SIR2 FUNCTION IN GENOME INTEGRITY

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ABSTRACT

The fission yeast *Schizosaccharomyces pombe* is a common model for studies of eukaryotic chromosome function. In this study, we have used the fission yeast model to investigate the function of the NAD-dependent histone deacetylase Sir2 in chromosome segregation and genome integrity. Novel yeast strains were generated and used in combination with an established genetic assay to assess Sir2 function using a colored colony growth reporter. Strain construction was achieved using growth selective marker genes and validated by polymerase chain reaction (PCR). Our results suggest that Sir2 plays an important role in maintenance of the Ch16 linear minichromosome reporter. Accordingly, we hypothesize that Sir2 may similarly play an important role in maintaining genome integrity during normal eukaryotic cell division.

KEYWORDS: Sir2, chromosome, genome integrity, fission yeast, *Schizosaccharomyces pombe*

Dedication:

I dedicate this dissertation first and foremost to my parents: Fiyah and Shiemah for their endless love encourage and support. Than for people who believes that I will do something great in my life, give me a hope that all my dreams will become true if I seek a right journey, helped me overcome the struggle and open the locked gates to achieve my goals: my sisters, brothers, friends and teachers. I wish, that I can list all of you by name here but I cannot and you are always in my mind. I reach to a dream which I have dreamt over my life, touch the stars and ready to achieve the next goal in my life.

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Table of Contents:

Page

List of Figures:

Page

List of Tables:

Page

Chapter 1: Introduction

1.1 Chromosome structure and function in eukaryotes

Within the eukaryotic nucleus, deoxyribonucleic acid (DNA) is extensively compacted, packaged, and organized. The eukaryotic genome is packaged within structures called chromosomes. Chromosomes have two principle functions: to guarantee that the DNA is efficiently copied and to ensure the integrity of the genomic material. Structural components of eukaryotic chromosomes include of the centromeres, which are at the center of the chromosomal body, and telomeres which are positioned to their exterior.

Chromosomes contain long strands of DNA that contain all hereditary information. They are the unit of DNA replication in living cells (Esteller, 2007). The structural and functional roles of chromosomes ensure that daughter cells receive the proper complement of DNA following cell division, or mitosis. Prior to cell division, eukaryotic chromosomes are duplicated and packaged within exceptionally macromolecular structures. The tight winding and high level of association in this supercoiled DNA ensures faithful segregation of the genomic material upon cell division (Esteller, 2007).

The genome size of eukaryotes varies broadly, from those of the yeasts (over ten million base pairs, or bp) to those of vertebrates (three billon bp or more). Plant genomes may be even larger still. In the Easter lily *Lilium longiflorum*, the genome comprises over three hundred billion bp. Likewise, the size and number of chromosomes in any specific species – termed the organism's karyotype – varies broadly (Zlatanova *et al.*, 1999). A minimum size appears necessary for stable maintenance of the eukaryotic chromosome. For example, yeast artificial chromosomes (YACs) from the budding yeast *Saccharomyces cerevisiae,* contain minimal centromeric and telomeric elements, yet have size of approximately 50 thousand bp.

A maximum limit may also exist for chromosome size. It has been suggested that the longest chromosome arm must not be longer than half the length of the spindle axis at telophase, thereby permitting faithful chromosome segregation upon cell division (Moyzis *et al.*, 1988).

The organization of eukaryotic chromosomes has direct consequences for DNA replication. DNA polymerase is an enzymatic factor required that is required for duplication of eukaryotic DNA. Without high replication fidelity, the DNA contents of eukaryotic chromosomes might be subject to a high rate of mutation or loss of genome integrity, bringing about the loss of hereditary data. Hereditary information is maintained in part by having lengths of redundant, non-coding DNA at the linear telomeric ends of the chromosome, which secure the remainder of the replicated strand from damage amid replication (Eric, 2002). Moreover, the DNA of eukaryotic cells is commonly supercoiled during cell division to facilitate chromosome segregation, yet must be uncoiled, or more loosely packaged, to permit transcription and replication. Thus, throughout the majority of the cell cycle, chromosomes exist in an uncoiled or diffuse state.

Mammalian chromosomes are maximally condensed at metaphase of the cell cycle, when a typical mammalian chromosome will have length that is approximately ten thousand fold shorter than if the same length of DNA were packaged as a decondensed helix. Packaging of the metaphase chromosome requires cooperative action of numerous proteins factors. The fission yeast genome is relatively simple, comprising only three chromosomes. A significant number of the factors directing cell division and genome integrity in the fission yeast are likewise found in humans. Cell cycle regulation and division are critical for development and replication of any cell. Notably, fission yeast centromeres are 50–100 kilobases (kb) in length long and contain repetitive DNA elements proximal to their central region, or core. The centromeric core provides

the template within which atypical characteristic histones variants and histone modifications are incorporated, directing assembly of protein complexes that mediate higher order centromeric functions. Increased histone methylation and decreased histones acetylation is typical of chromatin structure within the pericentromeric outer repeats of the fission yeast genome (Allshire *et al.,* 1995).

S. pombe is principally a haploid unicellular organism. In medium rich with supplements, wild type cells experience a mitotic division at regular intervals. However a mixture of conditions, or growth restrictive mutations, can be utilized to synchronize cells at different phases of the cell cycle or to otherwise characterize gene functions. Despite the observation that *S. pombe* exists most stably as a haploid organism, it is nonetheless possible to select and manipulate for diploid strains of this organism. Diploid cells may similarly be propagated by vegetative mitotic growth unless starved of nitrogen, whereupon they undergo meiosis to yield zygotic asci, from which haploid spores may in turn be selected (Allshire *et al.,* 1995)

1.2 Chromosome segregation in mitosis - roles of the centromere

Mitosis is the process by which eukaryotic cell divides to give rise to two daughter cells which each contain the same genetic complement as the parent cell (Moazed, 2001). The distribution of genomic material upon mitosis depends intricately on the structure of the eukaryotic chromosome. One chromosomal structure which is critical to chromosome segregation during mitosis is the centromere. The centromere was initially described by German researcher Walter Flemming in the 1880s as the "essential tightening" element of the chromosome. Researchers now regard the centromere as a distinct region of DNA upon which specific chromatin proteins are assembled, giving rise to the condensed structure of the

chromosomal center. The centromeric DNA template provides a point of nucleation for kinetochore assembly and serves as the site for sister chromatid cohesion (Peterson and Workman, 1999).

Lapses in centromere or kinetochore function are disastrous for cells and organisms. Such lapses can prompt distorted division and chromosomal shakiness, both of which are regularly seen in dangerous cells. The centromere is perhaps the most compacted region of the condensed dense mitotic chromosome. Yet, despite their name, in certain organisms, centromeres are not only found in the chromosomal center (Hsieh and Fire, 2000). For example, holocentric chromosomes may adopt multiple points of centromeric adhesion, whereas more typical metacentric chromosomes have centromeres at their center. In different chromosomes, centromeres are found at an assortment of positions that are specific to the individual chromosome. The position of the centromere within each chromosome therefore yields a meaningful point of reference to partitioning chromosomes into karyotype and for mapping the positions of genetic elements within the chromosomes themselves.

With a couple of special cases, eukaryotic chromosomes have a solitary centromere that guarantees their exact isolation amid mitosis. Chromosomes with aberrant centromeric function segregate haphazardly amid mitosis giving rise to aneuploidy daughter cells that contain more or less genetic material than the parent cell. The centromeres of human chromosomes are heterochromatic structures made up of repetitive arrangements known as alpha satellite DNA. A solitary unit of alpha satellite DNA is 171 base pairs long. Each repetitive element contains a 17-base-pair binding site for the centromeric histone variant CENP-B, termed the CENP-B box. An individual human chromosome can contain as many as 100 of more putative CENP-B boxes. Moreover, experimental evidence indicates that CENP-B binds as a dimeric complex, and so

may have the capacity to cross-connect two adjacent alpha satellite containing centromeric regions, thereby helping to form the higher-order structure of the chromosome (Irvine *et al.*, 2001).

One essential function of the centromere is to facilitate the assembly of the kinetochore, which is a protein complex essential to chromosomal segregation during mitosis. In electron micrographs of mitotic chromosomes, kinetochores show up as plate like structures made out of a few layers (Irvine *et al.*, 2001). The kinetochore forms the point of microtubule adhesion and assembly, thereby facilitating chromosome migration during mitosis. The mature kinetochore is a stunning complex comprising 80 or more proteins, by present appraisals (Irvine *et al.*, 2001). Recently, biochemical and hereditary studies have permitted researchers to recognize sub-complexes of proteins inside the kinetochore that are needed for the kinetochore's various functions. Many of these factors direct the functions of microtubules (e.g., microtubule connection, polymerization, and engine regulated developments). Other kinetochore proteins contribute to control of cellular growth checkpoints that regulate progression into anaphase (Kingston, 1999).

Kinetochores are dynamic assemblies of proteins whose components vary throughout the cell cycle. Except for CENP-A and a limited number of proteins factors internal to the kinetochore, the structure dismantles at the end of mitosis. CENP-A associated DNA elements remain parts associated with the centromere throughout the cell cycle, as the CENP-A histone variant comprises a central element of the centromeric nucleosome. During S phase, which occurs after mitotic division, the vicinity of CENP-A nucleosomes marks the position of the centromere on the replicated daughter strands. In keeping with the semiconservative nature of chromatin replication, CENP-A-containing nucleosomes are duplicated and incorporated within both

daughter strands. Taking after replication, a particular CENP-A stacking component is thought to enlist extra CENP-A to the centromeres. By this sort of mechanisms, centromeres can maintain constant positions on chromosomes throughout numerous rounds of cell division (Sullivan *et al.*, 2001). Notwithstanding their roles in kinetochore assembly, centromeres perform an additional vital role in mitosis by serving as the point of sister chromatid attachment. For precise mitoses, sister chromatids must stay connected until the spindle checkpoint has been passed. As noted elsewhere, the attachment of sister chromatids is mediated protein factors termed cohesins. Chromatid connection happens on an alternate region of the chromosome from the kinetochore. As a cell enters anaphase, cohesin structure is abruptly altered, and the cell's sister chromatids separate and segregate to divergent poles of the cell (Bird, 2002).

Genetic disorders may arise when chromosomes missegregate during formation of gametes by meiosis, yielding egg or sperm cells too many or too few chromosomes. Notable genetic disorders that arise due to developmental aneuploidy include human trisomy (an additional chromosome of a specific pair in a single cell) or monosomy (one chromosome less in each one cell). For instance, in trisomy 21, or Down syndrome, cells have an additional copy of chromosome 21. In Turner syndrome cells have one and only one X chromosome (monosomy X) and no Y chromosome. Thus, changes in chromosomal content have clear developmental and medical implications.

1.3 Chromatin and the regulation of gene expression

Genomes of higher eukaryotes are packaged within two types of chromatin: euchromatin and heterochromatin. Heterochromatin is a highly compacted material commonly encompassing telomeric and pericentric chromosomal regions, as well as regions of the genome which are

generally nonpermissive to transcription. Actively expressed genes that are introduced into regions of heterochromatin are inactivated, and obtain morphological properties of heterochromatin due to a phenomenon that has been termed position effect variegation. Heterochromatic components of the genome display comparable structural properties: hereditary inactivation, compaction, late DNA replication at S phase. Hereditary or adaptive inactivation and heterochromatin formation are driven by a particular epigenetic process, termed silencing, which may incorporate DNA methylation and/or posttranslational histone modification. The state of silencing is often inherited among specific cell types, and frequently, heterochromatin structure may be viewed as a morphological indication of gene silencing.

In the budding yeast heterochromatic epigenetic silencing is subject to control the silent information regulator (SIR) complex. The SIR complex is made up of the Sir2 histone deacetylase and the chromatin-connecting proteins Sir3 and Sir4. Reconstituted SIR heterochromatin has been used to investigate transcriptional mechanisms driving gene silencing. SIR complex binding is initiated through recruitment of Sir1, followed by recruitment of other SIR factors and nucleation of heterochromatin gathering. The SIR complex has additionally been demonstrated to be refractory to transcription by the elongating RNA polymerase II (Pol II). This Pol II extension obstruction may be mediated by even one nucleosome, yet is more successful when compounded across various nucleosomes, and is sensitive to a histone modifications that is are known to effect silencing.

Constitutive heterochromatin refers to those regions of chromosomes which stay heterochromatic throughout most or the all of the cell cycle. The remaining parts of chromosomes, those that are not incorporated within heterochromatin, are termed euchromatin (Heltz, 1928). Forms of heterochromatin that are by turn euchromatic are termed facultative

heterochromatin. Facultative heterochromatin may be embodied by the heterochromatinized X chromosome (Barr body) in placental vertebrates (Barr and Bertram, 1949; Mittwoch, 1964) and by the whole heterochromatinized set of chromosomes in mealy bugs (Hughes-Schrader, 1948) Facultative heterochromatinization of chromosomes may be triggered hereditary inactivation; in such cases, the homologous chromosome is generally not influenced. The hypothesis that heterochromatic genes are located in heterochromatin due to a functional requirement for a heterochromatic environment was initially proposed by Hilliker (1976).

