western blotting with anti-Flag antibodies.

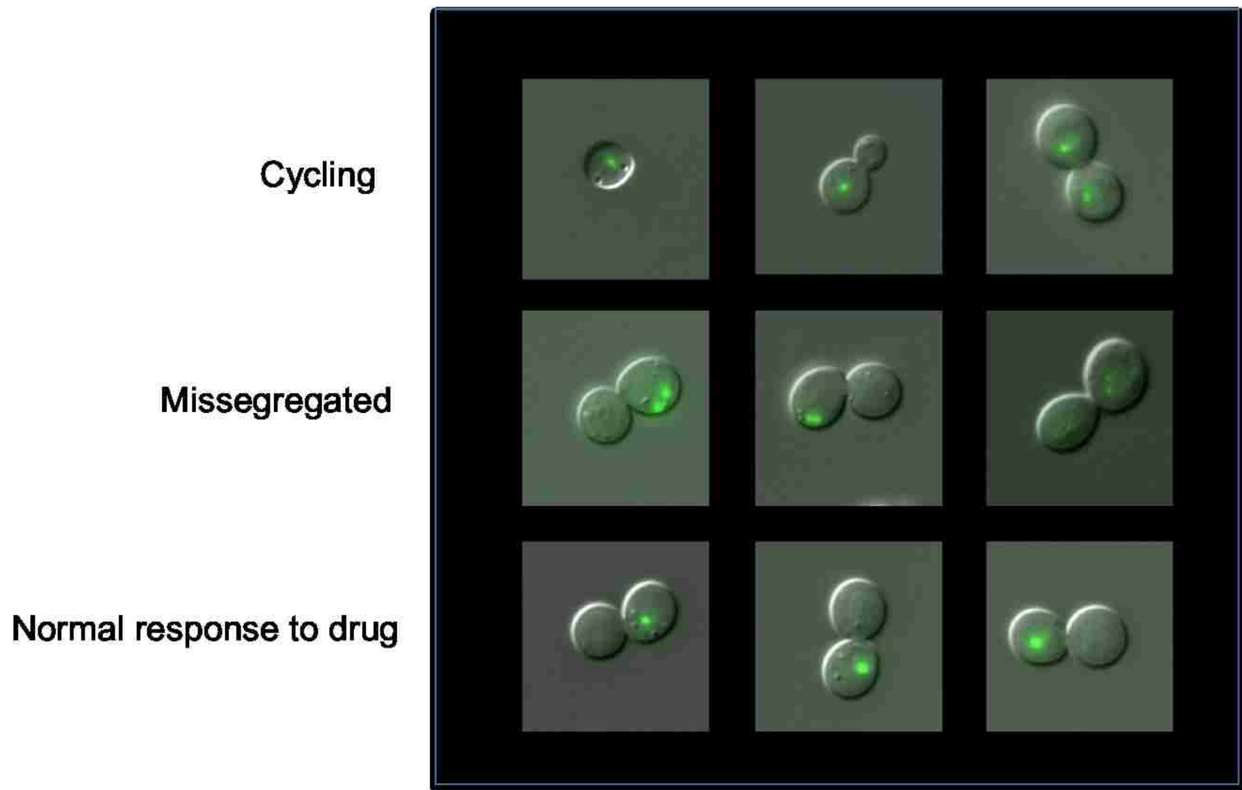

Figure 16. Diagram of the categories that cells were grouped into for counting with fluorescence microscopy.

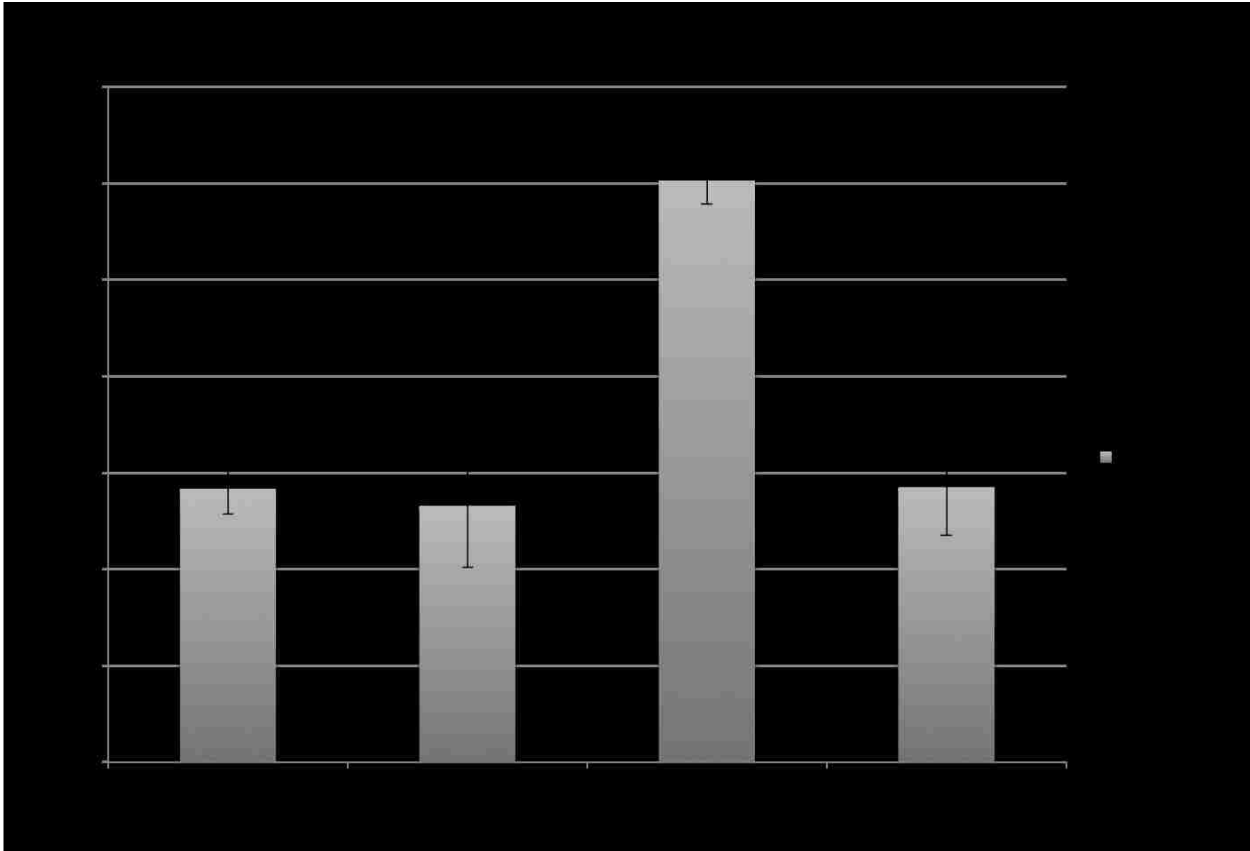

Figure 17. *hda1Δ* suppresses a chromosome segregation defect caused by *ndc10-1.* Strains were created with GFP-tagged *CEN4* to view chromosome segregation *in vivo.* Wild-type (IPY811), *hda1Δ* (IPY979), *ndc10-1* (IPY980), and *hda1Δ ndc10-1* (IPY981) were grown to mid-log phase and then viewed using fluorescence microscopy. 200 cells were counted and placed into one of the categories from Figure 16. The error bars indicate standard error from three independent experiments.

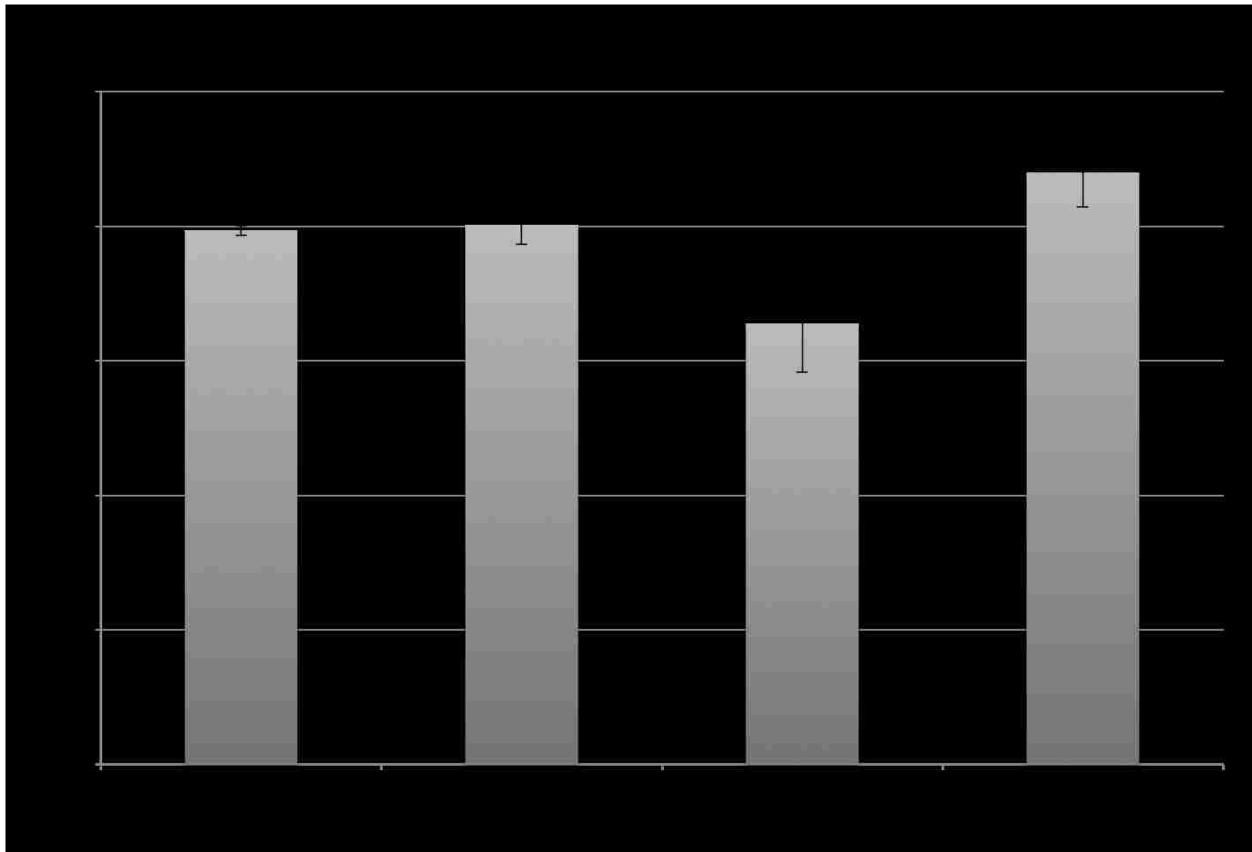

Figure 18. Recovery from benomyl assay. Wild-type (IPY811), *hda1Δ* (IPY979), *ndc10-1* (IPY980), and *hda1Δ ndc10-1* (IPY981) strains were grown to mid-log and then incubated with 50μg/ml benomyl for 6 hours. Following incubation, cells were counted and approximately 200 cells of each strain were plated on YPD plates and allowed to grow at the permissive (26°C) for 2 days followed by counting viable colony forming units (CFU's). Error bars indicate standard error from three independent experiments.

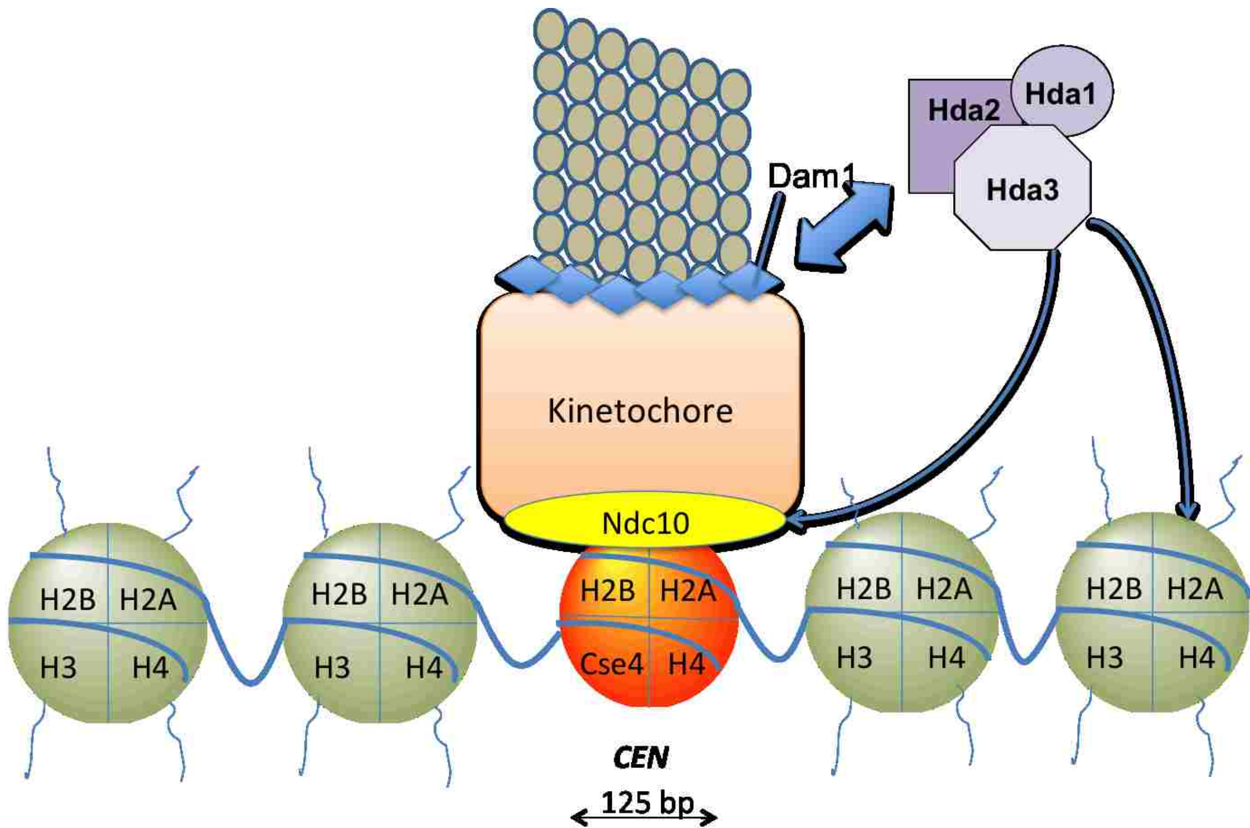

Figure 19. Schematic representing the yeast centromeric region with interactions between the Hda complex, chromatin, and kinetochore components.

