The Role of CAF-1 in Epigenetic Conversions at the Telomeres of Saccharomyces cerevisiae and its Control by Protein Kinases

by

Daniel Charles Barrie Jeffery

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ABSTRACT

THE ROLE OF CAF-1 IN EPIGENETIC CONVERSIONS AT THE TELOMERES OF SACCHAROMYCES CEREVISIAE AND ITS CONTROL BY PROTEIN KINASES

Daniel Charles Barrie Jeffery
University of Guelph, 2014

Advisor:
Professor K. Yankulov

Epigenetics is the study of heritable changes in gene expression that are not caused by changes in the DNA sequence. The expression profile of a given cell is regulated by the complex interactions of histone post-translational modifications, DNA methylation, nuclear organization and ATP-dependent histone exchange, as well as a multitude of interacting factors that come along with them. The inheritance of these epigenetic marks that are associated with silenced or active DNA are regulated by multiple mechanisms to ensure that they are faithfully copied after DNA replication. CAF-1 is a central figure in this process that reassembles nucleosomes at the replication fork. However, a mechanism for epigenetic conversions is necessary for cells to undergo differentiation and alter the epigenetic states of certain genes. This mechanism is currently unknown.

In this thesis, I use the telomeres of S. cerevisiae as a model to study the mechanism of epigenetic conversions. I developed an assay for the quantitative assessment of the frequency of epigenetic conversions in telomere position effect (TPE) variegation. Then, using this assay, I demonstrated that the deletion of CAC1, the largest subunit of the CAF-1 complex, causes a major reduction in the frequency of telomeric epigenetic conversions, indicating that CAC1 is a key factor in this process. I also present evidence that the cell cycle regulator CDK (Cdc28p) is a major regulator of CAF-1. I showed that CDK phosphorylates Cac1p at the S94 and S515 residues to recruit it to chromatin. Finally, I demonstrated the role of sub-telomeric elements in the control of TPE, showing that Core X and Y’ elements suppress extreme variations in gene silencing at telomeres.

With these data, I propose a model for the control of epigenetic conversions at regions of position effect variegation (PEV). This research represents the first comprehensive analysis of epigenetic conversions in a model organism and links epigenetic maintenance and conversions to cell cycle.
Dedication

This thesis is dedicated to my incredible wife, Alexandra, and to our little baby who is still on the way. Alexandra’s love, support, patience and encouragement have been absolutely essential to my work and the upcoming birth of our baby has been a great source of inspiration for me in many ways. With all my love, thank you both.

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List of Abbreviations

5-FOA, 5-fluoroorotic acid
A→S, active to silenced
AAR, O-acetyl-ADP ribose
ACS, ARS consensus sequence
Ago, Argonaute
ARS, autonomously replicating sequence
ASF1, anti-silencing function 1
CAC1, chromatin assembly complex subunit 1
CAF-1, chromatin assembly factor 1
CDK, cyclin-dependent kinase (Cdc28p)
C<sub>S→A</sub>, rate of conversions from silenced to active
Δcac1, CAC1 deletion
DDK, Dbf4-dependent kinase, (Cdc7p)
DNMT, DNA methyl transferase
dNTP, deoxynucleotide triphosphate
DSB, DNA double strand break
EB, extraction buffer
FACS, fluorescence activated cell sorter
FACT, Facilitates Chromatin Transcription
FOA<sup>R</sup>, 5-FOA resistance
FOA<sup>S</sup>, 5-FOA susceptibility
GFP, green fluorescent protein
HAT, histone acetyltransferase
HDAC, histone deacetylase
HIR, histone regulator
HP1, heterochromatin protein 1
HU, hydroxyurea
IP, immunoprecipitation
IPB, IP buffer
MBD, methyl CpG DNA binding domain
miRNA, micro RNA
MS, mass spectrometry
ORC, origin recognition complex
ORF, open reading frame
PAGE, polyacrylamide gel electrophoresis
PcG, Polycomb group
PEV, position effect variegation
PfEMP1, P. falciparum erythrocyte membrane protein 1
PIP, PCNA interacting protein domain
PRC, PcG repressive complex
PRE, PcG regulatory element
PTM, post-translational modifications
rDNA, rRNA gene clusters
RFB, replication fork barrier
RFC, replication fork clamp loader
RNR, ribonucleotide reductase
RPA, replication protein A
RT-PCR, reverse transcriptase PCR
S→A, silenced to active
SC, synthetic complete media
SC/FOA, SC with 5-FOA
SC+Can, SC with canavanine
SC-ura, SC lacking uracil
SDS, sodium dodecyl sulfate
SIR, silent information regulator
siRNA, small interfering RNA
STAR, sub-telomeric anti-silencing region
TPE, telomere position effect
TSG, tumour suppressor gene
YA, proportion of cells with active gene
YAC, yeast artificial chromosome
YPD, non-selective yeast media
YS, proportion of cells with silenced gene
General Introduction