The greater part of the genome (~99%) is incorporated within nucleosomes consisting of~146 bp of DNA wrapped around an octamer of histone proteins containing two molecules each of the histones H2A, H2B, H3 and H4 (Zlatanova *et al.*, 1999; Luger *et al.*, 1997). Nucleosomes are formed in a step-wise manner by first assembling two histone H3/H4 dimers as a tetrameric complex onto ~80 bp of DNA and afterward incorporating two histone H2A/H2B dimers to form an intact nucleosome around which ~146 bp of DNA wrap 1.7 times around the histone octamer. The nucleosomal model outlined above suggests nucleosome::DNA interactions are maintained by numerous contacts that are along the whole length of the nucleosomal DNA, with each of the eight histone particles intervening DNA contacts at different. It is additionally critical that this structure has the histone N-terminal tails distending from the nucleosomal core, as these tail regions are the site of various covalent modifications that regulate the structure and function of chromatin.

Histone tails are subject to a great number of posttranslational modification. Such modifications include lysine acetylation, lysine and arginine methylation, serine and threonine phosphorylation, lysine ubiquitination, lysine sumoylation, arginine deamination, proline isomerisationm and poly-ADP ribosylation (Kouzarides, 2007). These modifications may be

introduced by a diverse range of enzymatic activities, including, for example, histone acetyltransferases (HATs), histone methyltransferases (HMTs), kinases, and ubiquitin ligases. Contingent the transcriptional state, such modifications may be antagonized by competing enzymatic activities. For example, HDACs and HATs may work in an opposing fashion to regulate lysine acetylation state.

Specific histone modifications may help support either the active or the quiescent transcriptional state. Examples of such modifications include histone H3 lysine 9 acetylation (H3K9Ac), which is generally associated with actively expressed regions of the genome, and histone H3 lysine 9 methylation (H3K9Me), which is generally associated with repressive transcriptional states. Notwithstanding their influence on transcription factor binding to genomic DNA, the identity of histone modifications has an immediate impact on general chromatin structure. For instance, histone acetylation is hypothesized to neutralize limit the net positive charge present upon lysines in the histone tails, and to may thereby lead to a decreased level of chromatin compaction, as seen upon hyperacetylation of histone H4 lysine 16 (H4K16) and other residues (Shogren-Knaak M. *et al.*, 2006).Transcription and heterochromatic compaction are similarly controlled by incorporation of various histone isoforms, for example, H2AZ or H3.3 which may bind to DNA with lower affinity and thereby permit enhanced gene expression by the basal transcription machinery (Jin and Felsenfeld, 2007).

1.4 Histones and chromatin structure

Following Vincent Allfrey's spearheading studies in the early 1960s, histones have been recognized as being subject to post-translational modification (Allfrey *et al.*, 1964). We now realize that there are an extensive number of diverse histone post-translational modifications

(PTMs). Knowledge into how these changes could influence chromatin structure was significantly enhanced by determination of the high-resolution X-ray crystal structure of the nucleosome (Youngson and Robert, 2006). The structure demonstrates that very fundamental histone amino (N)-terminal tails can extend from their nucleosomal core and reach nearby nucleosomes. It appeared to be likely at the time that adjustment of these tails would influence between nucleosomal connections and in this way influence the general chromatin structure.

In addition to histone PTMs, chromatin structure appears to be controlled through active nucleosome positioning mediated by ATP dependent chromatin remodelers. The recruitment of such proteins is now thought to represent a major component of heterochromatin function. As alluded previously, histones are profoundly basic proteins found in eukaryotic cell cores that bundle and partition the DNA into structural units called nucleosomes (Youngson and Robert, 2006; Cox *et al.*, 2005). They are the protein segments of chromatin, analogous to spools around which DNA winds, by analogy, as a thread, and play a significant role in genome compaction and the regulation of gene expression. Without histones, the decondensed DNA in chromosomes would be long (a length to width ratio of more than 10 million to 1 in human DNA). For instance, every human cell has very long DNA; yet, wound on the histones it has around 90 micrometers (0.09 mm) of chromatin.

Eukaryotic genomes can by and large be isolated into two topographically unique domains (Xhemalce *et al.*, 2011). “Open” areas of the genome which are loosely condensed and permissive to gene expression are referred to as euchromatin. Interestingly, other genomic areas, for example, centromeres and telomeres, are generally transcriptionally silent regions refractive to transcription throughout the cell-cycle. These more "minimal" locales are alluded to as heterochromatin. This is admittedly a restrictive perspective, as recent work in the fruit fly

Drosophila melanogaster has demonstrated that there are at least five distinct subtypes of genomic regions which may be classified on the basis of chromatin structure centered about the identity their numerous protein components (Filion *et al.*, 2010). Nevertheless, euchromatin and heterochromatin comprise the two basic elements of genomic DNA packaging, and hence, the discussion below will be restricted to these two broad genomic organizational.

Both heterochromatin and euchromatin contain characteristic histone PTMs. Frequently, such regions are bounded by delimiting factors that may suppress transcription, which thereby play a role in defining chromatin incorporated regions (Ong and Corces, 2008). Without delimiting elements, heterochromatin might otherwise spread into and silence the euchromatic areas of the genome. Silent regions of the genome are enriched for certain histone modifications, including histone H3 lysine 9 methylation (H3K9Me), are depleted for others, including histone acetylation (Barski *et al.*, 2007). Moreover, a particular histone variant the H2A.Z, isoform is frequently at such genomic regions. How these components cooperate to establish heterochromatic boundaries remains far from clear; however, their significance is evident.

As alluded above, two different heterochromatic subtypes have been characterized: facultative and constitutive heterochromatin. Facultative heterochromatin comprises of genomic districts regions which are alternatively expressed and subject to silencing. For example, a whose expression is required in a specific developmental program may be expressed only during a specific time period of the organism's development. A notable example of facultative heterochromatin silencing is the dormant X-chromosome inside mammalian female cells, for which silencing is maintained by histone H3 lysine 27 trimethylation (H3K27Me3) and the Polycomb repressor complex (PRC), among other factors (Trojer and Reinberg, 2007). The catalytic H3K27 methyltransferase activity is contributed by the EZH2 methyltransferase, which

is a component of the trimeric PRC2 complex. Recent work has revealed insight into how H3K27me3 and PRC2 work to facilitate facultative heterochromatin formation through DNA replication (Hansen *et al.*, 2008). It appears that H3K27me3 may initiate recruitment of PRC2 to regions of active DNA replication, leading to deposition of further H3K27me3 through the activity of EZH2. Thus, the histone imprint is "repeated" within the recently incorporated histones and the facultative heterochromatin is maintained.

Constitutive heterochromatin incorporates genomic regions that are stably maintained in the quiescent state, for example, the centromeres and telomeres. It is characterized by moderately large amounts of H3K9 mono-, di- and tri-methylation (H3K9Me1, H3K9Me2, and H3K9Me3, respectively, and associated histone proteins HP1α/β or their homologs (Trojer and Reinberg, 2007). HP1 dimers bind to H3K9 methylated nucleosomes through their chromodomains. Moreover, HP1 proteins cooperate with Suv39, a major H3K9 methyltransferase in mammalian cells. As DNA replication proceeds, modified histones bearing H3K9 methylation, are incorporated within the duplicated chromatin. Since HP1 is closely associated with the H3K9 methyl mark that is thought to be established by Suv39 activity, it is tempting to hypothesize that the proteins create an input circle fit for keeping up heterochromatin situating after DNA replication (Banister *et al.*, 2001).

Relative to heterochromatin, euchromatin is a significantly less compact, leading euchromatin-incorporated regions of the genome to exhibit more dynamic transcriptional qualities. As with heterochromatin, not all euchromatin is the same. Certain regions of the genome which are packaged within euchromatin may be enriched for specific histone alterations, while different regions may have other qualities. The appearance of histone PTM-rich "islands" has been observed, which may ultimately direct transcription from associated regions of the

genome (Barski *et al.*, 2007). For example, dynamic transcriptional enhancers contain elevated amounts of H3K4Me, a mark that is associated with increased transcriptional activity (Hon *et al.*, 2009). Enrichment of H3K4me3, is commonly observed proximal to the transcriptional start site (TSS) of euchromatic genes (Barski *et al.*, 2007; Schneider *et al.*, 2004), and H3K36me3 is commonly enriched throughout the body of actively transcribed genes (Banister *et al.*, 2005). The causative mechanism leading to H3K4Me at enhancers remains somewhat obscure, yet work in yeast has given considerable into the functions and identities of the H3K4 and H3K36 methyltransferases.

The budding yeast Set1p H3K4 methyltransferase is thought to associate with serine 5 phosphorylated of C-terminal domain (CTD) of the activated RNA polymerase II (Pol II) holoenzyme , the initiating type of polymerase activity at the TSS (Ng *et al.*, 2003). Similarly, the budding yeast Set2p H3K36 methyltransferase has been linked to the serine 2 phosphorylated CTD of Pol II, promoting transcriptional elongation (Xiao *et al.*, 2003). In this manner, the two methyltransferases have distinct yet interrelated to functions that are ultimately critical for the activation of gene expression through histone posttranslational modification.

1.5 Histone mutations and their effects

Histones are the fundamental protein parts of nucleosomes. They are among the most highly conserved proteins and are subject to multiple forms of post-translational modification. Particular histone modifications have been shown to be critical in creating and maintaining chromatin structure, thereby regulating gene expression and the susceptibility of DNA to damage inducing agents. Modifications of the histone amino termini, for example, acetylation, phosphorylation, and methylation, have been proposed to underlie a chromatin-based

mechanisms underlying gene expression. As described above, such modifications can act synergistically or independently to effect recruitment of chromatin-related proteins, which ultimately control transitions between transcriptionally active and silent states.

The unicellular fission yeast *Schizosaccharomyces pombe* posses complex centromere architectures that are similar in many respects to those of metazoans, such as humans. Moreover, the fission yeast has a smaller genome than humans, and is readily manipulated using a number of genetic techniques that have been extensively adapted to the lab environment. Accordingly, the fission yeast model provides a useful framework with which to analyze the contributions of histone H3 and H4 N-terminal residues in the control of centromeric heterochromatin.

Fission yeast contain three copies each of the histone H3 and H4 gene cluster qualities at unique chromosomal loci within the genome. Moreover, strains of yeast bearing only a single copy of histones H3 and H4 have been shown to be viable, providing a tractable system for mutagenic analysis (Barbara, *et al.*, 2003).Histone proteins are thought to be among the most highly conserved proteins in eukaryotic cells. Amino acid conservation between the budding yeast (*Saccharomyces cerevisiae)* and human (*Homo sapiens)*histones are 92% (H4), 90% (H3), 71% (H2A), and 63% (H2B). The histone core regions are perhaps most highly conserved. This level of conservation reflects the critical parts of histone assemblies in the nucleosome. Histones other than H4 have undergone evolutionary divergence in higher organisms. Histones H1 and H2A comprise the biggest families, whereas histones H2B and H3 may exhibit comparatively fewer evolutionary variations. These histone variations may give rise to transcriptional activation, or heterochromatin silencing ((Brown, 2001; Kamakaka and Biggins, 2005), and may also have consequences for higher order genome structure. For example, the yeast H2A variant H2A.Z appears to limit silencing at telomere proximal regions and is found in nucleosomes

associated with active transcription during eukaryotic development (Meneghini *et al.*, 2003; Raisner *et al.*, 2005). A histone H3 variant, CENP-A, replaces the common H3 subunit in the histone octamers in the centromere of eukaryotes (Smith, 2002).

Histones are extensively modified by post-translational changes, for example, acetylation, methylation, and phosphorylation. These adjustments empower numerous capacities of the chromosome and imprint particular areas of DNA (Iizuka and Smith 2003; Millar and Grunstein, 2006). For instance, in budding yeast, H3 K56 acetylation encourages gene expression by recruiting the SWI/SNF nucleosome remodeling complex (Xu *et al.*, 2005), while hypoacetylation of H4 tails may stabilize gene silencing by permitting the stable association of the H4 N terminus with the silencing factor Sir3p (Grunstein, 1998).

In accordance with the histone code hypothesis, the physiological consequence of histone modification is mediated either by immediate physical regulation of nucleosomal structure or by specific downstream "effector" proteins (Strahl and Allis, 2000; Jenuwein and Allis, 2001). Increasing data is accumulating that suggest both hereditary and epigenetic variations msy drive the progression of numerous human malignancies (Wang *et al.*, 2007). For instance, aberrant DNA methylation is a often utilized by tumor cells to overcome the expression of tumor silencer factors that might otherwise limit malignancy and metastasis (Ones and Baylin, 2007).