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IV. INTERACTIONS BETWEEN THE HDA HISTONE DEACEYTLASE COMPLEX AND KINETOCHORE COMPONENTS

A. Introduction

During mitosis, cells undergo a highly regulated and precisely timed set of events to ensure that both daughter cells accurately inherit equal genetic material. Eukaryotic DNA is packaged into cells in the form of chromatin, a dynamic structure consisting of DNA, histone proteins, and non-histone proteins. The functional repeating unit of chromatin, the nucleosome, consists of an octamer of two each of the four core histones wrapped around twice by approximately 147bp of DNA (Luger et al. 1997; White, Suto, and Luger 2001; van Holde 1988). Because histones are so intimately associated with DNA, they participate in every cellular process involving DNA including replication, transcription, DNA damage repair, and chromosome segregation (reviewed in Williamson and Pinto 2012).

Also crucial to accurate chromosome segregation is the kinetochore, a large multi-subunit protein complex that assembles on each chromosome at the centromere and facilitates the binding of microtubules during metaphase. The binding of microtubules to kinetochores is monitored by the spindle assembly checkpoint (SAC). The SAC ensures that all kinetochores are bound to microtubules in a bi-oriented fashion, and that proper tension is being placed on the chromosomes. When the requirements for the SAC are satisfied, cohesin proteins that hold sister chromatids together are degraded allowing the chromosomes to be segregated to opposite ends of the cell.

One protein complex involved in the SAC is the chromosomal passenger complex (CPC), which consists of the Ipl1 kinase (Aurora B), Sli15 (INCENP), Bir1 (Survivin), and Nbl1 (Borealin). The CPC is an essential complex that is conserved from yeast to humans, and is involved in ensuring kinetochore bi-orientation by promoting kinetochore-spindle reattachments until adequate tension is obtained. The CPC has a dynamic localization throughout mitosis,

localizing to kinetochores at the onset of mitosis and moving to the spindle midzone in late anaphase. While they have a common localization throughout the cell cycle, they are not always together as a complex containing all members. Subcomplexes of chromosomal passenger proteins exist that carry out specialized functions. Bir1 and Sli15 form a separate complex that is active in linking centromeres to spindle microtubules (Sandall et al. 2006) and also regulating septin dynamics during anaphase (Thomas and Kaplan 2007). A separate complex consisting of Sli15-Ipl1 has been shown to promote turnover of mono-attached kinetochores to ensure correct bi-oriented microtubule-kinetochore attachments (Tanaka et al. 2002). Lastly, a Bir1-Sli15-Ipl1 complex is present in mitotic cells, although information about its function is limited (Thomas and Kaplan 2007).

The chromosomal passenger proteins are not the only tension-sensing proteins in the cell. Some of the function of the CPC is shared by Sgo1, which was originally identified to protect the centromeric cohesin Rec8 during meiosis (Kitajima, Kawashima, and Watanabe 2004) and to sense tension between sister chromatids during mitosis (Indjeian, Stern, and Murray 2005). It has been reported that Sgo1 is recruited to centromeric and pericentromeric regions through interactions with H3 G44 (Luo et al. 2010) and H2A S121 (S. A. Kawashima et al. 2010), and that its localization is dependent on the kinase Bub1 (Kitajima, Kawashima, and Watanabe 2004). Interestingly, overexpression of the CPC components Bir1 or Sli15 can suppress the increase-in-ploidy phenotype of *bub1Δ* (Storchová et al. 2011), indicating that Bub1-Sgo1 could potentially share parallel functions with the CPC.

Previous work identified two single amino acid substitution alleles of one of the genes that codes for histone H2A, *hta1-200* and *hta1-300,* which lead to increase in ploidy, chromosome loss, and cold sensitivity (Pinto and Winston 2000). A suppressor screen was

carried out to identify secondary mutations in other genes that could suppress the increase in ploidy phenotype of the H2A mutants. This screen led to the identification of the Hda histone deacetylase complex. Loss of function alleles, either partial or complete deletions, of any of the three members of this complex, *HDA1, HDA2,* and *HDA3,* are sufficient to suppress the chromosome segregation defects of the histone mutant (Kanta et al. 2006).

In this study, we explore interactions between the Hda complex, kinetochore components, and chromatin to shed light on the chromatin requirements for faithful chromosome segregation. We carried out a yeast two-hybrid library screen to search for proteins that interact with the Hda complex and identified Bir1, a component of the CPC. We also show genetic interactions between the Hda complex and the CPC and between chromatin and the CPC. To further study the role of Bir1*,* we generated a temperature sensitive allele. The allele that we isolated, *bir1-1,* is synthetically lethal in combination with *hta1-300*. This phenotype is unique to *BIR1,* as the other CPC components only show mild phenotypes in combination with the histone mutant. In a screen to identify high copy suppressors of *bir1-1,* we found *SGO1* (Shugoshin), a protein involved in the mitotic tension-sensing checkpoint. Interestingly, we find that *sgo1Δ* is also synthetically lethal with *hta1-300.* Our results suggest a network of interactions where centromeric chromatin and the CPC participate in the establishment and surveillance of kinetochore attachment and tension, functions that are modulated by the Hda histone deacetylase complex.

B. Materials and Methods

Yeast strains, genetic methods, growth, and media: The yeast strains used are listed in Table 1. Unless indicated, strains are isogenic to FY2, originally derived from S288C (Winston, Dollard, and Ricupero-Hovasse 1995). Strain construction and other genetic manipulations were carried out by standard methods (Guthrie and Fink 1991; Rose, Winston, and Hieter 1990). All yeast media, including YPD, synthetic minimal, omission media (SC), and media containing 5-fluoroorotic acid (5-FOA) were made as described previously (Rose, Winston, and Hieter 1990). Benomyl plates were made by adding benomyl (Sigma, St. Louis) to hot YPD to a final concentration of 10 μg/ml. Canavanine plates contain 60 μg/ml of canavanine sulfate (Sigma).

In general, genes were tagged with 13x*MYC* at the 3' end by PCR using plasmid GHB160 as template, with 3x*FLAG* at the 3' end by PCR using plasmid GHB342 as template, and with 3x*HA* at the 3' end by PCR using GHB159 as template as previously published (Longtine et al. 1998; Schneider et al. 1995). *BIR1* was tagged with 3x*FLAG* at the 3' end by PCR amplification of GHB342 using the primers oIP138 and oIP139. The PCR product was used to transform FY1333 and transformants were selected on YPD+G418. *hta1-300* was tagged with clonat resistance gene at the 3' end by PCR amplification of pAG25 using the primers oIP328 and oIP329.

Bacterial strains and plasmids: Plasmids were amplified and isolated from Escherichia coli strain DH5a, according to standard procedures (Ausubel et al. 1988).

Flow cytometry: DNA content of yeast cells was determined as described, using a Becton Dickinson (San Jose, CA) FACSCalibur instrument (Pinto and Winston 2000).

Immunoprecipitation: Protein extracts were prepared by resuspending 50ml of exponentially growing cultures in 500ml of RIPA buffer (10mM Tris-HCl pH 8, 250mM LiCl, 0.5% NP-40, 0.5% DOC, 1mM EDTA, 1X Roche EDTA-free protease inhibitor cocktail). Primary antibody was added and the slurry was incubated at 4°C for 2 hours. 15ml of Protein A Dynabeads (Dynal, Great Neck, NY) was added to the reaction and incubated for 2 hours at 4°C. Beads were washed 5 times with 1ml RIPA buffer, resusupended in 20ml of 2x SDS loading buffer (100mM Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 0.2% bromophenol blue, 200mM DTT), and incubated in boiling water bath for 5 minutes. 10ml of the immunoprecipitate was loaded on 4-20% gradient polyacrylamide iGels (NuSep) for SDS-PAGE, transferred to PVDF membrane, and analyzed by Western blot.

Western blot: Extracts were prepared by resuspending 1.5ml of an exponentially growing culture in 200ml of Rapid Protein Extract Sample Buffer (60mM Tris-HCl pH 6.8, 6M urea, 2% SDS, 5% b-mercaptoethanol, 0.0025% bromophenol blue) and incubating in a boiling water bath for 5 minutes. Following centrifugation, 10ml of the supernatants were loaded onto a 4-20% gradient polyacrylamide iGel (Nusep) for SDS-PAGE. Proteins were transferred to PVDF membrane, and the membrane was blocked with 5% nonfat dry milk in TBST (150mM NaCl, 100mM Tris-HCl pH 8.0, 0.5% Tween-20). Primary and secondary antibodies were diluted in 5% nonfat dry milk in TBST, and blots were developed with chemiluminescent substrate (Millipore). Blots were imaged using a FluorChem 8900 (Alpha Innotech).

Yeast Two-hybrid analysis: The yeast strain EGY48 was transformed with the bait plasmid pEG202 and the prey plasmid pJG4-5, both containing the genes of interest. Strains were plated

on SC-Leu+X-Gal media containing either glucose or galactose as the carbon source and incubated at 30°C for 2 days.

b-galactosidase assay: b-galactosidase activity was determined from liquid cultures using ONPG as substrate as previously described (Clontech Laboratories, Inc.).

Chromatin Immunoprecipitation: ChIP was carried out as previously described (Kanta et al. 2006). ChIP results were quantified by resolving the PCR products on 1.5% agarose gels stained with ethidium bromide. Gels were imaged using a FluorChem 8900 (Alpha Innotech), and relative band intensity was determined using AlphaEase FC software. The following primers were used for PCR: *CEN3* (oIP142, oIP143), *CEN1* (oIP140, oIP141), *CEN4* (oIP144, oIP145), *ENA1* (oIP193, oIP194), *TEL-VIR* (oIP150, oIP151), *PGK1* (oIP92, oIP93), and *HO* (oIP234, oIP235). The following primers were used for PCR walking away from *CEN3*: 5kb L (oIP204, oIP205), 2kb L (oIP206, oIP207), 1kb L (oIP208, oIP209), 0.5kb L (oIP210, oIP211), 0.25kb L (oIP212, oIP213), 0.25kb R (oIP214, oIP215), 0.5kb R (oIP216, oIP217), 1kb R (oIP218, oIP219), 2kb R (oIP220, oIP221), and 5kb R (oIP222, oIP223).

Generation of temperature sensitive mutant of *BIR1:* Temperature sensitive mutants were obtained as described (Muhlrad, Hunter, and Parker 1992). Conditions for mutagenic PCR using pIP92 as DNA template were as follows: 1x Platinum Taq Buffer (Invitrogen), 3mM $MgCl_2$, 100µM $MnCl_2$, 1µM each of the primers oIP226 and oIP227 (Table 2), biased dNTP's (100mM of three of the bases, 20mM of the biased base), and 5 units of Platinum Taq (Invitrogen). Four PCR reactions were set up, each one containing dNTP's with a different biased base (A, G, C,

T). The mutagenic PCR reactions were cleaned with a Qiagen MinElute PCR Purification kit and pooled together. pIP115 was gapped with SnaBI and SphI. The gapped plasmid and mutagenic PCR were used to co-transform the yeast strain x139-2A for *in vivo* recombination. Transformants were screened at 37°C for temperature sensitivity. Plasmid DNA was isolated and sequenced from transformants showing temperature sensitivity.

***bir1-1* high copy suppressor screen**:

IPY808 was transformed with a high-copy Yep13-based library (2μm-*LEU2*). Cells were plated on SC-Leu medium and incubated at 37°C and screened for transformants that were no longer temperature sensitive. Plasmid DNA was isolated sequenced from non-temperature sensitive transformants.