Purpose
The purpose of my research is to determine how epigenetic conversions are controlled at the telomeres in *Saccharomyces cerevisiae*, budding yeast, as a model for epigenetic change in general.

Significance
Epigenetic changes play an important role in development and disease in humans and many of the epigenetic modifications are conserved across eukaryotes. These epigenetic changes, consisting of histone modifications, chromatin remodelling and DNA methylation, represent a vast array of complex variations that interact to affect the expression of the genome in a precise and inheritable manner that does not alter the sequence of the DNA (1). These effects range from single-gene specific to genome wide and influence cellular processes from differentiation to disease (2-4). In fact, studies on epigenetic changes in cancer have made it increasingly apparent that they are as fundamental to the initiation and control of carcinogenesis as genetic changes (4-7).

In principle, epigenetic characteristics are copied faithfully during DNA replication (8), meaning that the epigenetic states of gene expression are maintained across the genome as cells divide. Furthermore, these epigenetic states have the capacity to be passed on to progeny through the gametes, indicating that alterations to the epigenome can even influence subsequent generations of individuals (9). However, programmed epigenetic changes are necessary for the proper function of a multi-cellular organism because each cell type must express a specific subset of the genes in its genome. This is apparent in the epigenetic profiles specific for each cell type in humans that are continuing to be characterized (10-14). These epigenetic profiles imply that the cells must have the ability to convert genes that are either expressed or silenced by epigenetic effects to the opposite epigenetic state during cell differentiation. Much work has been done to determine which genes are involved in the process of silencing and activating pluripotency and lineage specific genes, as well as the complexities of how they interact with one another (10,15,16). However, it is difficult to determine if these changes in the localization of trans-activators and repressors are in response to changes in epigenetic modifications or the cause of changes in epigenetic modifications (17). Therefore, it is necessary to establish a model system where epigenetic changes can be directly assessed, a key component of Chapter 2 in this thesis.

In summary, a definitive understanding for the basic mechanisms of epigenetic conversions would help us to understand key factors involved in carcinogenesis and the fundamental process of cellular differentiation. This is accomplished by using the telomeres of *S. cerevisiae* to examine the fundamental control of epigenetic conversions.
Chapter 1 - Review of Literature

1.1 Epigenetic Control

1.1.1 Heterochromatin and Euchromatin

What is chromatin?
Heterochromatin and euchromatin were initially defined as differentially staining regions of nuclear substance, with euchromatin lightly stained and heterochromatin stained heavily. It was later demonstrated that euchromatin is the site of active transcription while heterochromatin is generally transcription free (18). Thus, the idea emerged that chromatin structure may regulate gene expression.

Chromatin acts as a conserved means for the stable packaging of the large genomes of higher eukaryotes, where most of the DNA does not code for genes but rather for transposable elements and other repetitive sequences. The chromatin is made up of DNA which is wrapped around structural subunits called nucleosomes. Nucleosomes are made of the highly conserved, positively-charged, histone class proteins: H1, H2A, H2B, H3, and H4, along with a multitude of non-histone nucleosome-related proteins (19-21). (H2A-H2B)₂ and (H3-H4)₂ form two core tetramers that stably interlock at their carboxy-terminal and middle domains to form an octamer. Meanwhile, H1 acts to bind the DNA at the access points of the nucleosome core. The amino-terminal tails of the core histones are left open to multiple post-translational modifications (PTMs) that have been implicated as the determining factor for chromatin state (21).