In the current discussion, we focus on recent advances that connect oncogenesis to histone methylation, with modifications of histone H3 lysine 4 (H3K4) and H3 lysine 27 (H3K27) serving as paradigmatic samples. Here, we suggest that epigenetic shifts including histone alteration lead to misregulation of gene expression and developmental characters, which, in turn, may drive tumor formation and metastasis. Methylation of histones occurs at both lysine and arginine residues. The incidence of PTMs within a particular genomic region is generally

reversible. Its homeostasis is mediated by two opposing enzymatic activities, such as histone methylation "writers" and “erasers”, corresponding with histone methyltransferases and histone demethylases, respectively (Strahl and Allis, 2000; Ruthenburg *et al.*, 2007). As a representative examples, H3K4 methylation is “written” by the Set1 and methyl-L lysine (MLL) group of histone methyltransferases (HMTs) (Allis and Wysocka, 2007), and “erased” by the Lsd1 and Jarid1 family of histone demethylases (HDMs) (Klose and Zhang, 2007).

Within histone H3, methylation has been observed at numerous lysine (K) residues including H3K4, K9, K27, K36 and K79. Deposition of up to three methyl moieties at every lysine residue yields a sum of four methyl states for each residue: unmethylated, mono-, di- or tri-methylation. Each distinctive histone modification has been associated with different manifestations in the eukaryotic genome. For example, H3K4 trimethylation (H3K4me3) is unequivocally connected with transcriptional activity and transcription initiation, and is generally enriched proximal to the transcriptional start site of actively expressed genes, while H3K27 trimethylation (H3K27me3) is broadly connected with gene silencing quieting. Moreover, H3K4me3, H3K27me3, and related histone imprints are thought to underlie differences of developmental states for pluripotency and differentiation.

It has been suggested that a bivalent chromatin state serves as a system to hold chromatin pliancy and to keep the cell/chromatin state balanced at the early phases of embryogenesis and development (Bernstein *et al.*, 2006; Mikkelsen *et al.*, 2007). As epigenetics and histone modifications may ultimately direct with cell fate, it has been suggested that epigenetic deviation may similarly drive the formation of tumor producing cells (Feinberg *et al.*, 2006). For example, Fraga and associates have reported that global loss of trimethylation H4K16 and H4K20 is a hallmark of malignancy (Fraga *et al.*, 2005).

1.6 The Sir2 family of histone deacetylases

The Sir2 family of histone deacetylases was first identified in budding yeast. Sir2p, a histone deacetylase, was first identified as product of a genetic screen, in which it was the second Silent Information Regulatory (SIR) factor to be identified. The term "sirtuin" refers broadly to Sir2 homologs, and is most commonly applied to mammalian genes and enzymes. The identifier Sir2p is used within the yeast *Saccharomyces cerevisiae* (where it was initially identified), while mammalian homologs are typically referred to as Sirt1-Sirt7, with Sirt1being the mammalian ortholog closest in structure and function to the budding yeast Sir2p.

In the budding yeast *Saccharomyces cerevisiae*, deacetylation of histones in constitutively heterochromatic chromosomal regions requires Sir2p. Sir2p is a highly conserved histone deacetylase with a unique enzymatic mechanism that requires consumption of the nicotine adenine dinucleotide (NAD) cofactor. In the fission yeast *Schizosaccharomyces pombe*, Sir2 family deacetylases are needed for the formation of heterochromatin, however the specific contributions of sirtuins in this process have not been exhaustively determined.

It has recently been demonstrated that *S. pombe* Sir2 efficiently deacetylates H3K4Ac and H4K16Ac *in vitro* and *in vivo*. In *sir2* deficient cells, epigenetic silencing at the mating type loci, telomeres, and centromeric innermost repeats (imr) is substantially defective, while quieting at the centromeric outermost repeats (otr) and rDNA is subtly diminished. Moreover, fission yeast Sir2 appears important for hypoacetylation and methylation of H3K9 within constitutively heterochromatic regions, and for the association of Swi6 with such genomic loci *in vivo*.

Moreover, studies in budding yeast have suggested that Sir2 may play an important role in the control of cellular lifespan (Kaeberlein *et al.*, 1999). More recent studies have implicated Sir2 as an attractive target for mediating lifespan extension in higher organisms. Sir2 homologs

have been found in an extensive variety of organisms ranging from archaea to man (Brachmann *et al.*, 1995).

The capacity for Sir2 mediated replicative life span extension in yeast mother cells has been hypothesized to relate to silencing of the rDNA. The integrity of the 100–200 coupled rDNA repeats on chromosome XII requires functional *SIR2*, as the recurrence of recombination at that locus increments around 10-fold in *SIR2Δ* mutants (Gottlieb and Esposito 1989). One of the results of rDNA recombination is accumulation of extrachromosomal ribosomal DNA circles (ERCs) that once formed appear to be maintained within the mother cell (Sinclair and Guarente, 1997). ERCs hence become increasingly prevalent in mother cells as they age and may ultimately trigger senescence. In accordance with this hypothesis, one role of SIR2 in promoting replicative lifespan extension may be to limit the accumulation rDNA circles within actively dividing mother cells by promoting and maintaining the structure of silent heterochromatin. Sir2p mediated silencing requires deacetylation of specific lysine residues in the amino-terminal tails of histones H3 and H4 (Thompson *et al.*, 1994; Hecht *et al.*, 1995; Braunstein *et al.*, 1996). These and different lysines moieties are acetylated in actively expressed chromatin yet are deacetylated in transcriptionally silent heterochromatin (Braunstein *et al.*, 1993, 1996). Deacetylated histones apparently may condense to form a more compacted nucleosomal structure that is less permissive to transcription by Pol II (Luger *et al.*, 1997). These contemplations prompted the proposal that Sir2 could be a histone deacetylase.

Another potential mechanism linking the NAD-dependent histone deacetylase Sir2 with longevity concerns the relationship between metabolic rate and organismal development (Weindruch *et al.*, 1986). If the metabolic rate of an organism is limited, for instance, by bringing down caloric admission, or by bringing down growth temperature for certain creatures,

lifespan may be extended (Finch, 1990). Interestingly, this connection breaks are not conserved among differing life forms. For instance, rodents and bats have comparable metabolic rates, yet bats may live as much as 10-fold longer. Accordingly, every species seems to have a predetermined rate of longevity, which may be further directed by the rate of metabolism within a given organism over its lifetime.

1.7 Fission yeast as a genetic model

Several traits of the fission yeast *Schizosaccharomyces pombe* make it an especially attractive organismal model for studies of chromosome function. Fission yeast has a genome of around 12 Mb partitioned between only three chromosomes. The relatively large fission yeast chromosomes have characteristics ordinary of higher eukaryotes, including expansive centromeres involving repetitive elements, conserved heterochromatin proteins and epigenetic silencing components, extensive replication origins, and conserved telomeric factors. Thus, fission yeast chromosome structure differs markedly in relation to budding yeast, in which a marginally bigger genome is packaged within 16 chromosomes. Budding yeast has smaller point centromeres, which exhibit poorly conservation with those of man, and arguably, a more constrained or divergent set of telomeric silencing machineries than does fission yeast. Accordingly, fission yeast has developed into a highly tractable model for studies of eukaryotic chromosome structure.

Similarly, fission yeast is a suitable model for studying the assembly of silent heterochromatin, as many of the factors required within this process appear to be conserved with those of man. This transcriptionally form type of chromatin has a characteristic set of epigenetic changes, most notably methylation of histone H3 lysine 9 (H3K9me). This methylation is

performed by the Clr4 methyltransferase, and which promotes binding by the H3K9me chromodomain found in Swi6 (human heterochromatin protein 1, or HP1 homolog). Within heterochromatic domains, H3K9 methylation and Swi6 binding "secures" the chromatin into a transcriptionally silent state. This is the fundamental model for the major heterochromatin spaces in fission yeast: the centromeres, the telomeres, the mating sort loci, and the ribosomal DNA (rDNA). Notwithstanding the observation that Swi6 and H3K9me are characteristic components of constitutive heterochromatin structure, silencing machineries that maintain heterochromatin structure in the distinct regions of the genome have diverse features. The heterochromatin in the centromere proximal region is disturbed amid each cell division cycle, during S phase. This permits a short window for expression of non-protein coding centromeric DNA elements. These transcripts are thought to be processed by the RNAi protein Dicer into short dsRNAs, which may ultimately direct a specific RNAi dependent transcriptional silencing complex, RITS, to the site of transcription. RITS contains an RNAi effector protein, Argonaut (Ago1), and a chromodomain protein (Chp1), and a structural linker (Tas3).

As opposed to *N. crassa* and higher eukaryotes such as man, the fission yeast *S. pombe* appears to fail to exhibit any discernible DNA methylation. Since gene depleted DNA as often corresponds with the vicinity of heterochromatin in different eukaryotes, the vicinity of redundant DNA at fission yeast centromeres may have structural functions, for example, serving as a template for promoting nucleation of heterochromatin structure. In fission yeast, gene silencing may be observed using phenotypic tests like those developed within the budding yeast *S. cerevisiae*. Multiple tools have been developed with which to investigate epigenetic phenomena in fission yeast. However, it is not yet clear why *S. pombe* may depend

fundamentally on RNAi for quieting and heterochromatin structuring, while lacking the higher the DNA methylation machineries found in higher eukaryotes (Barbara *et al.*, 2003).

The genus *Schizosaccharomyces* (fission yeasts) is profoundly established among ascomycetous fungi made up of several species, the genus has been known to science for more than 100 years (Madigan and Martinko, 2005). Of the *Schizosaccharomyces* species *S. pombe*, has been used most broadly for wide-ranging scientific studies within the past half century. For this reason *S. pombe* has come to be practically synonymous with the term "fission yeast" (two related species are *S. octosporus* and *S. japonicus*). To datate, *S. pombe* has established itself as one of the most popular model fungal models – arguably second in terms of its scientific prevalence only to the budding yeast *Saccharomyces cerevisiae*.

As a cellular model that divides by fission rather than polar budding, the specialized mitotic adaptation of fission yeast are moderately few, as such much of the fundamental cell science observed in *S. pombe* is essentially applicable for conserved cellular processes of metazoans. Moreover, the sequenced genome of *S. pombe* holds little evidence of an expansive genome duplication event of the sort that happened in budding yeast and numerous different life forms. This "lower" eukaryotic unicellular life form has ~4,800 putative open reading frames (ORFs), the among smallest number for a standard eukaryotic model and significantly fewer than the ~5,600 putative ORFs within the budding yeast. As the bacterium *Streptomyces coelicolor* has >7,800 putative ORFs, it seems remarkable that as a eukaryotic organism *S. pombe* may possess fewer genes than some prokaryotes.

Around two-thirds of the anticipated fission yeast proteins have homologs in both budding yeast and nematode, and just 14% are found in neither budding yeast nor nematodes (a proxy for higher eukaryotic models). 'All against all' arrangement examinations demonstrate that

4,515 putative ORFs among the aggregate 4,876 putative ORFS within fission yeast have no homologs within the *S. pombe* genome itself, while the remaining 361 ORFs may have multiple homologs within the *pombe* genome (Forsburg, 2003). By comparison, in budding yeast, 5,061 predictive ORFs are unique, while 716 fall into gatherings that have two or more parts. Accordingly, the fission yeast genome can in this manner be said to be more reductionist when compared to that of budding yeast. While has observation has been anecdotally known for some time, however now we can put a number on repetitive qualities (Forsburg, 2003). One feature of the fission yeast genome is that 43% of its genes have introns (which are frequently rather short, representing a sum of 4,730 introns), far more are found in budding yeast (Sabatinos *et al.*, 2010). This distinction was initially established on the basis of gene-by-gene investigations, and has since been borne out by genome wide analysis. From such evolutionary genomic data, the two yeasts are held to have diverged from one another around 300-450 million years ago, and from metazoa and plants around 1,000-1,200 years ago. Remarkably, since the rates of evolutionary divergence appear to be accelerated within distinct lineages, the yeasts *S. pombe* and *S. cerevisiae* are arguably as dissimilar from one another as either yeast is from higher eukaryotes (Walker, 1998).

The morphology of the two yeasts is likewise altogether different: unlike the ovoid mother and daughter budding patterns exhibited by *S. cerevisiae, S. pombe* has more cylindrically shaped cells that partition along the major cell axis The quality thickness for the complete genome of *S. pombe* is one quality each 2,528 base sets, as contrasted with one quality each 2,088 base sets for sprouting yeast. In the initial report of the sequencing of the *S. pombe* genome, Wood and colleagues noted that the most highly conserved factors among sequenced eukaryotes incorporate those encoding proteins needed for the cytoskeleton, vesicular

compartmentalization, cell-cycle control, proteolysis, protein phosphorylation and dephosphorylation, and RNA processing (Wood *et al.*, 2002). These qualities appear to be in this way to have started with the presence of eukaryotic life. An aggregate of at least 60 genes have been discovered to be highly conserved in all eukaryotes and which are not present in prokaryotes. Clearly, this observation represents an enticing point of consideration and one that warrants further study. Intriguingly, the evolutionary transition from prokaryotic to eukaryotic life forms may have occurred over a longer timer period than the transition from single to multi-cell eukaryotes (Wood *et al.*, 2002).