Table 1. Yeast strains used in this study

EGY48	*MATa his3 trp1 ura3* $LexA_{op(x6)}$*-LEU2*
FY1331	*MATa trp1Δ63 ura3Δ0*
FY1333	*MATα leu2Δ0 ura3Δ0*
FY604	*MATα his3Δ200 leu2Δ1 ura3-52 trp1Δ63 (hta2-htb2)Δ::TRP1*
FY1819	*MATα his3Δ200 leu2Δ1 lys2-128δ ura3-52 trp1Δ63 (hta2-htb2)Δ::TRP1 hta1-200 <pSAB6>*
IPY69	*MATa his3Δ200 leu2Δ1 ura3-52 trp1Δ63 (hta2-htb2)Δ::TRP1 hta1-300 <pSAB6>*
IPY75	*MATa/α his3Δ200/his3Δ200 leu2Δ1/leu2Δ1 ura3-52/ura3-52 trp1Δ63/trp1Δ63 (hta2-htb2)Δ::TRP1/(hta2-htb2)Δ::TRP1*
IPY171	*MATa his3Δ200 leu2Δ1 ura3-52*
IPY311	*MATα leu2Δ0 ura3Δ0 BIR1-HA*
IPY384	*MATα ura3Δ0 trp1Δ63 BIR1-HA HDA1-MYC::KanMx*
IPY387	*MATα ura3Δ0 leu2Δ0 BIR1-HA HDA3-FLAG::KanMx*
IPY394	*MATa ura3Δ0 BIR1-HA HDA2-FLAG::KanMx*
IPY497	*MATα ura3Δ0 hda1Δ::URA3 BIR1-HA HDA2-FLAG::KanMx*
IPY498	*MATα ura3Δ0 leu2Δ0 BIR1-FLAG::KanMx*
IPY713	*MATa trp1Δ63 lys2Δ202 his3Δ200 leu2Δ1 ura3-52 bir1Δ::HIS3 <pIP92>*
IPY748	*MATα trp1Δ63 leu2Δ0 ura3Δ0 SLI15-MYC::KanMX HDA1-FLAG::KanMx*
IPY753	*MATα ura3Δ0 leu2Δ0 IPL1-MYC::KanMx HDA1-FLAG::KanMx*
IPY808	*MATa trp1Δ63 leu2Δ1 lys2Δ202 ura3-52 his3Δ200 bir1Δ::HIS3 pIP116 (bir1-1-LEU2-CEN)*
IPY858	*MATa leu2Δ0 or leu2-3,112 ipl1-2 hda1Δ::HIS3*
IPY859	*MATα ura3-52 or ura3Δ0 leu2Δ0 or leu2-3,112 ipl1-2*
IPY862	*MATα lys2-801 leu2Δ0 ura3-52 or ura3Δ0 sli15-3 hda1Δ::URA3*
IPY864	*MATa ura3-52 or ura3Δ0 trp1Δ63 his3Δ200 sli15-3*
IPY950	*MATα leu2Δ1 or leu2-3,112 ura3-52 or ura3Δ0 sli15-3 (hta2-htb2)Δ::TRP1 HDA1-MYC::KanMx HDA2-FLAG::KanMx*
IPY969	*MATα leu2Δ1 ura3-52 hda1Δ::HIS3*
IPY985	*MATa leu2Δ1 trp1Δ63 ura3-52 his3Δ200 (hta2-htb2)Δ::TRP1 hta1-300::clonat <pSAB6>*
IPY987	*MATa ura3Δ0 or ura3-52 trp1Δ63 his3Δ200 leu2Δ1 or leu2-3,112 (hta2-htb2)Δ::TRP1 hta1-300::clonat ipl1-2 <pSAB6>*
IPY988	*MATα ura3Δ0 or ura3-52 trp1Δ63 his3Δ200 leu2Δ1 or leu2-3,112 (hta2-htb2)Δ::TRP1 hta1-300::clonat ipl1-2 <pSAB6>*
IPY990	*MATα ura3Δ0 or ura3-52 trp1Δ63 leu2Δ1 or leu2-3,112 (hta2-htb2)Δ::TRP1 hta1-300::clonat sli15-3 <pSAB6>*
IPY1015	*MATa ura3-53 or ura3Δ0 leu2Δ1 or leu2-3,112 trp1Δ63 his3Δ200 (hta2-htb2)Δ::TRP1 ipl1-2 pSAB6*
IPY1016	*MATα ura3-53 or ura3Δ0 leu2Δ1 or leu2-3,112 trp1Δ63 his3Δ200 (hta2-htb2)Δ::TRP1 ipl1-2 <pSAB6>*
Wx38-2a	*MATα ura3Δ0 or ura3-52 leu2Δ1 or leu2Δ0 trp1Δ63 his3Δ1 or his3Δ200 <pSAB6 and/or pIP156>*
Wx38-2b	*MATa ura3Δ0 or ura3-52 leu2Δ1 or leu2Δ0 trp1Δ63 his3Δ1 or his3Δ200*

	(hta2-htb2)Δ::TRP1 sgo1Δ::KanMx <pSAB6 and/or pIP156>
Wx38-2c	*MATα ura3Δ0 or ura3-52 leu2Δ1 or leu2Δ0 trp1Δ63 his3Δ1 or his3Δ200 hta1-300::clonat <pSAB6 and/or pIP156>*
Wx38-2d	*MATa ura3Δ0 or ura3-52 leu2Δ1 or leu2Δ0 trp1Δ63 his3Δ1 or his3Δ200 (hta2-htb2)Δ::TRP1 sgo1Δ::KanMX hta1-300::clonat <pSAB6 and/or pIP156>*
Wx38-5a	*MATα ura3Δ0 or ura3-52 leu2Δ1 or leu2Δ0 trp1Δ63 his3Δ1 or his3Δ200 hta1-300::clonat <pSAB6 and/or pIP156>*
Wx38-5b	*MATa ura3Δ0 or ura3-52 leu2Δ1 or leu2Δ0 trp1Δ63 his3Δ1 or his3Δ200 sgo1Δ::KanMX*
Wx38-5c	*MATα ura3Δ0 or ura3-52 leu2Δ1 or leu2Δ0 trp1Δ63 his3Δ1 or his3Δ200 (hta2-htb2)Δ::TRP1 sgo1Δ::KanMX hta1-300::clonat <pSAB6 and/or pIP156>*
Wx38-5d	*MATa ura3Δ0 or ura3-52 leu2Δ1 or leu2Δ0 trp1Δ63 his3Δ1 or his3Δ200 (hta2-htb2)Δ::TRP1*

Table 2. Primers used in this study

oIP-92	5’-CACACTCTTTTCTTCTAACCA-3’
oIP-93	5’-CTTCAAGTCCAAATCTTGGACAGAC-3’
oIP-138	5’-GATGACAATCAATTGATCGATATTGCTAAGAAAATGGGCATTTTAAGG GAACAAAAGCTGG-3’
oIP-139	5’-AAAACTACAAAAAATACAAACCTTTAGCCTGTTTATCAAATTAGTCTA TAGGGCGAATTGGG-3’
oIP-140	5’-CTCGATTTGCATAAGTGTGCC-3’
oIP-141	5’-GTGCTTAAGAGTTCTGTACCAC-3’
oIP-142	5’-GATCAGCGCCAAACAATATGG-3’
oIP-143	5’-AACTTCCACCAGTAAACGTTTC-3’
oIP-144	5’-GCGCAAGCTTGCAAAAGGTCACATG-3’
oIP-145	5’-CGAATTCATTTTGGCCGCTCCTAGGTA-3’
oIP-150	5’-GCGTAACAAAGCCATAATGCCTCC-3’
oIP-151	5’-CTCGTTAGGATCACGTTCGAATCC-3’
oIP-193	5’-CACCTGACAGAAGAAAAAACAAGG-3’
oIP-194	5’-CACTTGATGAAGATATCTGCTT-3’
oIP-204	5’-GCGAACCCTTCTCCATTTGGCAAT-3’
oIP-205	5’-CCTCGAAGGCCATCAAGTAGAAAA-3’
oIP-206	5’-CCGAAGGCTGGTATGTGATTTGTT-3’
oIP-207	5’-GATGGGCCAAAATACTGGAATATCG-3’
oIP-208	5’-ACTGCTATTAAGCGCCACTT-3’
oIP-209	5’-TTCTAACCACTGTGTCATCCGT-3’
oIP-210	5’-CCGTATCATGGACGATTTCCTT-3’
oIP-211	5’-TTGTCAAGTTGCTCACTGTGATTT-3’
oIP-212	5’-CCATCCAATACCTTGATGAACTTTTC-3’
oIP-213	5’-CGCCATGCCATGTTTATGAA-3’
oIP-214	5’-CGTTTACTGGTGGAAGTTTTGCTC-3’
oIP-215	5’-GGGGCGGAAATTCATTTGAA-3’
oIP-216	5’-CAAATGAATTTCCGCCCCAT-3’
oIP-217	5’-CCAGTAGGTTTGTACTATAATGTGGGTG-3’
oIP-218	5’-ACGTGCATTAAATCTCACTGTCAC-3’
oIP-219	5’-TGCAGGTGCTATTTGACGACT-3’
oIP-220	5’-CGTCCAAACATGAAAGTGCTCCTT-3’
oIP-221	5’-CTGGCCTTCTTATCATACGTTGTC-3’
oIP-222	5’-GGAAAACGCATACCGCTAAAGAAG-3’
oIP-223	5’-CCGCTCCTTGTATTCTACCATTG-3’
oIP-226	5’-GAAGCCTATCAATAAGTGGA-3’
oIP-227	5’-CTTACTGTCCTACTACACCT-3’
oIP-234	5’-CATGATGAAGCGTTCTAAACGCAC-3’
oIP-235	5’-TAGCCGTGACGTTTGCGATGTCTT-3’
oIP-328	5’-GGGTATATAATTAACGGTAACATATGTCATGCATGATATAAATCAGGG GCATGATGTGACT-3'
oIP-329	5'-ACTCAATCATGTTCAAGTAAGCAACAGTGCCCAATGAACCTAAGCTCG TTTTCGACACTGGAT-3’

C. Results

Hda complex interacts with chromosomal passenger complex

To further characterize the Hda complex and to better understand it's role in chromosome segregation, we carried out a yeast two-hybrid library screen (Ausubel et al. 1988; Guthrie and Fink 1991). Using Hda2 as the bait and *lacZ* as a reporter gene, we screened a yeast library for proteins that could interact *in vivo* by looking for β-galactosidase activity on plates containing X-gal. Interestingly, from this screen we found an interaction with Bir1, the yeast homolog of human survivin and a member of the chromosomal passenger complex (CPC). The library clone of *BIR1* did not contain the complete open reading frame (ORF), therefore we confirmed the interaction by cloning the full *BIR1* ORF into our two-hybrid system and observed a comparable interaction (Figure 1).

To determine whether Bir1 interacted with Hda2 alone or the complete Hda complex, co-immunoprecipitation experiments were carried out between Bir1 and each of the three subunits of the Hda complex. The results indicated that Bir1 interacts with all members of the Hda complex, Hda1, Hda2, and Hda3 (Almutairi, Williamson, and Pinto, unpublished).

Since Bir1 is also part of a protein complex, the CPC, we asked if the Hda complex was interacting on its own or as part of the complex. Using strains carrying *HDA1-FLAG* in combination with either *SLI15-MYC* or *IPL1-MYC*, we performed co-immunoprecipitation. We find that the interaction with the Hda complex is not specific to Bir1, as Sli15 and Ipl1 also show interactions by co-IP (Figure 2).

To better understand the functional significance of the interactions between the Hda complex and the CPC, we explored genetic interactions. We created single and double-mutant

strains of *hda1Δ* and the temperature sensitive alleles *ip1-2* or *sli15-3*. We tested growth of the single and double mutants at 26°C (permissive temperature for all strains) on YPD and YPD containing 10 μg/ml benomyl, a microtubule-depolymerizing drug. At 26°C on YPD, there were no synthetic interactions for the double mutants *hda1Δ sli15-3* or *hda1Δ ipl1-2* (Figure 3). In YPD containing benomyl, the *ipl1-2* single mutant shows decreased viability that is partially suppressed by combining the mutation with *hda1Δ*. The *hda1Δ ipl1-2* double mutant also shows greater viability than *ipl1-2* alone on YPD at 29°C and 30°C. We also show that *hda1Δ* can partially suppress the temperature sensitive phenotype of *sli15-3* on YPD at 34.5^{o}C and 37°C. These genetic interactions are also seen in strains carrying *ip1-2* or *sli15-3* in combination with a deletion in HDA2 or HDA3, the other two components of the Hda deacetylase complex (data not shown). Thus, removal of the deacetylase activity associated with the Hda complex partially compensates for the growth defects caused by the *ip1-2* and *sli15-3* mutations.

Centromeric localization of Bir1

Work in our lab has previously identified a novel role for the Hda complex at centromeric regions of DNA (Kanta et al. 2006) (Williamson and Pinto, unpublished). Since we have also found that the Hda complex is present at pericentric regions up to 20kb away from the centromere, we asked whether Bir1 has a broad pericentric localization similar to the Hda complex, or if it localizes only very close to the centromere similar to kinetochore proteins. Using chromatin immunoprecipitation followed by PCR using primers walking away from *CEN3,* we show that Bir1 is enriched at the core region of *CEN3* and diminishes in the pericentric regions (Figure 4 and 5). Thus, Bir1 behaves like other components of the kinetochore, associated only with the core centromeric region, and differs from the extensive

localization of the Hda complex. We conclude that the Hda complex does not function in the targeting of Bir1 to the centromere-kinetochore complex.