Chromatin is the combination of DNA and core histones to form the nucleosome structural unit. PTMs have the ability to physically alter the conformation of the chromatin by adjusting the charge of the nucleosome, which affects the interaction or binding strength between the DNA and the histones (18), or in the case of H4K16 acetylation, prevents the formation of compact 30nm fibres (22). Furthermore, PTMs act as binding sites for numerous transcription regulating factors. Thus, the modifications to chromatin are able to determine the binding, accessibility and transcriptional activity of the genes associated with them (18). The particular pattern of epigenetic modifications ultimately determines whether a specific locus will be considered heterochromatic or euchromatic.

1.1.2 Epigenetic Marks: determining gene expression

Epigenetic marks of heterochromatin
Heterochromatin and gene silencing are characterized by histone hypoacetylation. Excluding some single-celled organisms like S. cerevisiae, histone deacetylation by multiple histone deacetylases (HDACs) allows for the methylation of histone H3K9 by SUV39h (and its homologs). This methylated lysine is then bound by Heterochromatin Protein 1 (HP1), which subsequently recruits additional HDACs and SUV39h. These enzymes would then have access to the adjacent histones, continuing the spreading of heterochromatin modifications until they reach a heterochromatin/euchromatin boundary, where histone acetyl-transferases (HATs) may also be recruited to halt the progression of the heterochromatin modification enzymes (20,23). Other
histone alterations, such as specific histone phosphorylation and ADP-ribosylation, as well as hypermethylation of 5-methyl cytosines (5mC) on the actual DNA sequence, also appear to induce heterochromatic states in certain organisms.

In *S. cerevisiae*, however, there is no HP1 and no DNA methylation. Hypoacetylation, specifically deacetylation of histone H4K16, is driven by the HDAC Sir2p (Silent Information Regulator)(24,25). The deacetylated nucleosomes allow for the recruitment of Sir3p and Sir4p (26,27), which subsequently recruit more Sir2p proteins (23,27) to continue a similar cycle of deacetylation to that driven by the HDACs, SUV39h and HP1 in humans. In general, *S. cerevisiae* heterochromatin is characterized by a lack of PTMs. The key exceptions are trimethylation of H3K36 by Set2p and monomethylation of H4K20. When present in the open reading frame (ORF) H3K36 is associated with active genes. However, if Set2p is mis-targeted to the promoter region it can cause repression (18). Additionally, H4K20me1 was recently shown to function in the silencing of sub-telomeres in *S. cerevisiae* (28). Although methylation of H3K9 and H3K27 are associated with silencing in mammals and other higher organisms, neither have been detected in *S. cerevisiae* (23,29).

**Epigenetic marks of euchromatin**

Euchromatin and active genes are characterized by the addition of histone PTMs. In *S. cerevisiae*, they are associated with general hyperacetylation of H3 and H4, with Sas2p specifically opposing Sir2p at H4K16, and hypermethylation of H3K4 and H3K79 by Set1p and Dot1p, respectively. Methylation of arginines (H3R2/17/26 and H4R3), phosphorylation at H3S10, and ubiquitination of H2BK120/123 and H2AK119 are also PTMs associated with euchromatin (18,20,23,29-32). Similarly, these PTMs are signals for active transcription in mammals and other higher organisms.