1.8 Minichromosome loss assay - theory and scope

In *S. pombe* an auxotrophic genetic reporter system commonly termed the minichromosome loss assay is used to study specific genetic manipulations which have detrimental effects on genome integrity. The assay provides a quantitative measure of minichromosome loss rate by rate at which cells containing an auxotrophic reporter gene within an extragenomic "minichromosome" exhibit characteristic color changes upon colony growth on nonselective medium. The minichromosome contains intact centromeric and telomeric DNA sequences, and provides for red white selection of cells containing the minichromosome, which contains the ade6-216 colored growth reporter in addition to other selectable auxotrophic markers such as *LEU2* or *ura4+* (Nimmo *et al.*, 1994; Allshire *et al.*, 1995).

For faithful chromosome segregation, kinetochores must be assembled on spindle microtubules and before anaphase onset (Tanaka, 2010). As cells enter mitosis, one of the two kinetochores of a sister-chromatid pair is at first caught by the sidelong surface of a microtubule nucleated from one of the two axle posts. Chromosomes are essentially recovered to the axle

shaft along the parallel surface of the microtubule by communication of the kinetochore with kinesin engines. In yeast, chromosomes might be recovered not just on the parallel surface of the microtubule by kinesin engines, but additionally by end-on connection to the depolymerizing microtubule end. Contemporary models of kinetochore function suggest required coupling of the kinetochore to the microtubule end by the DASH complex (Rieder and Alexander, 1990; Tanaka *et al.*, 2005, 2007; Franco *et al.*, 2007)

Transcriptional silencing is a general component for controlling the genome that can happen through modifications of substantial areas of chromatin structure. This phenomenon has been studied extensively in *Drosophila melanogaster*, in which situation of quality contiguous heterochromatic areas can bring about variegated gene expression, and in the yeasts *S. cerevisiae* and *S. pombe* (Weiler and Wakimoto, 1995). In *S. cerevisiae*, no less than three loci are known to be silenced: the mating-type loci, the telomeres, and the rDNA repeats (Loo and Rine, 1995; Sherman and Pillus, 1997; Lowell and Pillus, 1998). These silenced areas are inaccessible off to DNA-modifying enzymes and have an uncommon chromatin structure that is additionally unavailable to the transcriptional apparatus (Nasmyth, 1982; Gottschling, 1992; Singh and Klar, 1992; Kyrion *et al.*, 1993 Loo and Rine, 1994; Fritze *et al.*, 1997; Smith and Boeke, 1997; Singh *et al.*, 1998). Several *cis*- and *trans*-acting factors are required for transcriptional silencing in *S. cerevisiae*, including the four Silent Information Regulator (SIR) genes (Loo and Rine, 1995). Sir2 is extraordinary among the SIR genes in that it is needed for silencing at all three loci (Shore *et al.*, 1984; Ivy *et al.*, 1986; Aparicio *et al.*, 1991; Bryk *et al.*, 1997; Fritze *et al.*, 1997; Smith and Boeke, 1997; Sherman and Pillus, 1997).

It has been suggested that Sir2 controls level of histone deacetylation and accordingly drives the structural compaction of chromatin at constitutively heterochromatic loci (Braunstein

et al., 1993). However, higher order processes may also be involved. In fact, histones could be altered through acetylation as well as by methylation, phosphorylation, ubiquitination, and ADP ribosylation (Van Holde, 1989; Wolffe, 1992). Accordingly, it is conceivable if not probable that Sir2 may act not alone but as synergist directing control of one or more histone modifications at heterochromatic target loci.

The Sir2 homolog Hst4 was initially used in studies demonstrating the efficacy of the Ch16 minichromosome loss assay reporter system (Nimmo *et al.*, 1994). Ch16 bears the *ade6-216* transformation that supplements the *ade6-210* allele present at the chromosomal locus. Loss of the minichromosome in an *ade6-210* endogenous genomic background brings about red shaded yeast colony growth on standard rich yeast extract-containing (YE) plates supplemented with limiting amounts of adenine (not greater than 12 mg/L). The prevalence of chromosome loss is determined from the prevalence of half-sectored settlements, as depicted by Allshire *et al.* (1995). In the study presented by Nimmo et al, three separate isolates of the wild-type yeast strains and four isolates of the *hst4Δ* strains bearing the Ch16 minichromosome reporter were analysed, comprising a sum of 14,529 and 18,059 colonies, respectively. The mini chromosome loss assays were all performed at 30°C, though other studies have noted that chromosome loss is more pronounced at lower temperatures (Ekwall *et al.*, 1996). Indeed, the results reported here may reflect the temperature sensitive nature of the chromosome-loss phenotype.

Since the *hst4Δ* mutants had diminished viability related to loss of chromosomal integrity and centromeric silencing imperfections, it appeared plausible that the *hst4Δ* phenotypes may come about because of defects in chromatin structure, particularly at centromeres. No less than four genes known to be required for centromeric silencing are critical for chromosome integrity and centromere function. *clr4, rik1, swi6*, and *clr6* mutants exhibit chromosome-loss rates from

9-100 fold more prevalent than wild type strains (Allshire *et al.*, 1995; Ekwall *et al.*, 1995, 1996; Grewal *et al.*, 1998). To appraise whether centromeric silencing defects were connected with a defect in centromeric function in *hst4Δ* mutants, Nimmo and colleagues assayed maintenance of the Ch16 minichromosome by measuring colony color. This minichromosome reporter contains the *ade6-216* allele, which complements the chromosomal *ade6-210* allele in the *hst4Δ* mutant and wild type strains to promote functional adenine biosynthesis. In principle, cells that maintain the minichromosome throughout multiple cell divisions are *ade+* and grow to form white colonies, whereas loss of the minichromosome brings about red colony color. At the point when the chromosome loss occurs in the first cell divisions after plating on nonselective medium, colonies emerge that are both white and red, in variegated proportion.

To evaluate chromosome loss rates within the *hst4Δ* strain, Nimmo *et al.* crossed *hst4Δ* mutants with a strain containing the Ch16 minichromosome. Ch16-containing *hst4Δ* reporter strains lost minichromosomes in approximately 1.5% of cell divisions, a rate that was eightfold higher than in the wild type strain. In this way, *hst4Δ* was deemed critical for minichromosome maintenance and held to exhibit compromised genome integrity (Nimmo *et al.*, 1994).

Chapter 2: Materials and methods

2.1 Yeast strains and culture conditions

Schizosaccharomyces pombe also known as fission yeast is used as a model for eukaryotic genome function in part due to the relatively small size of its genome. The fission yeast genome is just 13.8 Mb in size and distributed in only three chromosomes containing single copy of any available gene. The small genome size of fission yeast makes it easy to manipulate the genome (Sabatinos and Forsburg.,2010). The fission yeast species are small in number compared to budding yeasts (*Saccharomyces cerevisiae*) and due to small genome size they are excellent to study cell differentiation, growth and division as well as chromosome dynamics and DNA replication. Fission yeast is used to study numerous cell biology problems and processes such as signal transduction, RNA splicing and cell morphology. Fission yeast shares some genes with humans that are not present in the budding yeast that makes them model experimental system (Sabatinos and Forsburg, 2010).

The fission yeast strains SPBA2, SPBA16, SPBA21 and SPBA3 were collected from "Alper lab" stocks that were stored at -80 ºC within a refrigeration unit kindly provided by the Department of Biology at Sacred Heart University. Strains SPBA2, SPBA16 and SPBA3 were stuck on freshly prepared YES medium (media preparation described below) and incubated for 2-3 days at 32ºC. The SPBA21 strain was initially struck on PMG-LEU (described below) to maintain the Ch16 *LEU2+* minichromosome, and incubated for 2-3 days at 32ºC. The collected strains and their genotypes are summarized in Table 1. The strains were stored by adding 1 volume of sterile 50 % glycerol to 1 volume of liquid PMG medium without. For storage of minichromosome reporter strains amino acid supplements were not added to the storage medium.

2.1.1 Preparation of malt extract (ME) medium

For preparation of 1 L of ME solution the components were added as malt extracts 30g, agar 20g, adenine 250mg, uracil 250mg, leucine 250mg, histidine 250 mg and arginine 250mg. The solution was autoclaved and poured in plates when still molten.

2.1.2 Preparation of yeast extract supplementary (ME) medium

For preparation of YES solution the following components were added in 4.8 L sterile water: glucose 150g, yeast extract 25g, arginine 1g, histidine 1g, leucine 1g, lysine 1g, and uracil 1g. The prepared medium was distributed in 10 bottles, each of them containing 480 mL of solution. Then added 10 g agar into each bottle and mixed well. The solution was autoclaved and stored at room temperature. These bottles were microwaved on demand. Adenine was added as a supplementary from liquid stocks.

2.1.3 Preparation of YES + geneticin medium

200 μg of geneticin (G418 disulfate salt, Sigma Life Science Co., St. Louis, MO) was dissolved in 20 mL diH2O and sterilized by filter sterilization technique. 1 mL of dissolved geneticin solution was added to each 500 mL of YES medium.

2.1.4 Preparation of YES-Ade medium

YES medium was prepared as described above, but without addition of adenine.

2.1.5 Preparation of *pombe* minimal glutamate (PMG) medium

For preparation of 4.5 L of PMG medium components were added as follows: 15.0 g phtalic acid, 11 g di-sodium orthophosphate, 18.75 g glutamic acid, and 100 g glucose. Then 5.0 mL of autoclave sterilized 1000X vitamins (1 g inositol, 1 g nicotinic acid, 0.5 g pantothenic acid and 1 mg biotin per 100 mL) were added. Then 0.5 mL of autoclave sterilized 10,000 X minerals (1 g H3BO3, 1 g MnSO4.4H_2O, 0.8 g $ZnSO_4$.7H_2O, 0.4 g $FeCl_3$•6H_2O, 0.3 g H_2MnO_4, 80 mg $CuSO_4$.5H_2O, 2 g citric acid, and 20 mg KI per 200 mL) were added. Afterwards 100 mL of 50X salts (26.75 g $MgCl_2$•6H_2O, 0.5 g $CaCl_2$•H_2O, 25 g KCl, and 1g Na_2SO_4 per 500 mL) were added. The prepared medium was distributed in 10 bottles, each of them containing 450 mL of PMG solution. For preparation of solid medium 10 g bacteriological agar was added in each bottle containing 450 mL PMG liquid medium. The pH of the medium was adjusted to 6.5 by addition of hydrochloric acid or sodium hydroxide. Bottles containing the PMG medium were then autoclaved at 121 ºC for minimum 35 min. Each bottle was supplemented with appropriate nutritional additives and/or stored under sterile conditions.

2.2 Mating and sporulation in fission yeast

For proficient mating of fission yeast the cells must arrest in the G1 phase under conditions of nitrogen starvation. Also, the mating and sporulation process of fission yeast is temperature sensitive and the G1 phase arrest of the cells is reduced with increasing temperatures. At high temperature most of the cells are arrested in G2 phase. Sporulation is restrained and the diploid formation is increased at high temperature. Thus, the recommended temperature for mating and sporulation is 25-29 °C (Sabatinos and Forsburg, 2010). *S. pombe* and *S. cerevisiae* require mostly same nutrients and culture conditions. However, they have

different growth rate and selective media. To test markers and ploidy the cultures are generally initiated on rich medium and replica plated. Different media are used for initial growth culture such as YE, YES or ME. Edinburgh minimal media 2 (EMM2) and its derivative pombe minimal glutamate (PMG) are synthetic and well defined media for experiments requiring nutrient stability. These media are suitable and used for maintenance of auxotrophic markers. To determine auxotrophy, appropriate supplements were added as required with simultaneous use of single dropout marker (Sabatinos and Forsburg, 2010). In order to generate single histone strains for use in the minichromosome loss assay strains were crossed as indicated in figure 1.

Manipulation of mating and sporulation of fission yeast is distinct from that of budding yeast. Fission yeast are most commonly either of two distinct mating types, h^+ and h^-. In fission yeast mating process and meiosis are united; thus, after crossing, sporulation proceeds spontaneously. Unlike budding yeast, which stably exist as both haploid and diploid karyotypes, care must be taken to maintain fission yeast diploids, i.e., through nutrient selection methods. In addition, upon mating, fission yeast diploids express both mating type genes, which means that haploid specific genes may be expressed in diploids also. These characteristics of fission yeast allow them to form tetraploids by mating of diploid cells (Sabatinos and Forsburg, 2010). In this study, mating and sporulation of fission yeast strains comprised a major component of the experimental methodology, and was performed in the manner described below.

2.3 Generation of yeast strains bearing single copies of histones H3 and H4, and Ch16

Using a sterilized coffee stirring stick, a small amount of an *h*+ strain was struck out on the side and in the middle of the ME plate. The portion plated to the side of the plate was used to assess self-mating, while the portion plated in the center was mixed with cells of the

complementary *h-* genotype. Then, a drop of sterile water was added in the middle spot in order facilitate the mixing with complementary strains. Using another sterile coffee stick, the *h-* strain was streaked on another side of same ME plate and then added on the drop of water containing already added *h+* strain. Both strains were mixed well and then incubated for 2-3 days in 32 ºC.