BIR1 **deletion analysis**

There has been some dispute in the literature regarding whether *BIR1* is an essential gene, with some groups reporting that it is essential and others reporting that is not essential. We addressed this discrepancy in our strain background (S288C) by creating a *BIR1/bir1Δ::HIS3* heterozygous diploid, then allowed that diploid to undergo sporulation and subsequent dissection and spore germination on YPD. Initially, the dissection showed 2:0 segregation, with the *BIR1* haploids alive and the *bir1Δ* haploids appearing to be dead. However, after longer incubation a subset of the *bir1Δ* segregants formed very small colonies. These survivors form heterogeneous colonies (Figure 6), appear very sick, and show dramatic aneuploidy by flow cytometric analysis (Figure 7). From this information, we conclude that *BIR1* is not essential in our strain background; however, the recovered *bir1Δ* strains are extremely sick and genomically unstable. Therefore, for the remainder of this work, we treat it as an essential gene.

In order to better study null phenotypes of *BIR1*, we generated a temperature sensitive allele by PCR mutagenesis (Figure 8). The resulting allele, *bir1-1,* has two point mutations at the C-terminus of the 954 amino acid protein, K887R and K950stop (Figure 9). To determine if one or both of these mutations were responsible for the temperature sensitivity we created strains carrying individual mutations, but neither retained the temperature sensitivity. Hence, both mutations are required in order for the resulting protein to be temperature sensitive.

Genetic interactions between CPC and histone H2A mutations

Having the *bir1-1* temperature sensitive allele allowed us to explore genetic interactions between the *bir1* mutant and the original histone H2A mutants that cause altered centromere chromatin structure and chromosome segregation defects (Pinto and Winston 2000). We crossed a strain carrying the histone H2A *hta1-300* allele covered with the wild-type plasmid (pSAB6) with a strain carrying the *bir1-1* allele in a plasmid and the chromosomal locus deleted (*bir1Δ::HIS3*). The germination efficiency of the dissected tetrads was very poor. Since Bir1 is known to play an essential role in meiotic chromosome segregation it is likely that the poor germination reflected some degree of insufficiency of wild type Bir1 in the diploid. Of over 50 dissected tetrads, none of the recovered meiotic segregants contained the double *bir1-1 hta1-300* mutant, although all other combinations were obtained. Thus, we conclude that there is synthetic lethality between *bir1-1* and *hta1-300*, indicating that the cell cannot tolerate the disturbances caused by the combination of the two loss of function alleles.

We also tested the combination of mutations in the other components of the CPC, the Ipl1 kinase and Sli15. Strains carrying the *hta1-300* and either of the temperature sensitive alleles *ipl1-2* or *sli15-3* were constructed. Both strains were viable at permissive temperatures and semi-permissive temperature (30°C), although some variation was observed in the *hta1-300 ipl1-2* strain (Figure 10). Neither of the double mutants showed a synthetic or suppressor phenotype on YPD medium containing 10µg/ml benomyl (data not shown). Therefore, the synthetic lethality is specific for the combination of *hta1-300* and *bir1-1*. However, we could not rule out a suppressor effect of the CPC mutants on the ploidy increase phenotype of the *hta1-300* allele. Therefore, we tested the double mutants *hta1-300 ipl1-2* and *hta1-300 sli15-3* for the papillation phenotype on canavanine containing plates and confirmed those phenotypes by measuring DNA

content by flow cytometry. The results of the canavanine assay show no suppression of the increase-in-ploidy phenotype (Figure 11). The *ipl1-2* and *hta1-300* strains are expected to diploidize but show some papillae, although reduced compared with the wild type, indicating that the strains are still in transition (after removal of the wild-type gene), as evidenced by the flow cytometry data (Figure 12). Since the *ipl1-2* and *hta1-300* share the increase-in-ploidy phenotype it was not expected to have a suppression effect, but rather a synthetic effect. In conclusion, we identified one novel phenotype, the synthetic lethality between *bir1-1* and *hta1-300.*

Analysis of high dosage expression of Bir1

Based on the synthetic lethality observed between *bir1-1* and *hta1-300* we hypothesized that the histone defect could result in deficient tension generated at microtubule-kinetochore attachment, a defect that would need the tension-sensing function of Bir1 for survival. If that were to be the case, then overexpression of Bir1 might overcome the histone defect and suppress the increase-in-ploidy phenotype. Thus, we set out to test the effect of Bir1 overexpression in the *hta1-300* mutant by introducing a 2μ plasmid that carries the wild type *BIR1* in high copy. The results of the canavanine assay shown in Figure 13 indicate that there is no suppression of the *hta1-300* ploidy defect by overexpression of Bir1. Although the ploidy phenotype may be related to inappropriate tension at the kinetochore, increasing Bir1 levels in the tested conditions is not sufficient no overcome the problem.

SGO1* is a high copy suppressor of *bir1-1

Because information on the role of *BIR1* has been limited to this point, we carried out a genetic screen to find other genes that can suppress the temperature sensitive phenotype of *bir1-1*. The goal was to discover genes that may clarify the function that Bir1 has in relation to the Hda deacetylase complex and centromeric chromatin. Cells carrying the *bir1-1* allele were transformed with a 2μ genomic library and checked for viability at the permissive temperature. From this screen we identified *SGO1* (shugoshin), a gene that has recently been implicated in sensing mitotic tension by acting through chromatin (Luo et al. 2010). We confirmed this interaction by constructing a yeast two-hybrid strain using Sgo1 as the bait combined with either full length Bir1, N-terminal half Bir1, or C-terminal half of Bir1 as the prey. These strains were used for β-galactosidase assays, and we find that the full length Bir1 interacts with Sgo1 (Figure 14). The N- and C-terminal halves of Bir1 showed interaction values comparable to the negative control, indicating that the intact Sgo1 protein is necessary to establish the interaction.

Because of its action through chromatin, we decided to explore genetic interactions between *sgo1Δ* and our original histone mutant, *hta1-300*. We created double mutant strains that carry *sgo1Δ hta1-300,* both covered by plasmids containing the *URA3* auxotrophic marker and wild type copies of *SGO1* and *HTA1,* respectively. After growth on selective media allowing the cells to lose these plasmids, and subsequent plating on media containing 5-Fluororotic acid (FOA) to counter select for the cells that lost the plasmid, *sgo1Δ* and *hta1-300* single mutants are able to grow, however the *sgo1Δ hta1-300* double mutant is inviable (Figure 15). This indicates that *sgo1Δ* is synthetic lethal with *hta1-300.*

High copy *BIR1* does not suppress *sgo1Δ hta1-300*

Based on the observations that the double mutants *bir1-1 hta1-300* and *sgo1Δ hta1-300* show synthetic lethality, *SGO1* overexpression can suppress the temperature sensitivity of *bir1-1*, and *BIR1* overexpression can suppress the benomyl sensitivity of *bub1Δ* (the kinase associated with Sgo1), we asked the question if overexpression of *BIR1* can suppress the synthetic lethality of *sgo1Δ hta1-300*. The double mutant *sgo1Δ hta1-300* strain covered with *URA3*-marked plasmids containing wild type copies of *SGO1* and *HTA1* was transformed with a *LEU2*-marked high-copy (2μ) plasmid containing *BIR1*. These strains were checked for viability on media containing 5-FOA, which allows selection of cells that have lost the *URA3*-marked (*SGO1* and *HTA1)* plasmids (Figure 16). Under the conditions that we tested, overexpression of *BIR1* does not suppress the synthetic lethality of *sgo1Δ hta1-300.*

Discussion

We have previously shown that a mutant of histone H2A that causes chromosome segregation defects can be suppressed by deletion of the Hda histone deacetylase complex (Kanta et al. 2006). In an attempt to further characterize the Hda complex and understand its novel role in chromosome segregation and centromeric function, we carried out a yeast two-hybrid screen to look for interacting proteins. From this screen we identified *BIR1,* and subsequently confirmed both physical and genetic interactions between the Hda complex and the CPC. Deletion of the Hda complex does not affect centromeric localization of Bir1. Since the Hda complex is not essential for cell viability, it is not unexpected that the Hda complex does not target Bir1 to centromeres. Additionally, we showed by chromatin immunoprecipitation that Bir1 is localized only to the core centromeric regions while the Hda complex is present up to 20 Kb away from the centromere in the pericentric regions. These results suggest that the interaction between Bir1 and the Hda1 complex may be transient, where Bir1 or other component of the CPC may the posttranslationally modified and the subject of deacetylation by the Hda complex.

To address a conflict in the literature regarding the issue of whether *BIR1* is an essential gene in *Saccharomyces cerevisiae*, we constructed the *bir1Δ* strain and showed that although *BIR1* is not essential strictly speaking, very few *bir1Δ* segregants of meiosis survived and formed viable colonies. However, these survivors are very sick, grow extremely slow, and have significant aneuploidy and in some cases polyploidy. These findings help to reconcile the differences observed by others in their analyses of *bir1Δ* strains. It is very likely that the extreme sickness we observed can become lethal in other strain backgrounds. Since the surviving *bir1Δ* were so sick and genomically unstable, we continued our analysis of *BIR1* by generating a

temperature sensitive mutant, *bir1-1*. This mutant contains two point mutations (K887R and K950stop) at the C-terminus of the protein. The positioning of these mutations is not surprising considering that only the final ~80 amino acids of Bir1 are required for viability (Widlund et al. 2006).

Our attempts to generate a *bir1-1 hta1-300* double mutant were not successful, and our results suggest that the yeast cell cannot tolerate the combination of both loss of function alleles. Interestingly, this synthetic lethality does not extend to the other members of the CPC. Double mutants of *ipl1-2 hta1-300* are slightly sicker than the single mutants and *sli15-3 hta1-300* shows no synthetic phenotype. We can infer that *BIR1* has a distinct role in chromatin dynamics that is separate from that of other members of the CPC, at least with respect to its interaction with chromatin.

Based on the synthetic lethality between *bir1-1* and *hta1-300*, we thought that overexpressing *BIR1* could potentially suppress the increase-in-ploidy phenotype of the *hta1-300* and *hta1-200* mutants. However, our experiment showed that an increased dosage of *BIR1* had no effect on the ploidy defects associated with other histone H2A mutants. Although the synthetic lethality suggests that Bir1 and chromatin have functions in the same pathway, namely the association of centromeres to the microtubules and the establishment of biorientation, the fact that Bir1 acts in combination with other CPC proteins may explain why the overexpression of Bir1 is not sufficient to suppress the histone H2A defects. Alternatively, the tension sensing function of Bir1 is required but not sufficient to compensate for the defects caused by the altered centromeric chromatin present in the histone mutants.

Because information on Bir1 is limited with respect to chromatin and chromatin modifiers, we sought to identify interactions with other proteins that could help us better

understand its role. Using the *bir1-1* allele that we generated, we carried out a high copy suppressor screen to look for other genes in high copy that could suppress the temperature sensitive phenotype. From this screen we found *SGO1,* and confirmed a physical interaction with Bir1 by two-hybrid analysis. Also from this screen, we identified *IPL1* and *SLI15*, the other components of the CPC. This is complementary to the finding that overexpression of *BIR1* or *SLI15* suppresses the benomyl sensitivity of *sgo1Δ* (Storchová et al. 2011).

It's conceivable that *SGO1* and *BIR1* share similar or partially overlapping functions in the cell, and providing the cell with more Sgo1 compensates for a less functional Bir1. Considering that Sgo1 has been implicated in sensing mitotic tension by acting through chromatin (Luo et al. 2010), we tested genetic interactions between *sgo1Δ* and *hta1-300* and found that the two mutations are synthetic lethal.

In *Schizosaccharomyces pombe*, which has two homologs of shugoshin (Sgo1 and Sgo2), Sgo2 is required for efficient targeting of the chromosomal passenger proteins to the centromere. Furthermore, both SpSgo2 and Bir1 are mutually necessary for their centromeric localization (Vanoosthuyse, Prykhozhij, and Hardwick 2007; Shigehiro A Kawashima et al. 2007). From this information, together with our results, we believe that Sgo1 and Bir1 possibly have overlapping functions in the tension-sensing machinery of the cell. It's possible that the overlapping function of Bir1 and Sgo1 could be as part of the same pathway, or as a parallel pathway that accomplishes a similar task. The fact that high copy *SGO1* can suppress *bir1-1* indicates that these overlapping functions are likely in parallel pathways.

Recently, it has been shown that Sgo1 acts in combination with the kinase Bub1 to phosphorylate H2A S121, and this affects tension at all kinetochores and pericentromeric regions (Haase et al. 2012). If Bir1 functions in a parallel pathway to Sgo1, then it is possible that Bir1

interacts with the aurora kinase Ipl1, similar to the Sgo1/Bub1 interaction, to control tension at another location. If Sgo1 recognizes a particular region of the nucleosome surface, as suggested by Luo et al. (2010), then Bir1 might interact with a different nucleosomal region.