1.1.3 Epigenetics and nuclear organization

Another important consideration for epigenetic effects is the chromosomal organization of the genome within the nucleus, which influences gene expression and is itself influenced by gene expression (33). In general, positioning of DNA at the nuclear periphery correlates with gene silencing while gene-rich chromosomes are found nearer to the interior. However, chromosomes are actually distributed into specific territories during interphase, which exhibit a multitude of complex interactions that may depend on and/or determine cell type (34). Recent evidence suggests that the epigenetic profile of a cell may help to establish the territorial organization of the chromosomes and how they interact with the nuclear envelope, based off of the transcriptional machinery and ATP-dependent remodelling proteins associated with different densities of gene activity (35). Therefore, while the locus-specific effects of epigenetic marks may appear to be straightforward, it is clear that epigenetics does more than simply turn on and off small regions of the genome but can also have major effects on the cell as a whole. It should also be noted that there are indeed exceptions to the above rules of epigenetic characteristics. Certain genes have actually been shown to require heterochromatin marks for proper activation and transcription (36) so it is clear that particular PTM patterns do not simply activate or shutdown all genes.
1.2 Inheritance of Epigenetic Marks

1.2.1 DNA Replication

DNA replication represents one of the major challenges for the inheritance of epigenetic marks. As the replication process doubles the amount of DNA present, the chromatin too must be doubled and assembled onto the new sister DNA molecules without losing the PTM patterns on the histones associated with their specific regions of DNA. Thus, a certain amount of interplay between the elements and factors involved with DNA replication and epigenetic inheritance is to be expected. In fact, there is a well-established correlation between replication origin firing and rate of transcription (37).

**Eukaryotic origins of replication**

Eukaryotic origins of DNA replication display a significant inter-species diversity. In higher eukaryotes this diversity reaches a point where origin locations are difficult to identify by homology search (38). An exception to this difficulty is presented by *S. cerevisiae*. In this organism, the first functional origins have been identified by screens for DNA elements which confer DNA replication on plasmids (39-41). Comparison between these Autonomously Replicating Sequences (ARSs) has shown that they encompass approximately 200bp of DNA in length and contain perfect or one-base mismatches to the 11 bp ARS consensus sequence (ACS) 5’-WTTAYRTTTW-3’ (where W=A/T; Y=C/T; R=A/G). Linker scanning substitutions in several ARSs have determined that the ACS is the sole essential element for DNA replication (42-45). However, auxiliary B1, B2, and B3 elements within ARSs are also necessary for efficient DNA replication (42). Utilization of these readily identifiable origins in *S. cerevisiae* has helped to establish the mechanisms of DNA replication in eukaryotes.

**Initiation of replication in *S. cerevisiae***

A wealth of information has been accumulated on the mechanisms by which ARSs initiate DNA replication (Fig. 1.1). The Origin Recognition Complex (ORC), which is built of six different Orc proteins, binds the ACS/B1 elements to nucleate the formation of the pre-replicative complexes (46,47). Shortly after mitosis, Cdt1p and Cdc6p recruit the heterohexameric MCM complex to the ARS-bound ORC. Thus, ARSs are poised to initiate DNA replication upon receiving a regulatory stimulus. This stimulus is provided in early S-phase by two protein kinases, DDK (Dbf4-dependent kinase, also known as Cdc7p) and CDK (Cyclin-dependent kinase, Cdc28p in *S. cerevisiae*) (46). It seems that the critical event in the stimulation of origins is the phosphorylation of MCM4 by the DDK (48). However, other components of the pre-initiation complex are also phosphorylated with similar timing, including Cdc45p, which along with GINS, must bind to the MCM complex in order for replication fork progression to occur (47). These events culminate in the activation of the MCM helicase, in the unwinding of origin DNA and in the assembly of the DNA replication machinery (49,50). It is believed that the same events take place at all origins of DNA replication, but at different times throughout S-phase. The early firing origins are usually located in the central portion of the chromosomes, while the later firing origins are found at the periphery (51), suggesting that the chromatin landscape may actually be involved in the regulation of origin firing.
Figure 1.1 - DNA replication origin activation in *S. cerevisiae*.

ORC binds the *ACS/B1* elements. In early G1 phase, ORC recruits Cdc6p and Cdt1p. In turn, Cdc6p and Cdt1p load the hexameric helicase complex MCM2-7. In the G1/S transition, DNA replication initiation factors Dbf4-dependent kinase DDK (also known as Cdc7p) and Cyclin-dependent kinase CDK (Cdc28p) phosphorylate the MCM2-7 complexes to trigger their helicase activity. Cdc45p and the GINS complex are recruited to MCM2-7 and elongation machinery assembles for the progression of DNA replication.