The first crossing between SPBA2, which lacks multiple auxotrophic markers, and SPB16 strain, which bears sole copies of genes encoding histones H3 and H4, generated *his+arg+* progeny bearing both H3.1/H4.1 and H3.3/H4.3 deletion. The spores of this crossing were first patched on nonselective YES medium, grown at 32 °C for 2-3 days, and subsequently replica plated upon PMG-ARG and PMG-HIS media. Progeny which grew on both PMG-ARG and PMG-HIS (*his+ arg+* strains) checked for mating type by PCR using primers oBA11 and oBA12, as described below (See table and section). The parents were used as – and + controls during. The results obtained by oBA17 and 29 primer showed that these progeny did as detected by using parents as – and + PCR controls. The sole histone status (H3.1/H4.1- H3.3/H4.3-) was checked by using oBA20 and oBA21 primer for H3.1/H4.1 and oBA22 and oBA23 primer for H3.3/H4.3 using the parents as positive and negative PCR controls. Neither locus H3.1/H4.1 nor H3.3/H4.3 was not amplified in PCR due to replacement with *his+* and *arg+* auxotrophic markers, respectively. Once the progeny status was confirmed with PCR, a single colony was isolated, spread on YES and incubated for 4-5 days in 32 ºC. To retrieve more amount single colony was spread again on another YES and incubated for 4-5 days in 32 ºC. To check auxotrophic status the progeny was grown in selective medium with suitable controls on PMG-ARG, and PMG-HIS. Strains were then numbered, catalogued and stored at 80 ºC. The cross described above generated strains SPBA31, strains SPBA32 and SPBA33.

Similarly, the second crossing was carried out between strains SPBA21 and SPBA3 strains to generate strain with minichromosome reporter. Both SPBA21 and SPBA3 are *his-arg-* Genotyping by PCR check was performed for parents by using oBA18 and oBA19 primers using pBA1 (plasmid without *LEU2*) pBA26 (plasmid with *LEU2*) as negative and positive controls, respectively. After the crossing the strains were replica plated onto PMG-HIS plate and PMG -ARG plates. The progeny that were able to grow in PMG-HIS and PMG- ARG plate were selected. Single colony was picked from these plates and streaked on PMC-LEU plate and incubated for 4-5 days in 32ºC. Then spread it again on PMG-LEU and incubated for 4-5 days in 32ºC. The growth was observed in selective medium with suitable controls on PMG -ARG, and PMG-HIS. In addition, the progenies were checked by PCR using oBA11 and oBA12 primer using parents as – and + PCR controls. Strains were then catalogued and stored at 80 ºC. This crossing generated strains SPBA34, SPBA35 and SPBA36.

SPBA35 and SPBA32, which were generated in first and second crossing, were used for the third crossing. After the crossing the spores were patched in LEU plates and replicated in PMG -ARG -HIS -LEU plates. The progeny which were his+, arg+ and leu+ were used for PCR check with oBA11 and oBA12 primer using parents as – and + PCR controls. They showed sole histone status (H3.1/H4.1 -H3.3/H4.3) from the PCR results with the primers oBA20 and oBA21 for H3.1/H4.1 status and primers oBA22 and 23 for H3.3/H4.3 status using the parents as positive and negative controls. The progeny was checked by PCR for *LEU2* status with oBA18 and oBA19 primer using pBA1 (plasmid without *LEU2*) as negative and PBA26 (plasmid with *LEU2*) as positive controls in addition SPBA3 as negative control and SPBA21 as positive control. The mating type of progeny was checked by PCR using oBA11 and oBA12 primers using parents as – and + PCR controls. A single colony was picked from these plates, streaked on

a PMG-LEU plate and incubated for 4-5 days in 32 ºC. Then it was spread it again from a single colony on PMG-LEU and incubated for 4-5 days in 32ºC. The growth was observed in selective medium with suitable controls on PMG -ARG, and PMG-HIS. Progeny serial number was recorded for each strain and the strains were stored -80 ºC. This crossing generated strains SPBA50, SPBA51, SPBA52 and SPBA53.

The last crossing was carried out between SPBA50 strain generated from the third crossing and SPBA11 strain collected from the lab. This crossing was carried out to generate sir2D strain with minichromosome reporter. After the crossing the spores were patched and replicated in PMG-HIS, PMG- ARG, PMG-URA and YES + Geneticin plates. The progenies with genotype His+, Arg+, Ura+ and GEN+ were generated and named as SPBA58 and SPBA59. This crossing also generated progeny with genotype His+, Arg+, Ura- and GEN+ named as SPBA60. The progeny with genotype His+, Arg+, Ura+ and GEN+ was named as SPBA61. The mating type of progeny was checked by PCR using oBA11 and oBA12 primers using parents as – and + PCR controls. PCR was conducted to determine the sir2 status. The oBA10 and oBA15 primers were used for PCR with SPBA11 as negative and SPBA50 as positive control. PCR results confirmed that progenies SBPA 58, SPBA59, SPBA60 and SPBA61were negative for *sir2*. Single colony was picked from these plates and streaked on PMC-LEU plate and incubated for 4-5 days in 32ºC. Then spread it again on PMG-LEU and incubated for 4-5 days in 32ºC. The growth was observed in selective medium with suitable controls on PMG -ARG, PMG-HIS, PMG-URA and YES +GEN. Strains were then numbered, catalogued and stored at 80 ºC. This crossing generated strains SPBA58, SPBA59, SPBA60 and SPBA61.

2.4 Morphological identification of yeast strains

Fission yeast divides by medial fission and appears as rod shaped cells during vegetative growth. Strains that successfully met produce banana0shaped or zig-zag zygotes, which can be observed under microscope. The cells are generally 8–14mm in length and 4 mm in width. They mainly grow in length compared to width (Sabatinos and Forsburg,2010). For identification the strains were observed under microscope at 40x power to check their appearance. The strains that showed successful crossing were used in the next step for glusulase treatment.

2.5 Glusulase treatment and random spore analysis

The glusulase treatment degrades the cell wall and separates clumps of cells that are held together by cytoplasmic membranes. This help for identification of the cells. For instance, bud and its parent cell appear as two distinct cells after completion of cytokines is even if they have not completed cell wall separation (Hartwell et al., 1973). Random spore analysis (RSA) is commonly employed technique for analysis of large numbers of spores. For random spore analysis the asci are incubated in diluted glusulase solution to release spores. The glusulase treatment kills the vegetative cells that make it more efficient for identification. These techniques also reduce the need for overwhelming tetrad analysis (Sabatinos and Forsburg, 2010).

2.5.1 Prepration of Glusulase

200 µg of Glusulase was dissolved in 20 mL diH2O and sterilized by filter sterilization technique. After mating, a small amount swage of crossing was added to 300 µl of glusulase containing liquid medium and incubated overnight at 37 ºC. In the next morning, the overnight treated samples were centrifuged for 1 minute at high speed. The supernatant was discarded and

the cells were resuspended in 500 µl of sterile water and mixed well. Then centrifuged again and the supernatant was discarded. The cells were resuspended again in 500 µl of sterile water and used for serial dilution. The collected sample was serially diluted as 1:2, 1:20 and 1:200. The diluted sample of each concentration was plated in separate plate in appropriate medium and incubated at 32 ºC for 3to5 days.

2.5.2 Strain selection for auxotrophic markers

The most commonly used auxotrophic markers for fission yeast are involved in adenine, glutamic acid, histidine, leucine, lysine, and uracil biosynthesis. Some additional markers are also available. Prior to auxotrophic growth selection most strains were struck out for growth as single colonies on YES. Afterword, the colonies were replica plated on minimal growth medium with and without supplement and incubated for 2 to 3 days. After incubation, the plates were investigated for growth (Moreno, Klar and Nurse,1991).

The desired spores were screened from the spores collected with random spore analysis by growing in selective media. Around 100 colonies were picked from the random spore plates and incubated in the same medium at 32 ºC for 3 to 4 days. After incubation the patch plate was replicated using velvet clothe in selective growth medium such as PMG-LEU, PMG–ARG, PMG–HIS, PMG-ADE, PMG-URA and YES+GEN. Afterword the plates were incubated at 32 ºC for 3 to 4 days. The incubation duration was adjusted as per the requirement for example, PMG –LEU medium was kept for 8 to 10 days. Then single colony was selected, spread on another plate and then incubated at 32 ºC for 4 to 6 days to collect the desired progeny. From each crossing around 8 progeny were selected to increase the probability of obtaining desired strain.

2.6 Minichromosome loss assay

The strains SPBA58 and SPBA59 were used to investigate the effect of *sir2* deletion by minichromosome loss assay. The wild type strain SPBA46 and SPBA49 (*swi6D*) were used as negative and positive controls for minichromosome instability, respectively. All strains were propagated on minimal media lacking leucine (PMG-LEU) to maintain the minichromosome [Ch16 *LEU2*+]. A small amount of yeast (<20 μg) was dissolved in 5 mL of liquid PMG medium without any additional supplement, and resuspended in solution. The inoculation and resuspension process was performed using sterile technique in order to avoid possible contamination. The resuspended sample was diluted with liquid PMG at a ratio of 2:3 mL. The optical density was measured at 600 nm using the spectrophotometer to determine approximate cell concentration.The samples were serially diluted by adding 100 μl of the sample with 900 μl of sterile water. The samples were diluted to get the concentration of 5.0 x 10^3 cells/mL. Each strain was placed on PMG limited ADE and YES-ADE and incubated at 32 ºC for 5 days. After incubation the plates were placed at 4 °C for 2 to 3 days to intensify the red color. The % loss of minichromosomeswas determined by counting around 1000 colonies for each strain. A schematic depiction of the principle for the minichromosome assay is depicted in Figure 2.

2.7 Genetic screening by polymerase chain reaction (PCR)

Polymerase Chain Reaction (PCR) is used for a variety of tasks, such as molecular cloning, the detection of hereditary diseases, the identification of genetic fingerprints, and paternity testing. Characteristically, PCR comprise of a series of 20 to 40 repeated temperature cycles. Each of these cycles has 2 to 3 distinct temperature steps. Before the cycling steps a

"hold" step is generally added that hold the thermal cycles at high temperature (usually 90 - 96 °C). The temperature used in each cycle and the period of time depends on a numerous parameters. These parameters include the type and amount of DNA polymerase used, the concentration of salt and metal ions, the working concentration of deoxynucleotide triphosphates (dNTPs) within the PCR reaction mixture, and the melting temperatures of oligonucleotide primers employed.

In order to determine whether the colonies generated within this study bore specific genetic traits, strain genotypes were evaluated by PCR. The primers that were used for PCR are summarized in Table 2, along with their sequence and genomic targets.

2.7.1 Lyticase treatment of *S. pombe*

200 µg lyticase from *Anthrobacter lutease* (Sigma Aldrich Co., St. Louis, MO) was dissolved in 20 mL diH2O and sterilized by filter sterilization technique. A toothpick-tip sized swadge (<5 µg) of yeast cells were treated with 15 µL lyticase for 60 minutes at 37°C to liberate genomic DNA. Then the samples were heated at 100 °C for 2 minutes to stop the enzymatic reaction, and the enzyme yeast mixture was diluted with (15 µL) sterilized water.

2.7.2 PCR and thermal cycling conditions used in this study

The PCR mixture was prepared by mixing lyticase treated cells (2µl) with 10 µL of 2X PCR Pre Mix (Syd Labs Inc., Boston MA). Then 4 µL of sterilized water and 2 µl of each primer (0.25 µM final) was added to the reaction mixture. The PCR was performed using a protocol consisting of 30 cycles. Each thermal cycle consisted of an initial DNA denaturing step (95 °C x 30 sec.), followed by an annealing step (55 °C x 10 sec.) and a DNA polymerase mediated

extension step (72 °C x 1 min). Thermal cycling was performed within a BioRad Gene Cycler Thermal Cycler (BioRad Inc., Hercules CA).

2.8 Agarose gel electrophoresis

Agarose gel electrophoresis is used to separate, identify, and purify DNA fragments on the basis of size. Agarose gels are prepared by melting the solid agarose in a buffered solution, until the solution becomes transparent. The melted solution is then poured into a tray and allowed to solidify. After cooling the agarose forms an extensively cross-linked sieving matrix. The density of the matrix depends upon the concentration of the agarose. Upon application of an electric field the negatively charged DNA migrate towards the anode. The DNA fragments present in the gel are stained with ethidium bromide that produce glow in UV light. This allows determining the location of DNA fragments within the gel. Very small amount of DNA (1 ng) can be detected by this method with direct visualization of the gel in ultraviolet light (Sambrook and Russell, 2001).