Both of these pathways appear necessary for the survival of the histone mutants, since mutations in either *bir1* or *sgo1* make the H2A mutants inviable. It is likely that histone mutations that affect centromeric chromatin, like the H2A alleles, cause genomic instabilities as a result of microtubule misattachments that are sensed and partially alleviated by the functions of Bir1 and Sgo1. The involvement of the Hda1 histone deacetylase complex is still intriguing and presents an opportunity to further investigate the role acetylation-deacetylation at the centromere-microtubule interphase (Figure 17).

Figure 1. Bir1 interacts with Hda2 by two-hybrid. Two-hybrid strains (EGY48) were constructed by transforming with the bait plasmid containing Hda2 (pIP87) and either full length Bir1 (pIP100) or the Bir1 fragment obtained from the library (plasmid) screen as the prey. Strains were streaked on X-gal medium containing either glucose (control) or galactose as the carbon source and incubated at 30°C for two days.

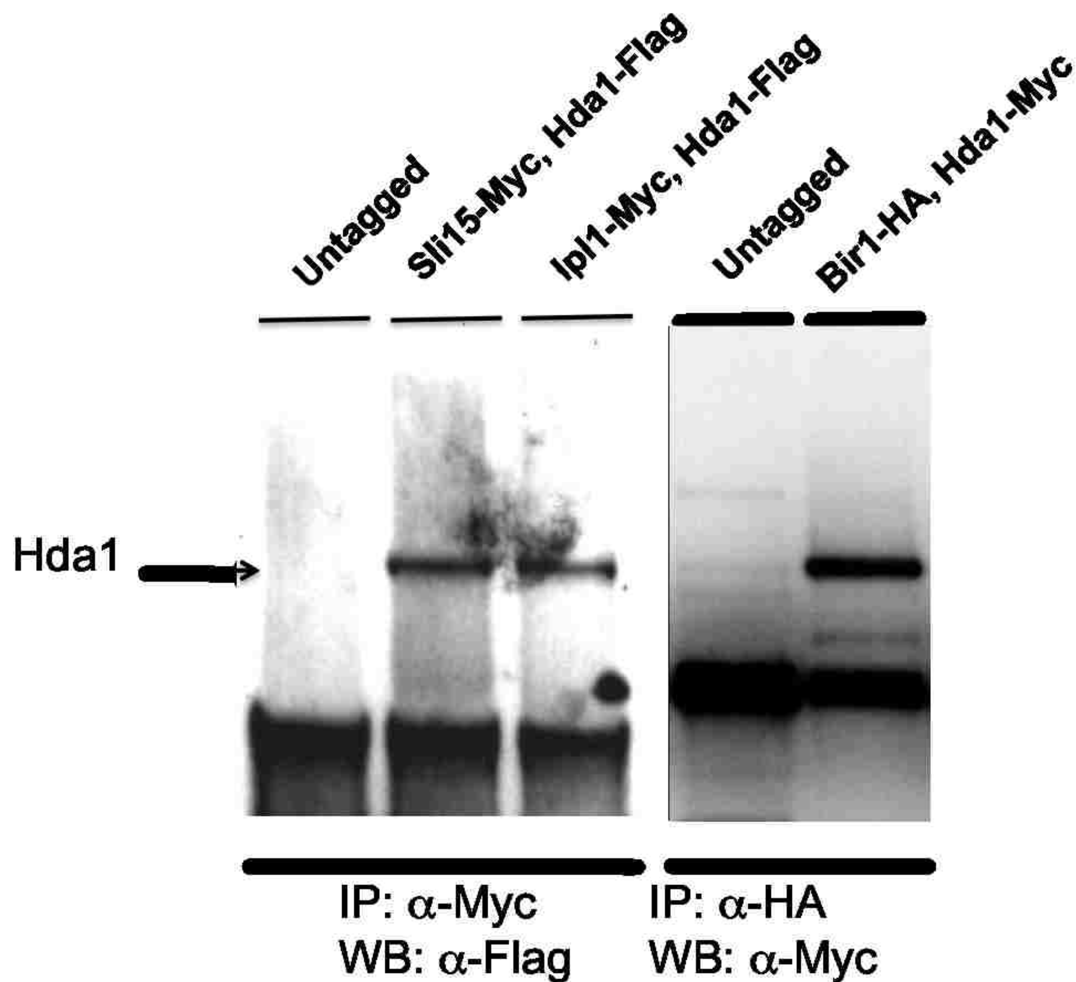

Figure 2. Hda1 interacts with chromosomal passenger proteins by co-immunoprecipitation. Protein extracts from wild-type strains untagged (FY1333) or double tagged *SLI15-MYC HDA1-FLAG (*IPY748), *IPL1-MYC HDA1-FLAG (*IPY753), and *BIR1-HA HDA1-MYC* (IPY384) were immunoprecipitated with either anti-Myc or anti-HA antibodies, separated by SDS-PAGE, followed by western blotting with either anti-Flag or anti-Myc antibodies.

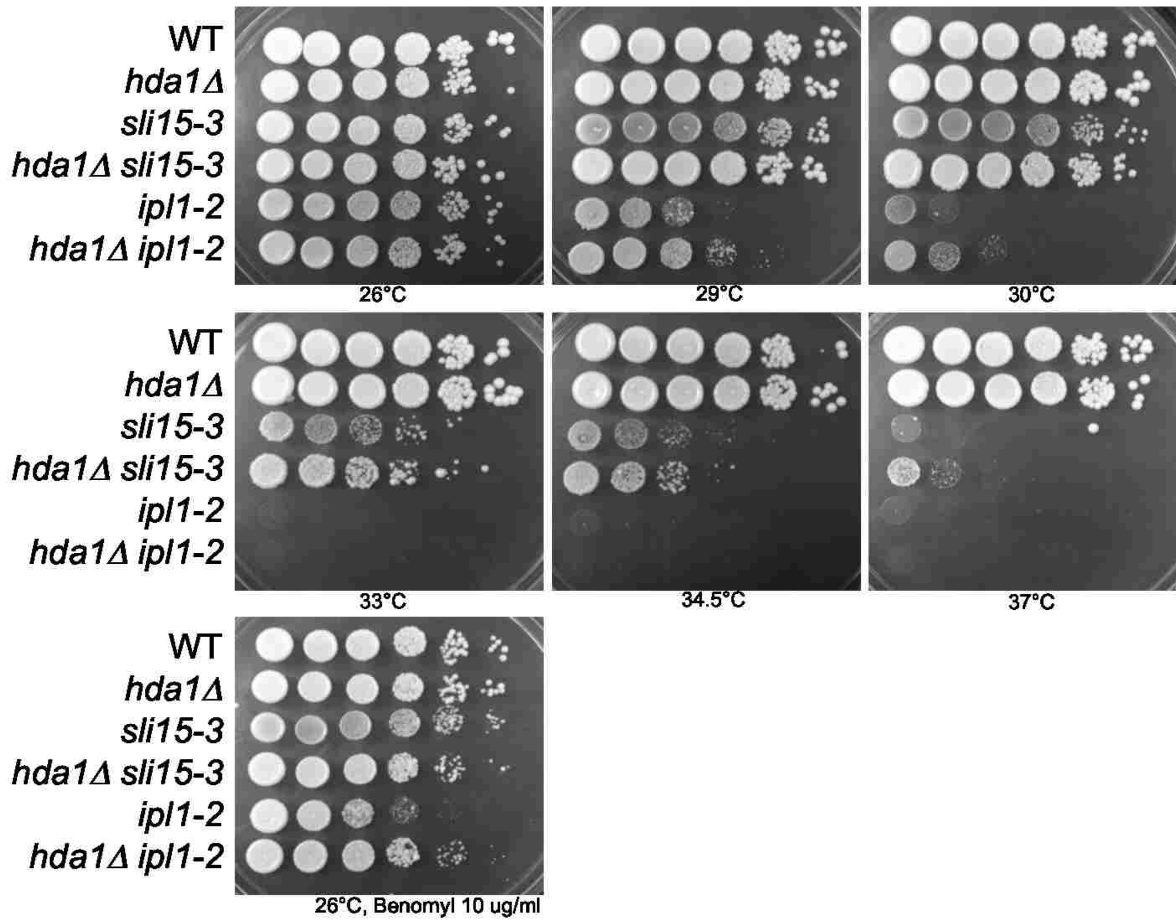

Figure 3. Suppression of *ipl1-2* and *sli15-3* by *hda1Δ*. Double mutants were generated by crosses between *hda1Δ* strains and *ipl1-2* or *sli15-3* strains. Serial dilutions (108–10^3cells/ml) were spotted (4μl) onto YPD or benomyl plates and incubated at the indicated temperatures for 2 days. The genotypes correspond to the following strains: wild-type (IPY171), *hda1Δ* (IPY969), *sli15-3* (IPY864), *hda1Δ sli15-3* (IPY862), *ipl1-2* (IPY859), and *hda1Δ ipl1-2* (IPY858).

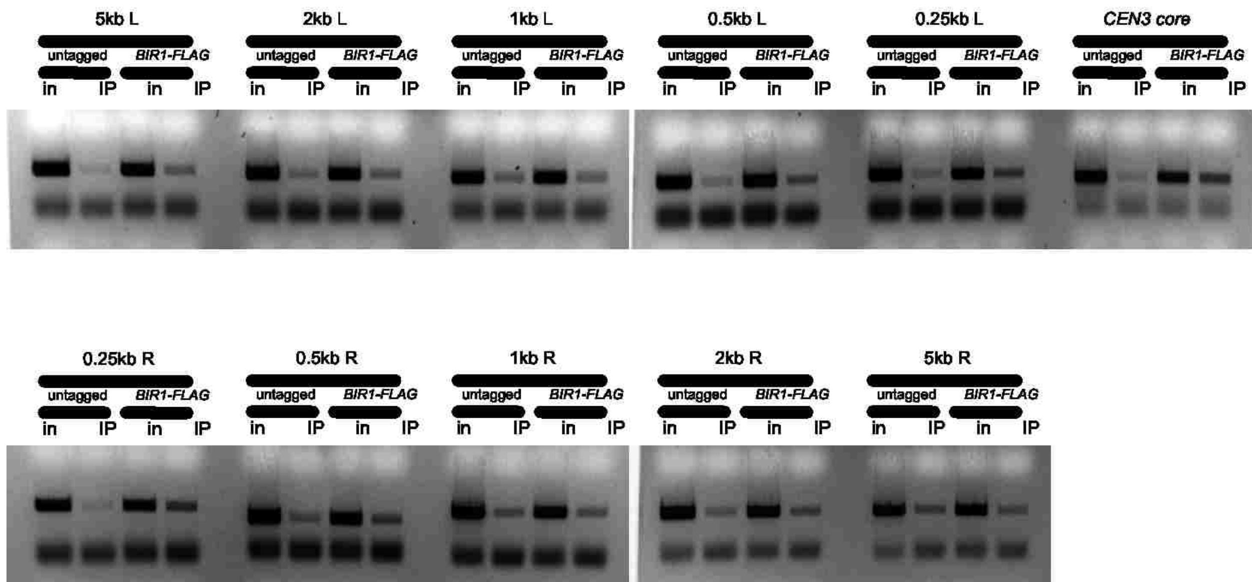

Figure 4. Pericentric localization of Bir1. Chromatin extracts from an untagged strain (FY1333) and a strain carrying *BIR1-FLAG* (IPY498) were immunoprecipitated with anti-Flag antibodies followed by DNA purification. PCR primer walking was performed up to 5kb left or right of the core region of *CEN3*.