**Replication fork progression**

The progression of replication requires the unwinding of the DNA double helix by the MCM helicase activity, which is catalysed by the formation of the replisome complex as S-phase begins. Strict cell cycle regulation prevents the reformation of the preRC so that DNA is replicated only once per cell cycle. The replisome complex is made up of the Replication Clamp Loader (RFC), PCNA (the sliding clamp), and the single-stranded DNA binding proteins Replication Protein A (RPA), which move along with the replication fork during the swift 5’ to 3’ duplication of each template DNA strand by DNA polymerase-ε for continuous leading strand synthesis and DNA polymerase-δ for synthesis of the fragmented lagging strand. It should be noted that PCNA is essential for the tethering of the replication machinery during DNA replication and may in fact also interact with histone chaperones during leading strand synthesis, lagging strand synthesis, ligation of Okazaki fragments and after replication (52).
1.2.2 Chromatin Disassembly and Reassembly during DNA replication

Chromatin disassembly ahead of replication
The process of faithfully reproducing epigenetic profiles after DNA replication is achieved by the incorporation of both ‘old’ and ‘new’ histones after nucleosome disassembly and subsequent reassembly. The disassembly process is accomplished as the replication fork progresses forward. It disrupts the chromatin ahead of the replication machinery as the DNA is unwound and displaces the nucleosomes. H2A-H2B histones are removed as dimers, dissociating from both the (H3-H4)\textsubscript{2} tetramer and the DNA, while the H3-H4 histones remain as a tetramer that retains its association with the DNA (23,53). This process of nucleosome displacement likely also involves several ATP-dependent chromatin remodelling proteins, which are quite active throughout the cell cycle for nucleosome exchange and incorporation of histone variants (18). Several of these enzymes appear to be required for replication through chromatin (54). For example, if WTSF, which forms a complex with ISWI and SNF2h, is depleted, it slows the rate of S-phase overall (55) and ACF-SNF2h must be present for replication through heterochromatin to occur (56). However, it is still uncertain whether these remodelling proteins act ahead of the replication fork for nucleosome disassembly or if their presence is required for activity after the replication fork has passed (54,57).

Asf1p (Anti-Silencing Function 1), a highly conserved histone chaperone responsible for the trafficking of nearly all new histones as they are brought to chromatin, has been implicated in the disassembly of nucleosomes as well (54). It was shown to evict histones in \textit{S. cerevisiae} (58), though the binding of Asf1p to H3-H4 precludes the formation of the (H3-H4)\textsubscript{2} tetramer (59) and it is clear that old (H3-H4)\textsubscript{2} tetramers are not split during replication (60-62). However, Asf1p may be able to remove histones by transiently disrupting the H4 tail of the (H3-H4)\textsubscript{2} tetramers without splitting the tetramer, according to the crystal structures obtained of the H3-H4 dimer in complex with Asf1p (59).

Chromatin assembly after replication
The process of chromatin assembly is summarized in Figure 1.2. Another conserved histone chaperone, FACT (Facilitates Chromatin Transcription), is responsible for the transfer of the old H2A-H2B dimers during DNA replication, according to \textit{in vitro} Xenopus (African clawed frog) and genetic \textit{S. cerevisiae} experiments (54,63), similar to its role in transcription. Furthermore, FACT has been shown to associate with the MCM complex during DNA replication in \textit{S. cerevisiae} (64) and mammalian cells (65). However, without the synthesis of new histones, replication progression stops (66).