2.8.1 Preparation of TBE buffer

1 LTBE agarose gel electrophoresis buffer (10X) was prepared by adding 121.1 g Tris base, 61.8 g Boric acid and 7.4 g EDTA (disodium salt) in sterile water.

2.8.2 Preparation of ethidium bromide for DNA detection

Ethidium bromide (10,000X) was prepared for agarose gel pre-stain. For preparation of 1 mg/mL solution, 30 mg Ethidium bromide was added in 30 mL sterile ultrapure water.

2.8.3 Preparation of 1% agarose gel slab for use in gel electrophoresis

1 g molecular biology grade agarose (US Biological Co., Salem MA) was measured and added to a glass Erlenmeyer flask, to which 100 mL TBE Buffer was previously added, and mixed thoroughly. Agarose was then melted in a microwave until the solution became clear, and the solution was allowed the solution to cool to approximately 50 °C (warm to touch, but no longer visibly vaporizing). Ethidium bromide (5-10 µl of the 10,000X stock) was added to the solution melted agarose gel and mixed well. Then the ends of the casting tray were sealed and the melted agarose solution was poured into the casting tray. The combs were inserted at appropriate place to form the wells. Once the gel is solidified the combs were pulled out carefully. The gel was placed in to the electrophoresis chamber and sufficient amount of 1X TBE buffer was added to submerge the gel completely

2.8.4 Agarose gel sample loading

Each sample was loaded in separate wells in to the gel. Similarly, DNA ladder was loaded in to the gel. Turned on the power supply and DNA fragments were resolved by electrophoretic separation at 100 V for 40 min.

2.8.5 DNA visualization by UV transillumination

After permitting sufficient electrophoretic separation, the gel electrophoresis power supply was turned off and the gel was removed from the electrophoresis chamber while wearing gloves gloves. The gel was visualized using a UV transilluminator (Edvotek Inc., Washington DC) and photographed using a digital camera. The sizes of the DNA bands were estimated by comparing them with the DNA ladder, where applicable.

Chapter 3: Results

Genetic crosses used to generate strains analyzed within this study are shown in Figure 1. Strain genotypes and primers used in strain validation are shown in Tables 1 and 2, respectively. Figure 2 presents the theoretical basis of the minichromosome loss assay. PCR Validation of strain genotype is presented in Figure 3.

The impact of *sir2* deletion on genome integrity was assessed using the minichromosome loss assay. As shown in Figure 4A, all experimental strains tested gave rise to pink and white colonies over the course of the experiment. The total numbers of colonies were counted as presented in Table 3. This data was in turn used to calculate the percentage of colonies that appeared pink for each cell background, as shown in Table 4. All experiments were performed in duplicate; values shown in Figure 4B represent experimental averages plus or minus standard error of the mean, where $n = 2$ experimental replicates.

As shown in Figure 4, wild type yeast bearing the Ch16 minichromosome loss reporter (SPBA46) gave rise to 18.9 % pink colonies over the course of the experimental assay when cultured on non-selective adenine-limited medium. This value was limited in comparison to yeast lacking the heterochromatin factor Swi6 (SPBA49), which showed 59.4% of pink colonies under equivalent culture conditions. Intriguingly, strains which lacked Sir2 (SPBA58 and SPBA59) showed presence of 64.9% and 61.1% pink colonies respectively, indicating an increased rate of minichromosome loss relative to wild type controls.

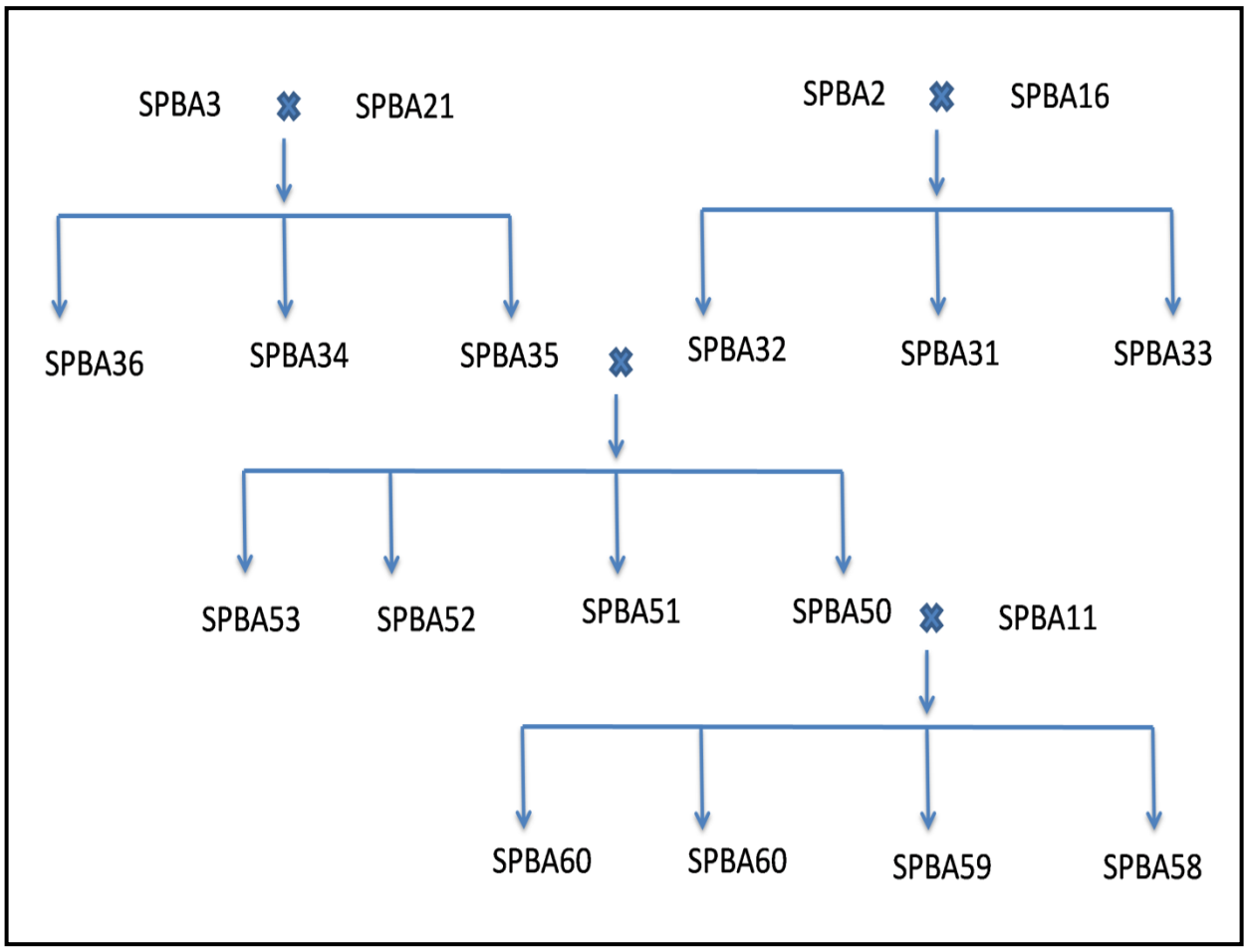


Figure 1: Schematic representation of fission yeast crosses used in this study.

Legend for Figure 1: Schematic representation of fission yeast crosses used in this study. Parent strains and progeny are indicated. Parental crosses are indicated by an "x", with hereditary lineages indicated by lines and arrows. Note that genotypes for the strains indicated are further described in Table 1.

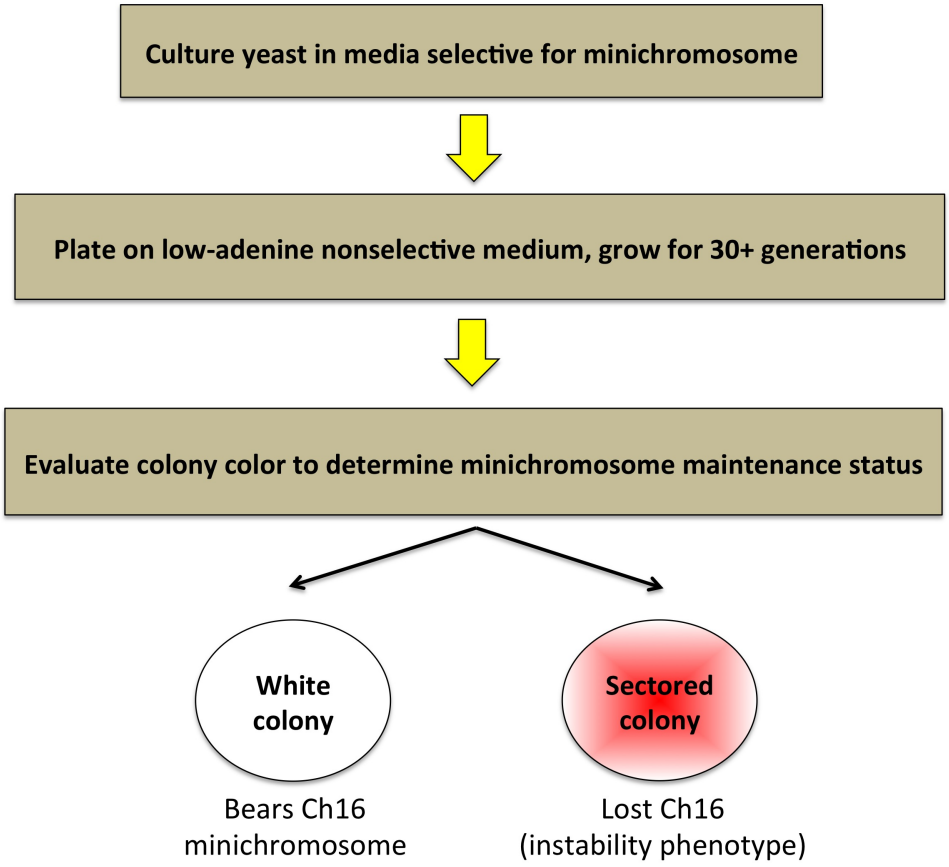
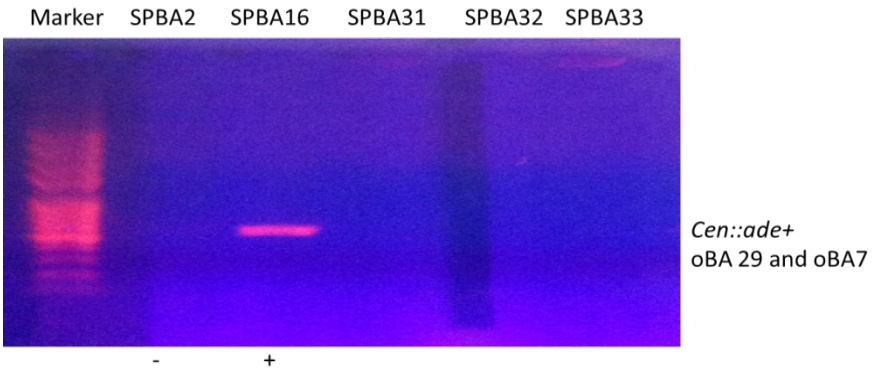


Figure 2. Principle of the minichromosome loss assay

Legend for Figure 2: Principle of the minichromosome loss assay. All strains were propagated on minimal media lacking leucine (PMG-LEU) to maintain the linear minichromosome [*Ch16 ade6-216 LEU2+*], which contains centromeric and telomeric DNA sequence elements from *S pombe* chromosome 3 and two auxotrophic selection markers. After selective growth on PMG-LEU, each strain was placed on YES medium with limiting amounts of adenine and incubated at 32 ºC for 5 days. After growth, pink and white colonies were counted.

A.



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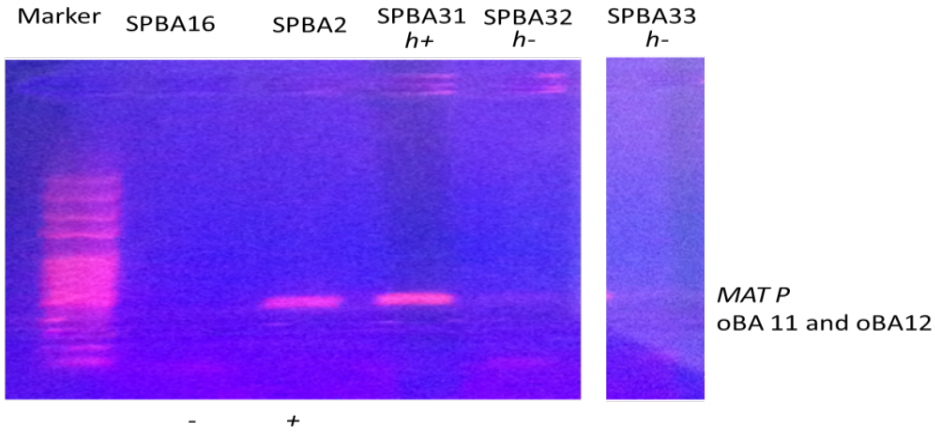


C.