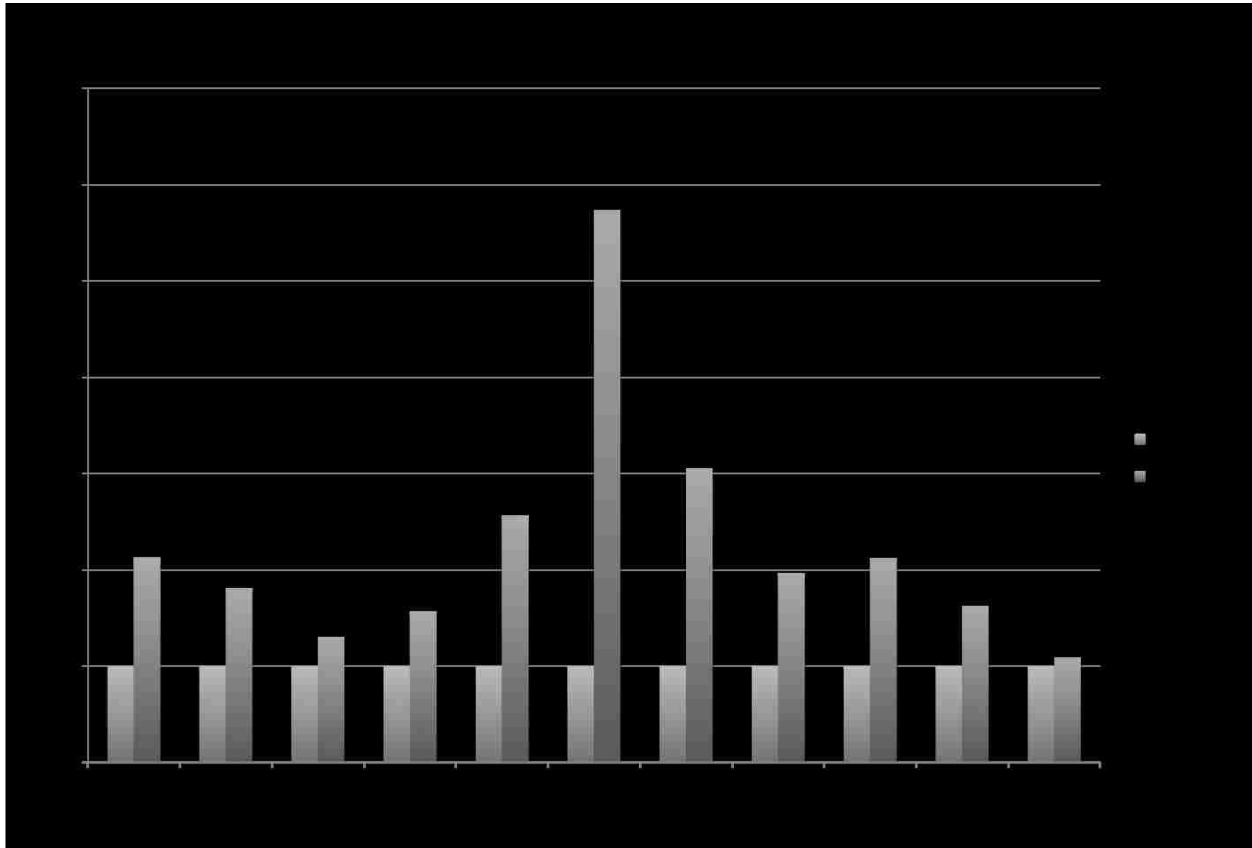

Figure 5. Relative PCR band intensity from figure 4 was analyzed using AlphaEase FC software.

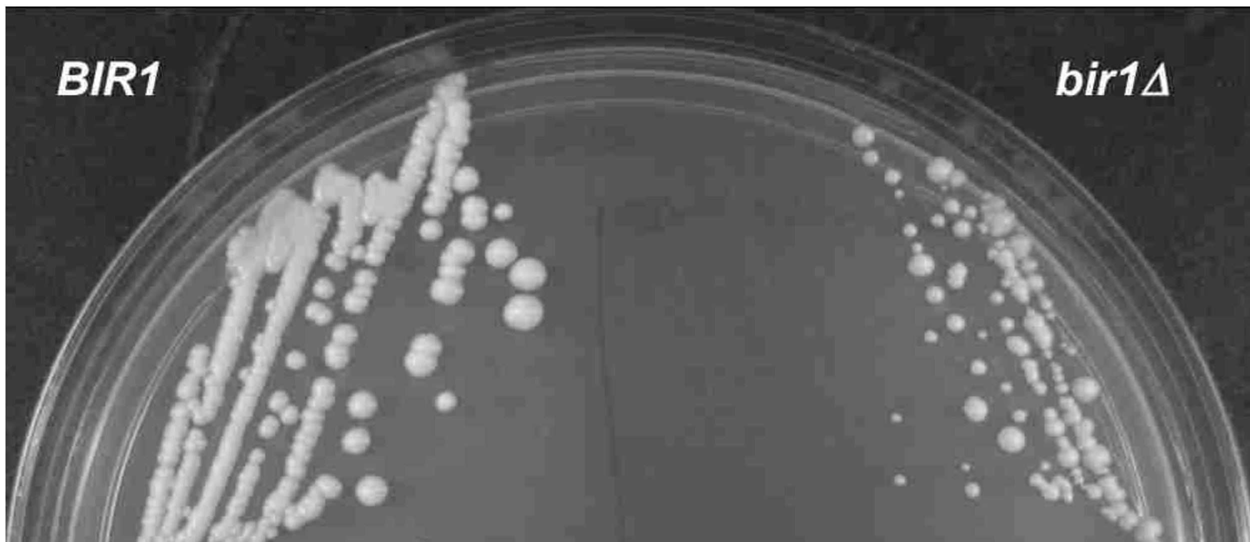

Figure 6. Growth phenotype of *bir1Δ*. A *BIR1/bir1Δ* heterozygous diploid was constructed and allowed to undergo sporulation. The left side of the image shows a $BIR1^{+}$ haploid segregant, and the right side shows one of the rare *bir1Δ* "survivors."

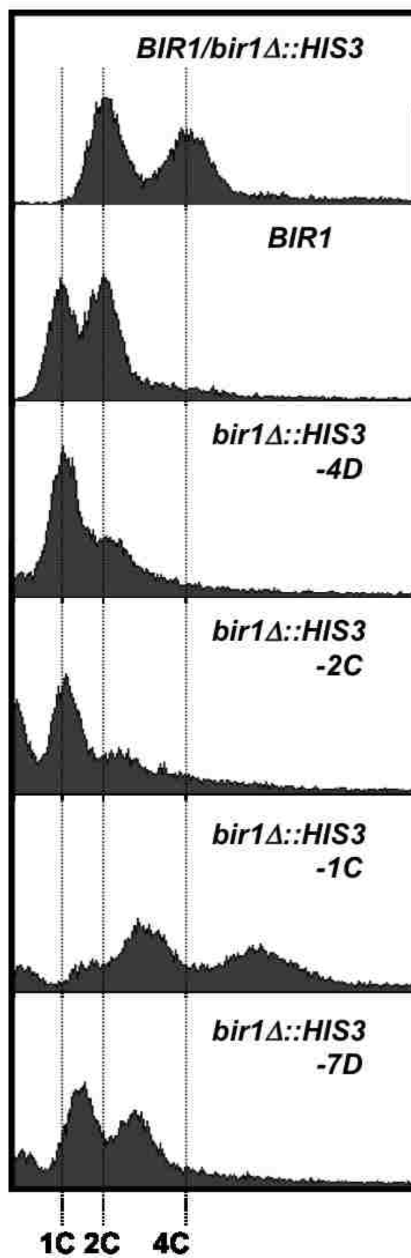

Figure 7. DNA content of *bir1Δ* survivors. FACS analysis was performed on the *BIR1/bir1Δ* heterozygous diploid, *BIR1*$^{+}$ haploid, and four of the *bir1Δ* survivors.

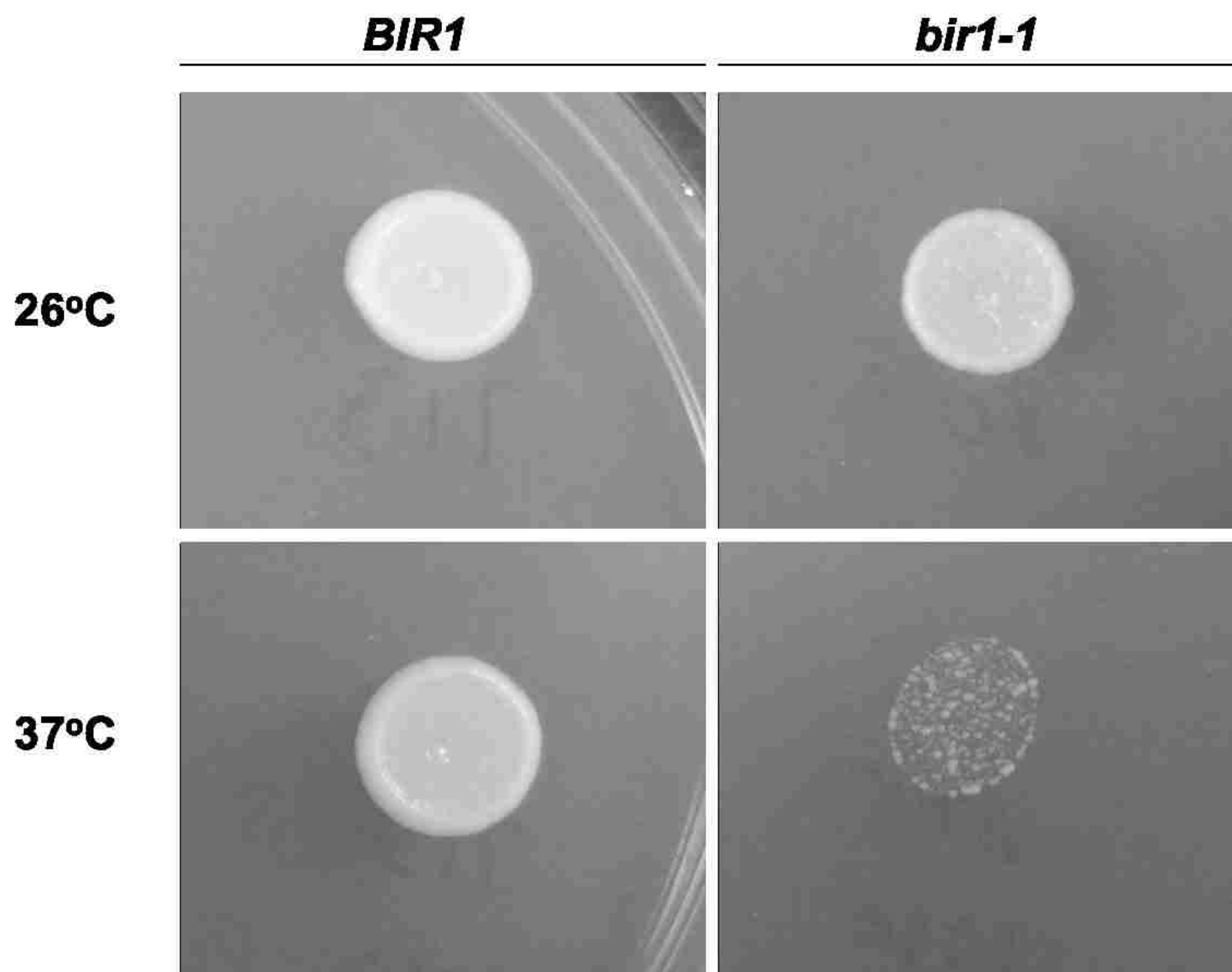

Figure 8. *bir1-1* is temperature sensitive. Strains carrying either wild-type *BIR1* (IPY713) or *bir1-1* (IPY808) were grown in liquid YPD overnight at the permissive temperature (26°C). 5μl of the overnight culture was spotted on YPD plates and incubated at the indicated temperatures for 2 days.

Figure 9. Schematic showing the location of the mutations in the *bir1-1* allele.

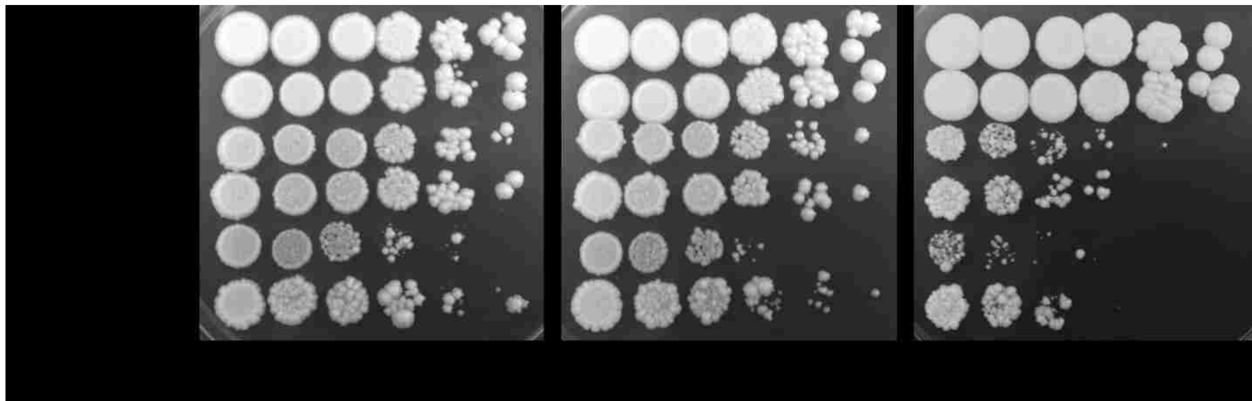

Figure 10. Synthetic phenotype of *hta1-300 ipl1-2.* Double mutants were generated by crosses between *ipl1-2* and *hta1-300* strains. Serial dilutions (108–10^3cells/ml) were spotted (4µl) onto YPD plates and incubated at the indicated temperatures for 2 days. The genotypes correspond to the following strains: wild-type (FY604), *hta1-300* (IPY985), *ipl1-2* (IPY1015 and IPY1016), and *hta1-300 ipl1-2* (IPY987 and IPY988).