New histones are synthesized in the cytoplasm and transported to chromatin with the help of a series of histone chaperones (67,68). This transport culminates with Asf1p (69,70), which binds H3-H4 dimers and passes them to either CAF-1 (Chromatin Assembly Factor-1) (71) or Rtt106p (72). Both of these histone chaperones accept two H3-H4 dimers and assemble them into a tetramer (73-75) before depositing the histones onto the DNA (60,76). There is also evidence that CAF-1 is aided by Asf1p, which can synergize with CAF-1 \textit{in vitro} for deposition of histones during DNA repair (77) or replication (69). Though both Asf1p and CAF-1 can be deleted individually in \textit{S. cerevisiae} (69) the double mutants show severe loss of genome stability (78).
CAF-1 is a highly conserved heterotrimeric complex made up of three subunits called p150, p60 and p48 in humans (79). These subunits are referred to in S. cerevisiae as the chromatin assembly complex proteins Cac1p, Cac2p, and Cac3p, respectively. CAF-1 preferentially adds new H3-H4 histones to DNA (80) and is found with H3, H3.1 and H4 histones (81), not H3.3, a histone variant specific to replication-independent histone exchange in metazoans.

Interestingly, in mammalian cells it has been observed that the DNA replication initiation kinase DDK (Cdc7p/Dbf4p) phosphorylates the p150 subunit of CAF-1 and promotes the binding of p150 to PCNA, thereby targeting CAF-1 to the replication fork and advancing histone assembly (82,83). Human CAF-1 contains a PCNA Interacting Protein (PIP) domain and a dimerization domain.
domain. The phosphorylation of p150 promotes the interaction of CAF-1 with PCNA by disrupting dimerization in human cells (82,84). However, though the PCNA and p150 homologues in *S. cerevisiae* have been shown to interact with each other (85,86) via a PIP domain on Cac1p, it remains to be seen whether Cdc7p/Dbf4p regulates this or if dimerization is involved. Finally, CAF-1 does not uptake histones if human cells are treated with replication inhibitors (70) suggesting that tight regulation may be in place to prevent non-replication interference with PCNA or coordination by DDK to ensure that the PCNA-CAF-1 interaction occurs during replication (54). The kinase control of Cac1p in *S. cerevisiae* is the major topic of Chapter 3 in this thesis.

The old (H3-H4)$_2$ tetramer or two new H3-H4 dimers for each nucleosome are assembled onto either of the leading or lagging strands of DNA (23,60,87-89). Then two H2A-H2B dimers are assembled on the flanks of the (H3-H4)$_2$ tetramer (90,91). There are still several questions about the repositioning of the old histones, specifically the (H3-H4)$_2$ tetramers, since they must be assembled before the H2A-H2B dimers. It is unknown whether their repositioning is random, sequence specific or semi-conservative because they are not clearly marked like new histones, which quickly become unmodified (52,60). This means that it is unknown if the old (H3-H4)$_2$ tetramers are placed on the exact same sequences of DNA that they occupied prior to replication or are simply assembled as soon as the histone chaperones have access to the DNA. The former would require a mechanism for delaying assembly, reading, and then recognizing the correct sequence of DNA, since the complementary sequences of the leading and lagging strands are not replicated simultaneously (52). It is also unclear whether there is specificity for which old (H3-H4)$_2$ tetramers move to which of the two DNA molecules or if their distribution is random.

**Replication-Independent Chromatin Assembly**

Besides histone exchange during DNA replication, there is also replication-independent chromatin assembly that occurs throughout the cycle. In the case of replication-independent deposition of histones, ASF1 passes the histones to the histone regulatory complex HIRA (92), which carries homology to the *S. cerevisiae* histone regulators Hir1p and Hir2p, and has been shown to function in a similar manner to CAF-1. Also, whereas CAF-1 is specifically targeted to the DNA by PCNA, it is unknown how HIRA selects the region of chromatin it will act upon (81). Perpetuating this mystery, studies in *S. cerevisiae* provided evidence suggesting that incorporation of histone H4 can occur at any region of the genome, regardless of transcriptional activity, cell cycle, or silencing (93). However, other evidence suggests that replication-independent exchange of histone H3 can only occur in coding regions that are highly transcribed (94), but can occur in promoter regions even if they are inactive (95), while further evidence suggests that some of these locations may actually be gene-specific, independent of transcription rates (96). The main distinguishing factor between replication-dependent and replication-independent histone exchange is the H3.1 and H3.3 histone variants, which are specific to new histones in the replication-dependent and independent processes, respectively (97). However, in *S. cerevisiae* these histone variants are not present and the H3 used is actually more similar to the H3.3 variant, making it somewhat more difficult to compare the two mechanisms of histone dynamics (98). In both cases they arrive as dimers with H4 (81,99).
1.2.3 Reestablishment of Epigenetic Marks