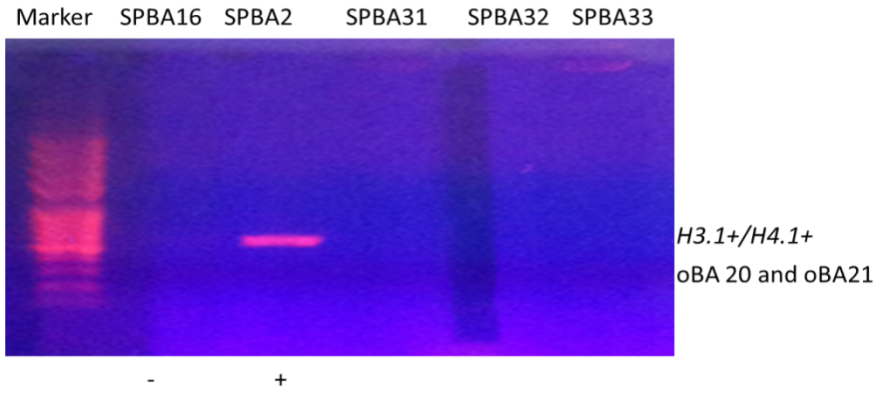
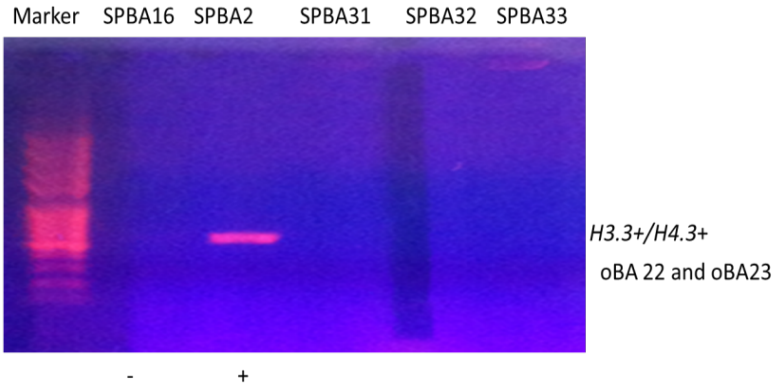


Figure 3: PCR analysis of strains used in this study

D.



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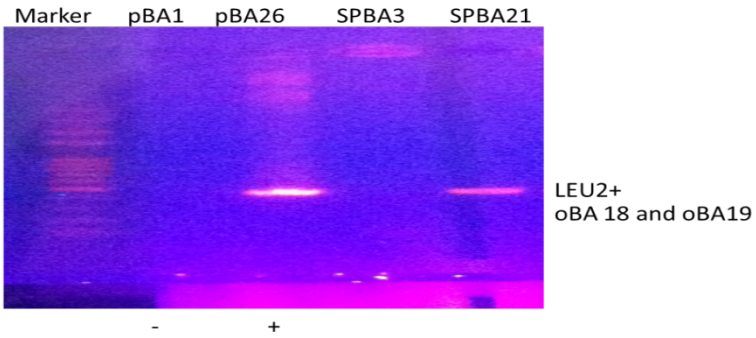

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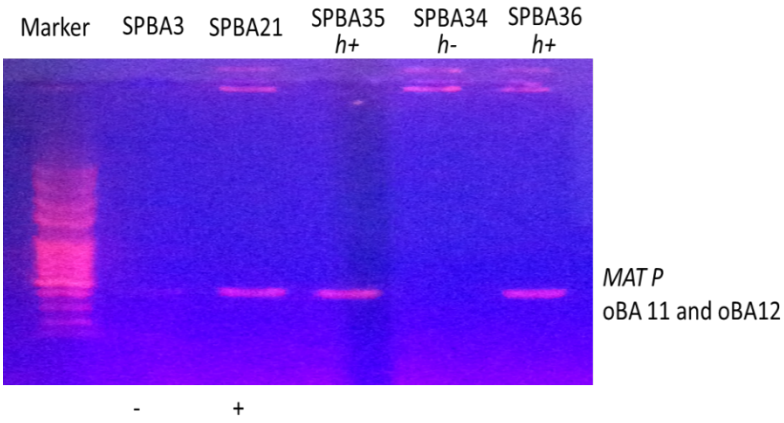
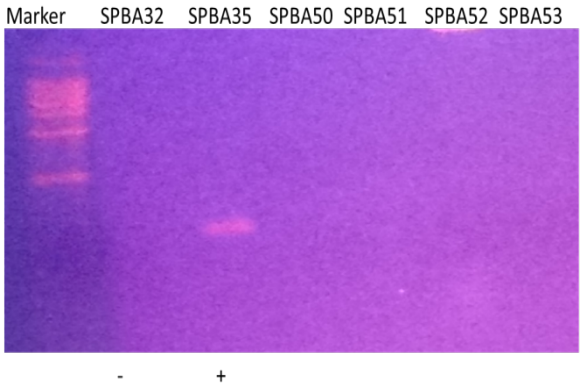


Figure 3: PCR analysis of strains used in this study (ctd.)

G.



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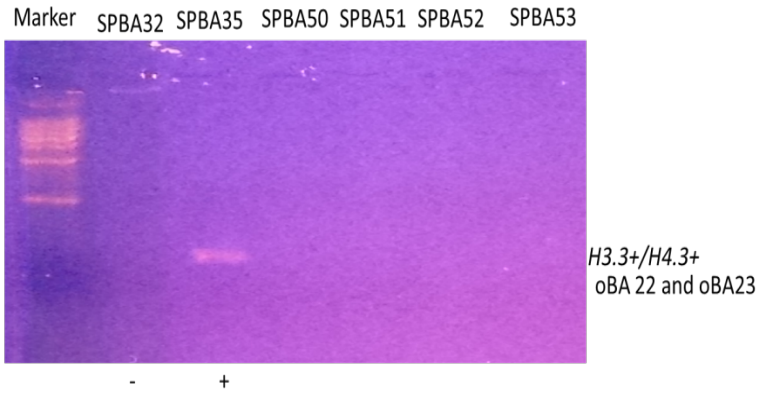

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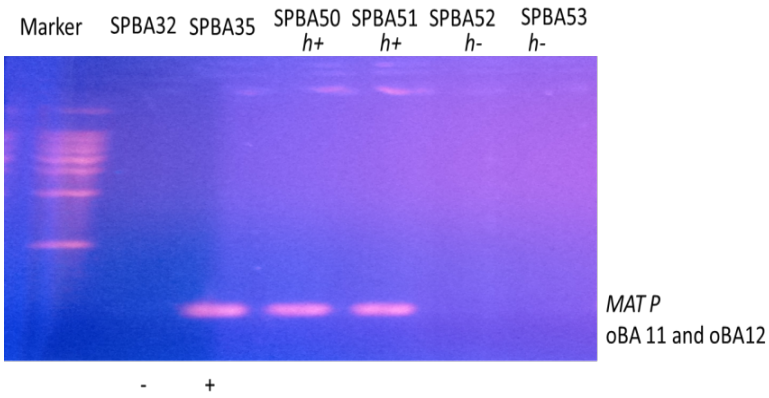
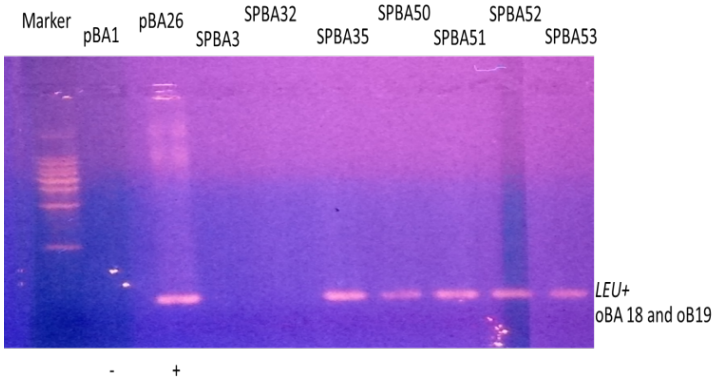


Figure 3: PCR analysis of strains used in this study (ctd.)

J.



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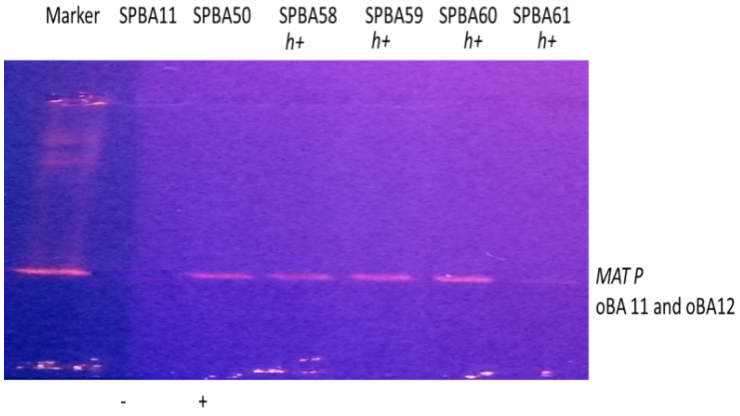


L.

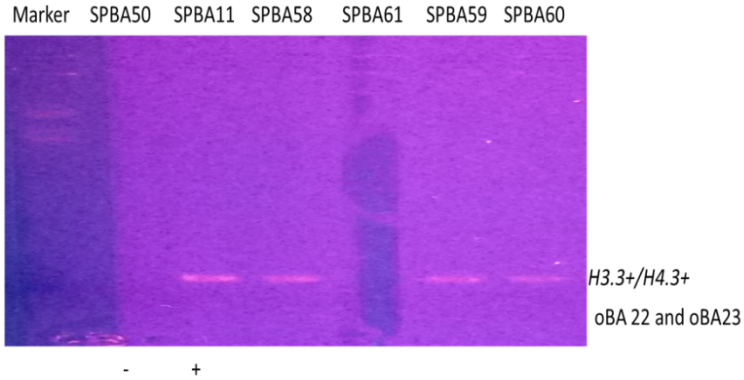
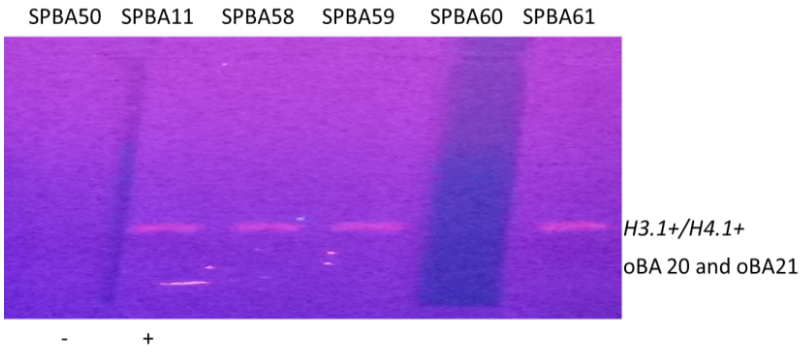


Figure 3: PCR analysis of strains used in this study (ctd.)

M.



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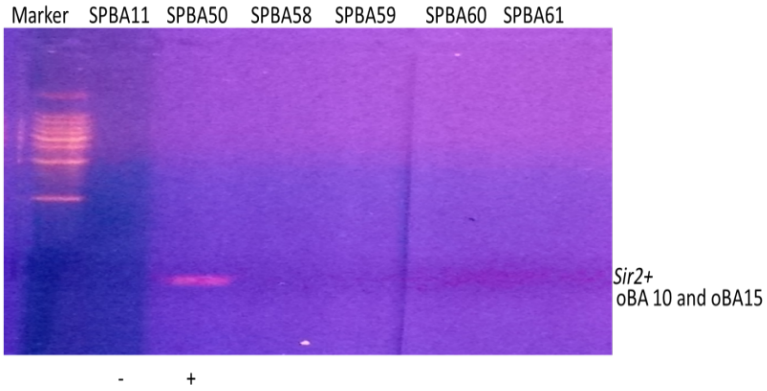
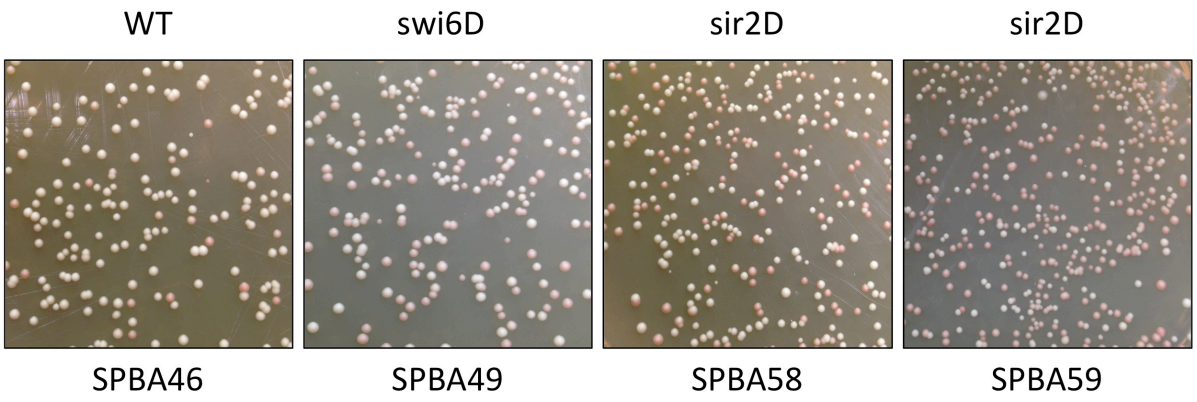


Figure 3: PCR analysis of strains used in this study (ctd.)