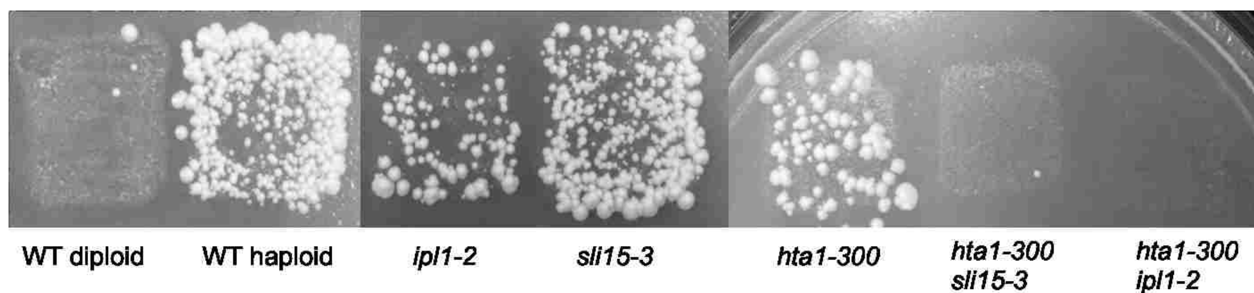

Figure 11. Canavanine assay for ploidy. Strains with indicated genotypes were streaked on SC-Arg medium containing canavanine and exposed to 5000 μJoules of UV radiation. Growth indicates the strain is haploid and no growth indicates the strain is diploid or beyond. Genotypes correspond to the following strains: WT diploid (IPY75), WT haploid (FY604), *ipl1-2* (IPY1015), *sli15-3* (IPY950), *hta1-300 sli15-3* (IPY990), and *hta1-300 ipl1-2* (IPY988).

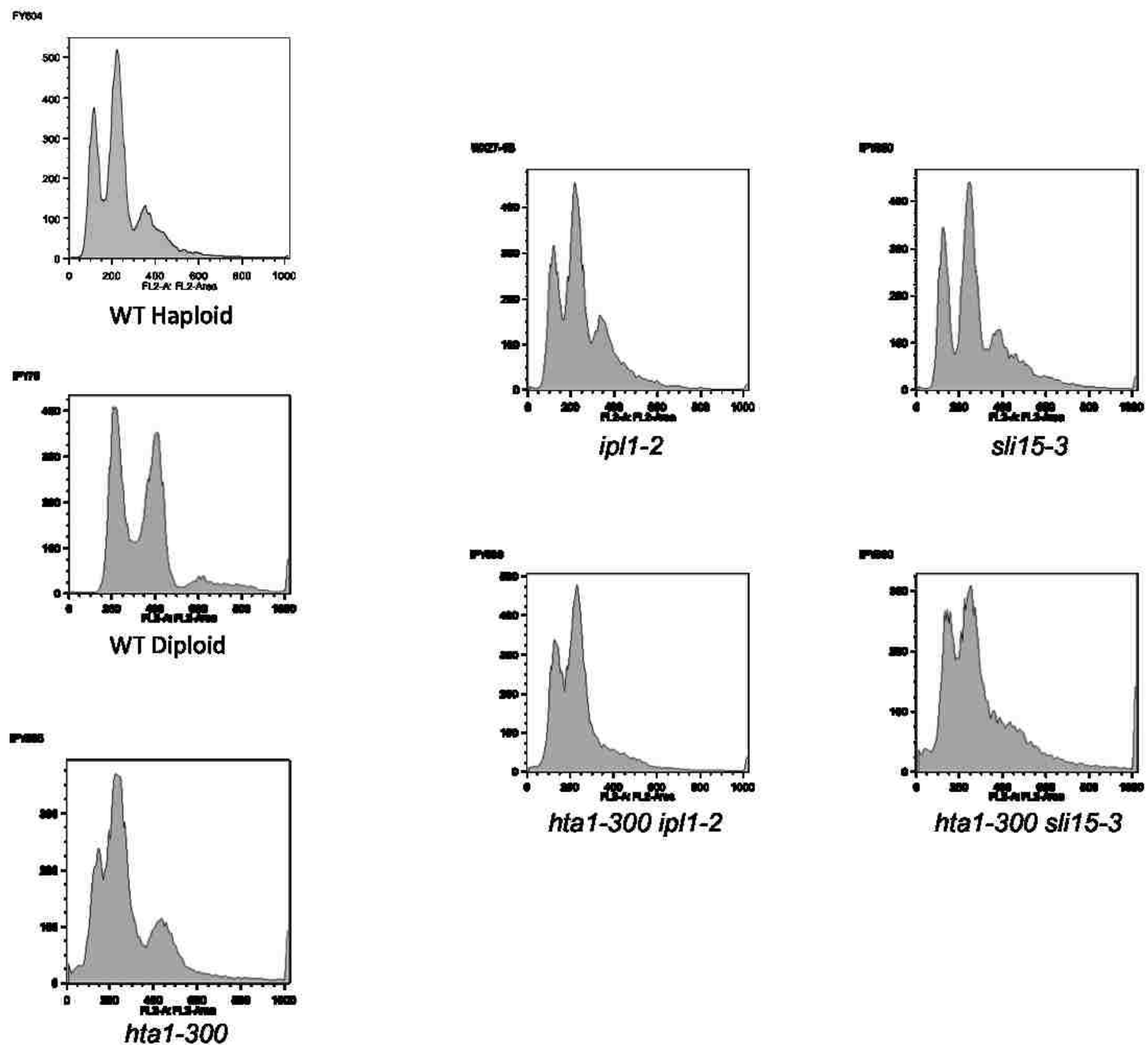

Figure 12. DNA content by flow cytometry. Strains from figure 11 were prepared for flow cytometry and analyzed using a Becton Dickinson FACSCalibur instrument. Genotypes correspond to the following strains: WT diploid (IPY75), WT haploid (FY604), *ipl1-2* (IPY1015), *sli15-3* (IPY950), *hta1-300 sli15-3* (IPY990), and *hta1-300 ipl1-2* (IPY988).

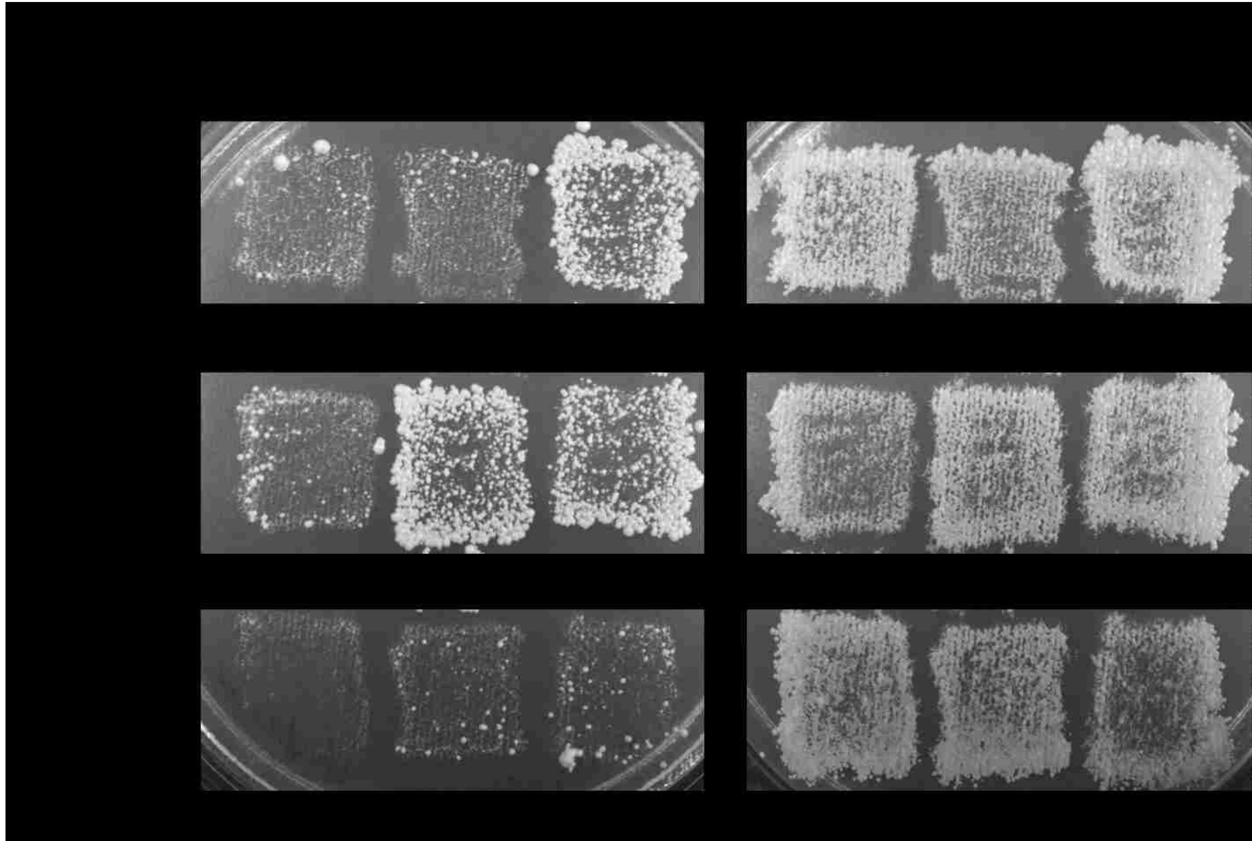

Figure 13. Overexpression of *BIR1* does not suppress the increase-in-ploidy phenotype of *hta1-300* or *hta1-200.* WT (FY604), *hta1-300* (IPY69), and *hta1-200* (JH492) strains were transformed with either YEP181 (empty vector) or pIP140 (*BIR1-2μ-LEU2)*, followed by analysis with the canavanine assay for ploidy.

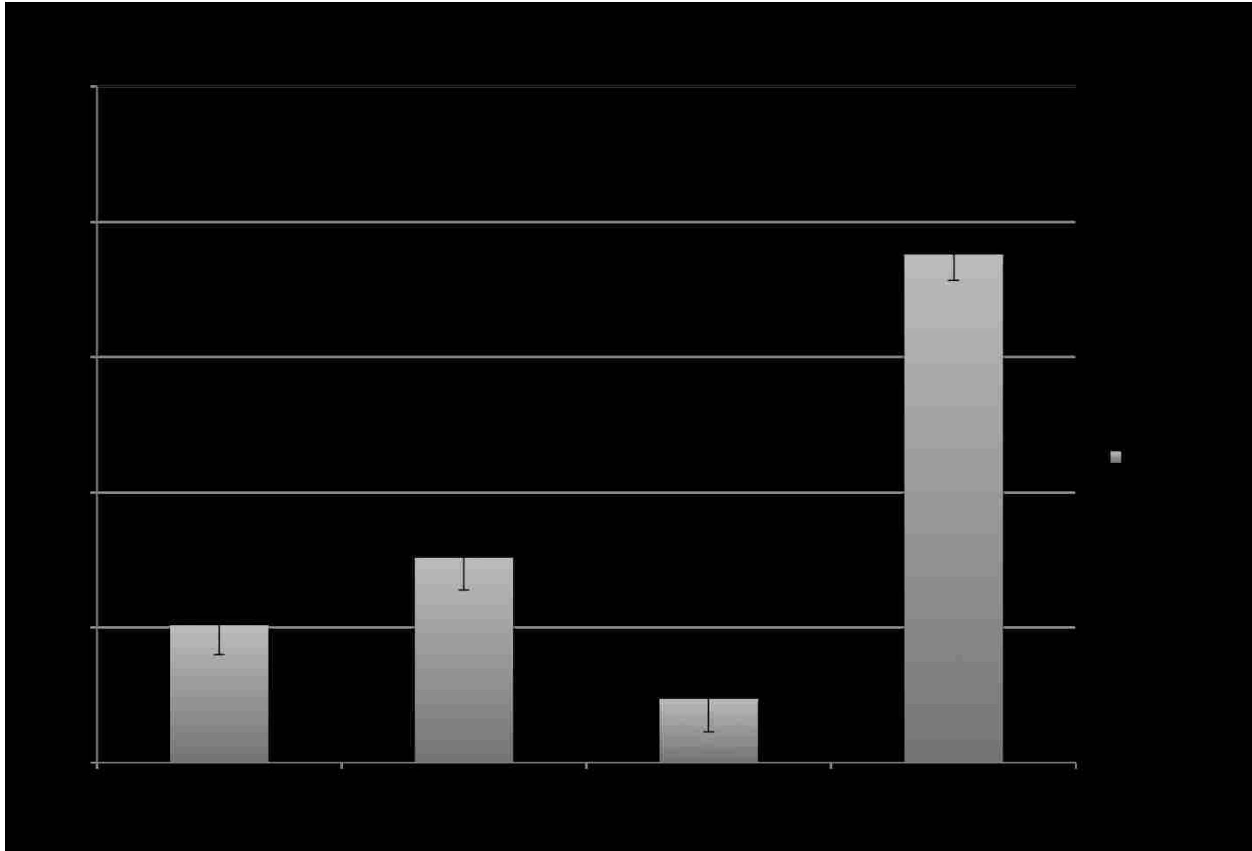

Figure 14. Bir1 interacts with Sgo1 by two-hybrid. Two-hybrid strains were constructed using Sgo1 as the bait (pIP155) and either C-terminal Bir1 (pIP98), N-terminal Bir1 (pIP99), or full length Bir1 (pIP100) as the prey. These strains were assayed for B-galactosidase activity.

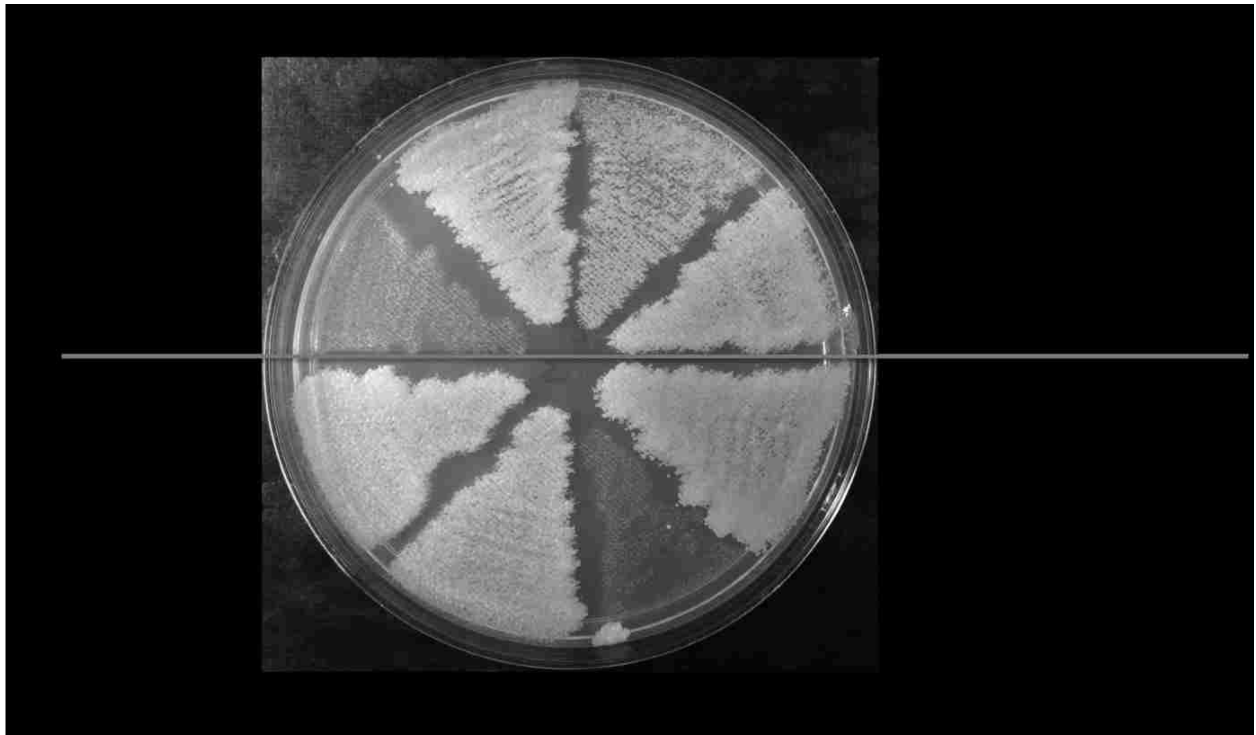

Figure 15. *hta1-300* is synthetic lethal with *sgo1Δ.* Double mutants were obtained by crossing *hta1-300* and *sgo1Δ* single mutants covered by pSAB6 (*HTA1-URA3-CEN*) and pIP156 (*SGO1-URA3-CEN),* respectively. Strains were streaked on 5-FOA medium to check for the ability to grow in the absence of the wild-type *SGO1* and *HTA1* plasmids.

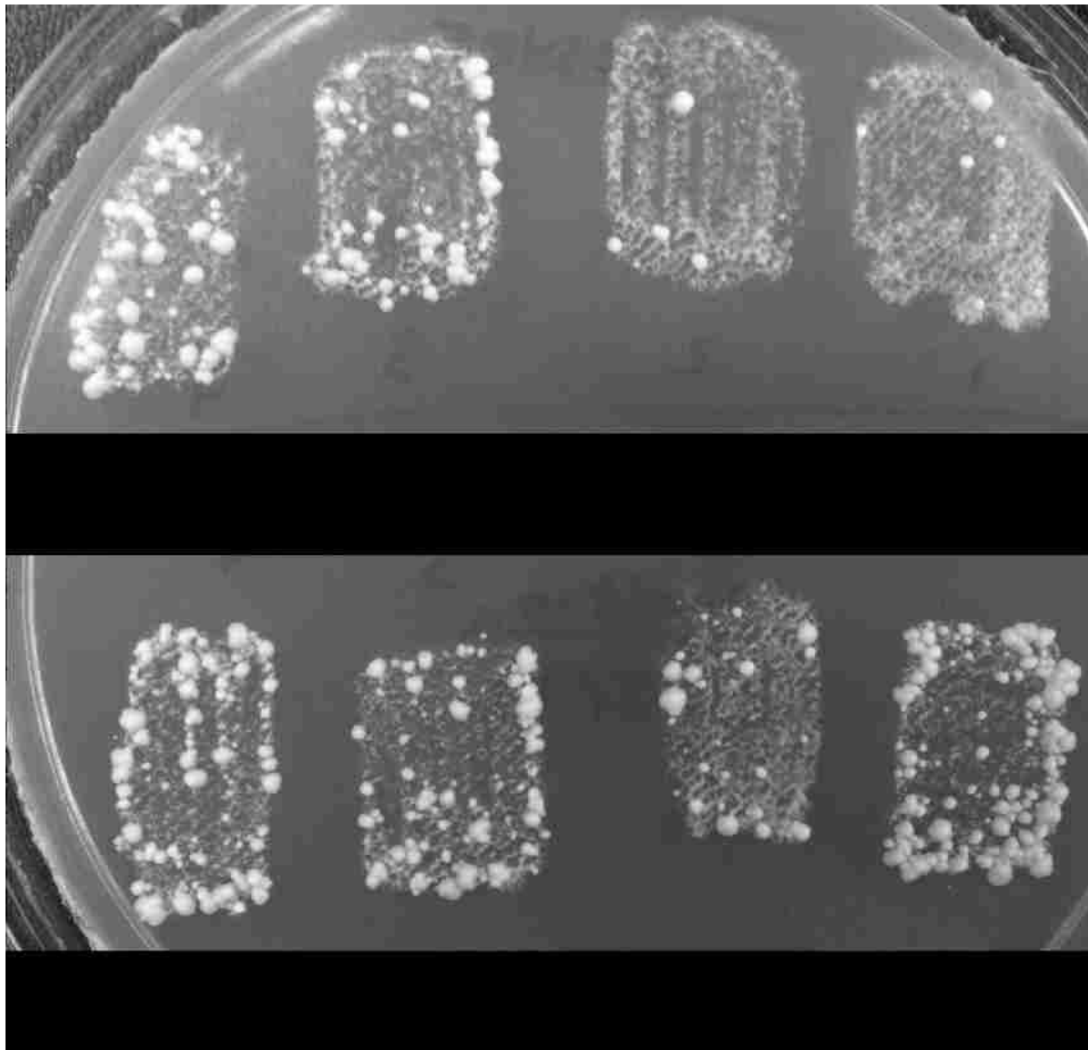

Figure 16. Overexpression of *BIR1* does not rescue the synthetic lethality of *hta1-300 sgo1Δ*. Strains carrying *hta1-300 sgo1Δ* covered by pIP156 (*SGO1-URA3-CEN)* and pSAB6 *(HTA1-URA3-CEN)* were transformed with pRS425 (empty vector) and pIP114 (*BIR1-LEU2-2μ),* followed by plating on 5-FOA medium to check for the ability to grow in the absence of the cover plasmids.

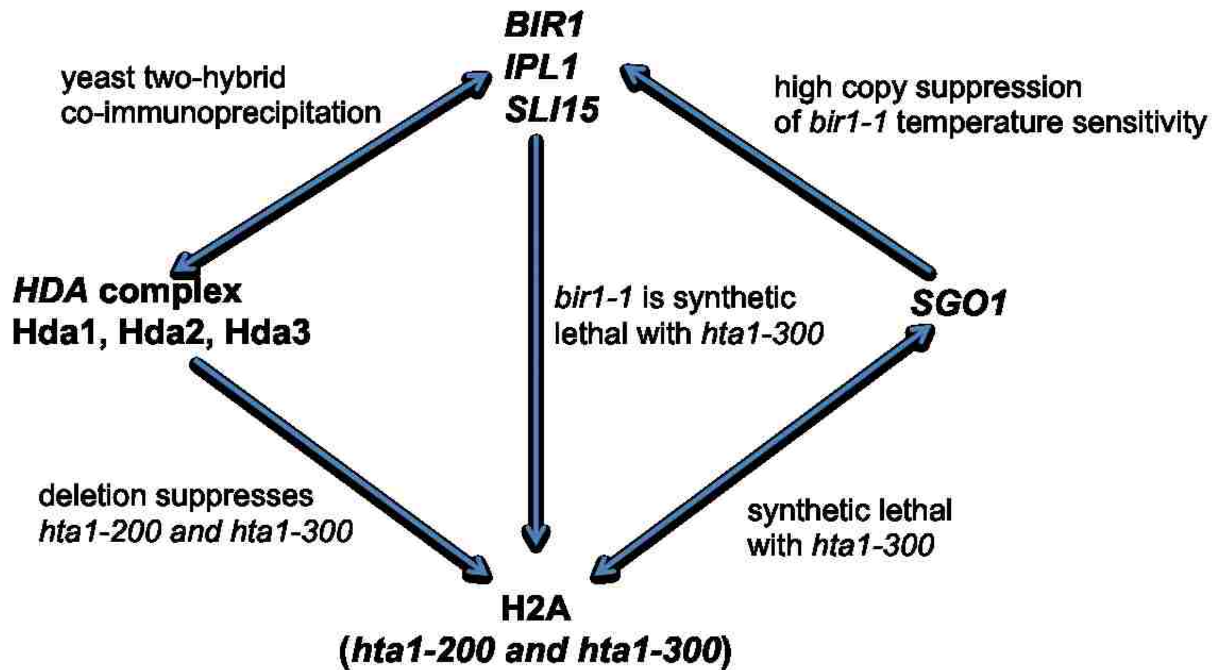

Figure 17. Schematic showing the interactions between chromosomal passenger proteins, chromatin, Hda complex, and Sgo1.

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V. CONCLUSION

All eukaryotes must carry out basic cellular processes, such as DNA replication, transcription, translation, and chromosome segregation, in order for survival. These basic cellular processes are well conserved from simple eukaryotes, such as the budding yeast *Saccharomyces cerevisiae*, to complex eukaryotes, such as metazoans. Using the simple eukaryote *S. cerevisiae* as a model organism, we aim to understand the role that chromatin plays in chromosome segregation.

Previous work identified a single point mutation in *HTA1*, one of the genes coding for histone H2A, which causes chromosome segregation defects ranging from aneuploidy to polyploidy. Subsequently, it was determined that mutations in each of the genes encoding the three members of the histone deacetylase (Hda) complex, *HDA1, HDA2,* and *HDA3*, could suppress the increase-in-ploidy phenotype of the histone H2A mutant. This work characterizes the Hda complex and establishes a novel role for it in centromere function and chromosome segregation.

We have found that the original mutant alleles of the Hda complex that were isolated as suppressors of the histone H2A mutant behave as null alleles, as comparable suppression can be obtained by deletion of the Hda complex. Additionally, we show that the Hda complex localizes to centromeric and pericentromeric regions of DNA in a cell cycle independent manner and is able to deacetylate centromeric chromatin.

Strengthening our proposal of a role for the Hda complex in centromere function we present biochemical and genetic data indicating that the Hda complex interacts with kinetochore components. We show that the Hda complex interacts with Dam1, a non-histone protein that has been shown to be post-translationally modified by the histone methyltransferase Set1 and the Aurora kinase Ipl1. We also show that deletion of the Hda complex suppresses a mutant form of

the inner kinetochore component Ndc10 by restoring normal chromosome segregation. In addition to deacetylating centromeric chromatin, it is possible that the Hda complex modulates the activity of kinetochore components by deacetylation of kinetochore proteins.

Interestingly, we have found that the Hda complex interacts with the chromosomal passenger complex (CPC), which is involved in the spindle assembly checkpoint. A mutant allele of one of the CPC components, *bir1,* is lethal in combination with the histone H2A mutation. Because information on Bir1 is limited with respect to chromatin, we carried out a genetic screen to identify high copy suppressors of the *bir1* mutant. From this screen we identified Sgo1, another component of the cell's tension sensing and spindle checkpoint machinery that has been shown to interact with chromatin. We tested genetic interactions between *sgo1* and the histone H2A mutant, and found that deletion of *SGO1* is also lethal in combination with the histone H2A mutant. These results suggest that the histone H2A mutant causes genomic instabilities as a result of microtubule misattachments or tension deficiencies that are sensed and partially alleviated by the functions of Bir1 and Sgo1.

Using the model organism *Saccharomyces cerevisiae*, we have furthered the body of understanding of the molecular mechanisms of chromosome segregation, a process highly conserved in eukaryotes and essential for maintaining genomic integrity. We conclude that centromeric chromatin and chromatin modifiers such as the Hda histone deacetylase complex interact with kinetochore components and checkpoint proteins in the establishment of a functional centromere-kinetochore complex. Furthermore, we propose that the Hda complex may have kinetochore or spindle checkpoint proteins as targets for deacetylation, modifications that may be relevant in the establishment or maintenance of centromere-microtubule interactions.

VI. APPENDIX

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