Legend for Figure 3: PCR analysis of strains used in this study. PCR products were resolved by agarose gel electrophoresis, stained using ethidium bromide, and subject to UV transillumination. Experimental details may be found within the materials and methods. Applicable genotypes and oligonucleotide primer sequences are summarized in Tables 1 and 2, respectively. **A. PCR check for *ade6* gene inserted within the outer repeats of centromere 1 (*cen::ade6*).** SPBA2 and SPBA16 served as negative and positive controls, respectively. **B. PCR check for *MAT1p*.** SPBA16 and SPBA2 served as negative and positive controls, respectively. **C. PCR check for the intact *H3.1+/H4.1+* locus.** SPBA16 and SPBA2 served as negative and positive controls, respectively. **D. PCR check for the intact *H3.3+/H4.3+* locus.** SPBA16 and SPBA2 served as negative and positive control, respectively. **E. PCR check for *S. cerevisiae LEU2*.** Plasmids lacking or bearing the *S. cerevisiae LEU2* gene (PBA1 and PBA26) served as negative and positive controls, respectively. **F. PCR check for *MAT1p***. SPBA3 and SPBA21 served as negative and positive controls, respectively**. G. PCR check for the intact *H3.1+/H4.1+* locus**. SPBA32 and SPBA35 served as negative and positive controls, respectively. **H. PCR check for the intact *H3.3+/H4.3+* locus.** SPBA32 and SPBA35 served as negative and positive controls, respectively. **I. PCR check for *MAT1p***. SPBA32 and SPBA35 served as negative and positive controls, respectively**. J. PCR check for *S. cerevisiae LEU2*.** Plasmids PBA1 and PBA26 served as negative and positive controls, respectively. **K. PCR check for *MAT1p***. SPBA11 and SPBA50 served as negative and positive controls, respectively. **L. PCR check for the intact *H3.3+/H4.3+* locus.** SPBA50 and SPBA11 served as negative and positive controls, respectively. **M. PCR check for the intact *H3.1+/H4.1+* locus**. SPBA50 and SPBA11 served as negative and positive controls, respectively. **N. PCR check for *sir2+***. SPBA11 and SPBA50 served as negative and positive controls, respectively.

A.



B.

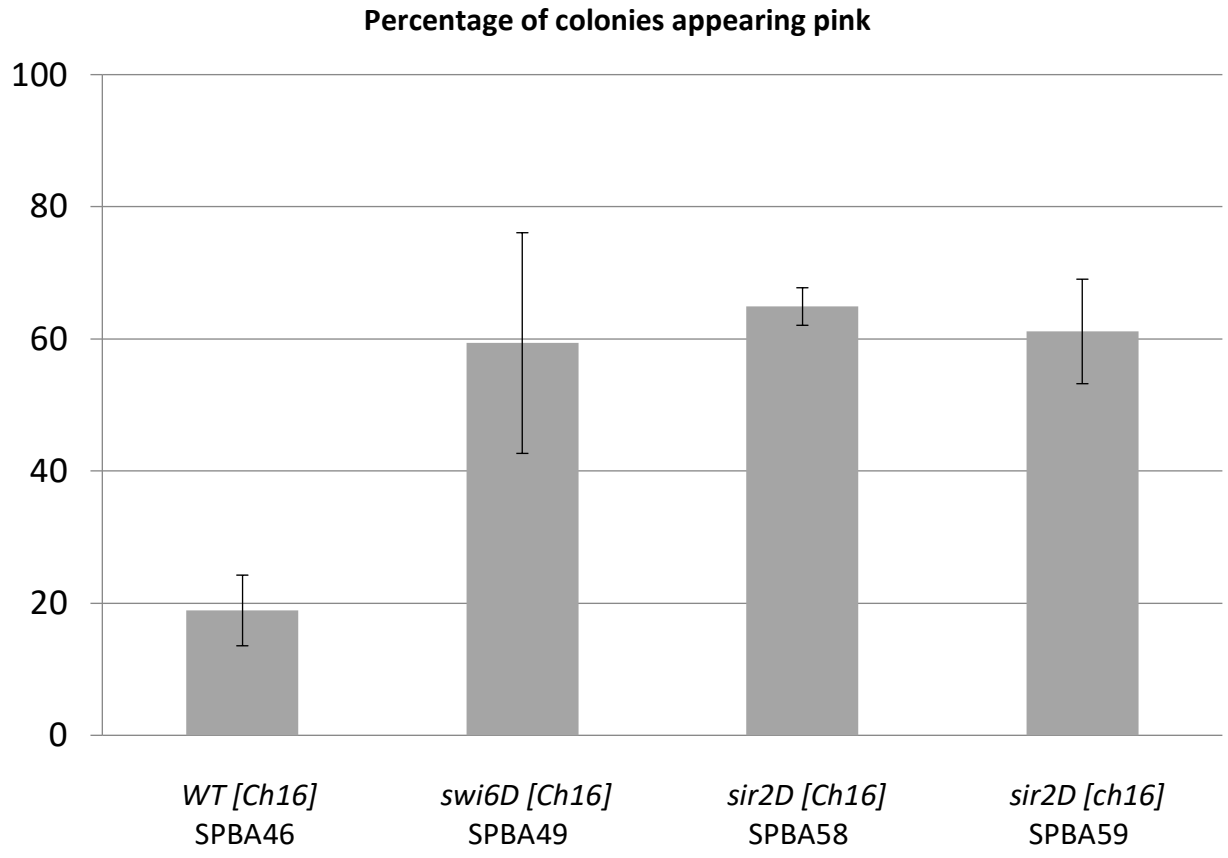


Figure 4: Results from the minichromosome loss assay

Legend for Figure 4: Results from the minichromosome loss assay. A. Photographs of experimental cell cultures. Cells of the genetic background indicated were cultured on nonselective medium containing limiting amounts of adenine for 5 days at 32 °C and then photographed using a digital camera. Additional details of the experimental methodology may be found within the materials and methods. Pink colony growth is indicative of defective adenine biosynthesis. **B. Quantitation of colored colonies.** Total numbers of pink and white colonies were counted. Data presented in Tables 3 and 4 data was used to construct the graphs in panel B. Each vertical bar represents the percentage of observed pink colonies grown on adenine-limited medium. Error bars indicate standard error of the mean (n=2 experimental replicates).

Strain	Genotype
SPBA2	*h+ ade6-210 arg3-D4 his3-D1 leu1-32 ura4-D18*
SPBA3	*h- ade6-210 arg3-D4 his3-D1 leu1-32 ura4-D18*
SPBA11	*h- sir2D::kanMX6 otr1R(SphI)::ura4+ ura4-DS/E ade6-210 leu1-32 his3D*
SPBA16	*h- H3.1/H4.1::his3 H3.3/H4.3::arg3 ade6-210 otrsph1::ade6 his3D arg3D*
SPBA21	*h- ade6-210 leu1-32 ura4DS/E [Ch16 ade6-216 m23::LEU2+]*
SPBA31	*h+ H3.1/4.1::his3+ H3.3/4.3::arg3+ his3D arg3D ura4D-18 leu1-32*
SPBA32	*h- H3.1/4.1:: his3+ H3.3/4.3::arg3+ his3D arg3D ura4D-18 leu1-32*
SPBA35	*h+ ade6-210 arg3-D4 his3-D1 leu1-32 ura4- [Ch16 ade6-216 m23::LEU2+]*
SPBA46	*h- ade6-210 leu1-32 ura4DS/E [Ch16 ade6-216 m23::LEU2+]*
SPBA49	*swi6D::arg3+ ade6-210 arg3D his3D leu1-32 ura4DS/E [Ch16 ade6-216 m23::LEU2+]*
SPBA50	*h+ H3.1/4.1::his3+ H3.3/4.3::arg3+ his3D arg3-D4 ade6-210 leu1-32 ura4- [Ch16 ade6-216 m23::LEU2+]*
SPBA54	*h+ ade6-210 arg3-D4 his3-D1 leu1-32 ura4- [Ch16 ade6-216 m23::ura4]*
SPBA58	*h+ sir2D::kanMX6 otr1R(SphI)::ura4+ ura4-DS/E ade6-210 leu1-32 his3-D arg+ [Ch16 ade6-216 m23::LEU2+]*
SPBA59	*h+ sir2D::kanMX6 otr1R(SphI)::ura4+ ura4-DS/E ade6-210 leu1-32 his3-D arg+ [Ch16 ade6-216 m23::LEU2+]*

Table 1: Yeast strains used in this study

Oligo	Sequence	Target
oBA12	TGTTTAGCGCACTTTGATTTTCCAGTC	*S. pombe MAT1p*
oBA13	CTTCAATTCTCACAAAACCGC	*S. pombe MAT1p*
oBA16	GGCGACCATAGACATAACTG	*S. pombe ade6*
oBA17	CTACTCTTCTCGATGATCCTGTA	*S. pombe cen1 OTR*
oBA20	GGCGACGCTGTCTATTTGTT	*S. pombe h3.1* -211->-191
oBA21	ATGGCAGAAGATTGGAAACG	*S. pombe h3.1* +269->249
oBA22	TACCACGGCCAGACATATTG	*S. pombe h3.3* -252->-232
oBA23	GACCAAGCGTTGGAAAGGTA	*S. pombe h3.3* +216->196
oBA18	CTAAAGGTACTGACTTCGTTGTTG	*S. cerevisiae LEU2*
oBA19	GGTGTTCTTGTCTGGCAAAGAGG	*S. cerevisiae LEU2*
oBA10	ACAATTCAACGCGTCTGTGAG	*KANMX6* cassette
oBA15	GATCTTCAGTTCAGTAGATCATTGCC	*S. pombe sir2*

Table 2: Oligonucleotide primers used in this study

Genotype	Strain	Pink Colonies	White Colonies
WT [Ch16 ade6-216 m23::LEU2+]	SPBA46	64 141	406 440
swi6D::arg3 [Ch16 ade6-216 m23::LEU2+]	SPBA49	184 540	247 170
sir2D::kanMX6 [Ch16 ade6-216 m23::LEU2+]	SPBA58	408 480	281 200
sir2D::kanMX6 [Ch16 ade6-216 m23::LEU2+]	SPBA59	513 330	230 290

Table 3: Quantitation of colony color in minichromosome loss assay

Genotype	Strain	% Colonies Pink	SEM
WT [Ch16 ade6-216 m23::LEU2+]	SPBA46	18.9	5.3
swi6D::arg3 [Ch16 ade6-216 m23::LEU2+]	SPBA49	59.4	16.7
sir2D::kanMX6 [Ch16 ade6-216 m23::LEU2+]	SPBA58	64.9	5.7
sir2D::kanMX6 [Ch16 ade6-216 m23::LEU2+]	SPBA59	61.1	7.9

Table 4. Percentage of colonies appearing pink in minichromosome loss assay

Standard error of mean, SEM = standard deviation from experimental data / $\sqrt{n}$

Where n = number of experimental replicates.

Chapter 4: Discussion and conclusion

The Ch16 minichromosome loss assay is held to represent a means for investigating the contribution of specific genes to chromosome segregation in *S. pombe* (Allshire et al., 1995). When Chl6 is lost from dividing cells, resultant progeny for pink rather than white colonies on rich medium containing limiting amounts of adenine. If minichromosome loss occurs in the first division, then half of the colonies retain the minichromosome, whereas the other half lacks the minichromosome. In this experiment, the minichromosome loss was compared for wild type yeast strains bearing the Ch16 minichromosome, as well as strains bearing deletion of the heterochromatin factor *swi6* and *sir2* histone deacetylase deletion mutants.

In this study, we observed that wild type cells bearing the Ch16 reporter appear to exhibit a decreased rate of minichromosome loss relative to strains lacking either swi6 or sir2. Earlier studies have demonstrated that the heterochromatin protein Swi6 is involved in silencing at constitutively heterochromatic mating type loci, telomeres and centromeres (Allshire et al., 1995). Here, we have demonstrated that like Swi6, Sir2 appears to play an important role in promoting mitotic stability of the Ch16 minichromosome. However, these results remain to be validated, and the potential mechanism of action for Sir2 within this process remains to be established. We therefore hypothesize that Sir2 may have roles in promoting genome integrity that have yet to be conclusively determined. Future studies will thus seek to determine the role of Sir2 in chromosome segregation and genome integrity in greater detail.

Chapter 5: References

Chapter 1: Introduction

1.1 Chromosome structure and function in eukaryotes

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Chapter 2: Materials and Methods

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