The question of how epigenetic marks are retained after replication when half of the histones present are new is still an area of much speculation, in part because the exact mechanisms of post-replication nucleosome assembly have not yet been determined. It has been established that old (H3-H4)$_2$ tetramers are not split during replication (60-62) or mixed with new H3-H4 dimers (100) and that they carry PTMs with them from the old to the new DNA during replication (54,99). Therefore, it is presumed that these tetramers are at the core of how epigenetic marks are maintained and that the new histones are altered to match (23). Currently, there appear to be three main mechanisms for maintaining epigenetic states through replication: 1) post-replication or gradual reestablishment, 2) replication-coupled reestablishment, and 3) pre-replication priming.

Post-replication reestablishment of histone marks

Post-replication or gradual reestablishment of histone marks refers to scenarios where certain PTMs do not quickly return to pre-replication levels, but rather build up throughout the cell cycle and reach pre-replication levels before S-phase begins again. Known cases include H4K20me1, which is gradually re-established after replication (101), H3K79 methylation, which is re-established throughout the cell cycle (102), and H3K9 and H3K27 methylation, which are reduced in S-phase and then gradually become re-established before the next S-phase (61). It is interesting to note that of these examples only H3K79 and H4K20 methylation applies to S. cerevisiae and that the H3K79 PTM is a mark for active chromatin (18) while H4K20me1 is rare in S. cerevisiae and mostly associated with the sub-telomeres where other silencing marks are abundant (28). It is possible that the presence of abundant silencing marks, like in the sub-telomeres of S. cerevisiae as described below, or DNA methylation in higher organisms, negates the requirement for immediate reestablishment of these marks or enables the possibility of using these PTMs as stabilizers of transcriptional repression or activity. In support of this, DNA methylation patterns are quickly maintained by the DNA methyltransferase DNMT1 and its interaction with PCNA (103). In fact, MBD1, a methyl CpG DNA binding domain protein in mammals, associates with several enzymes involved with H3K9 methylation to induce transcriptional repression, including the methyl transferase SUV39h-HP1 complex (104) and the monomethyl transferase SETDB1 (60,105). As for the active methylation marks, it has been shown that parental histones with active marks may be sufficient to maintain the ‘activity’ of a gene until transcription starts (54). Then, once the gene is transcribed the new histones are able to be marked accordingly (106,107).

Replication-coupled reestablishment of histone marks

For those epigenetic marks that require strict and swift recovery in order to be effective, replication-coupled reestablishment mechanisms are in place, which apply post-translational modifications to the new histones either during nucleosome assembly or immediately after. While it may be possible for the active epigenetic marks from old histones alone to maintain activity of a gene until transcription begins, silencing appears to require more rigorous control (54). In S. cerevisiae, it is possible that the SIR complex (Sir3p/Sir4p/Sir2p) remains associated with the deacetylated (H3-H4)$_2$ tetramer as it is depositioned during replication, whether it remains directly bound or is quickly detached and reattached while the histones are transported.
The histone deacetylation activity of Sir2p could then deacetylate the new (pre-acetylated) adjacent nucleosomes, and spread silencing effects (23). However, silencer elements are also required for the inheritance of silencing (80,108) so they may be needed to direct Sir3p/Sir4p to those deacetylated nucleosomes in silencing areas rather than elsewhere in the genome (23).

In several cases, PTM machinery is directly associated with the replication machinery, providing a mechanism for swift targeting of newly assembled nucleosomes or histones in the process of assembly, as with DNMT1 and PCNA for DNA methylation (60). In mouse cells, CAF-1 was shown to associate with both PCNA and HP1 during replication (109). PCNA also associates with HDACs (110), G9a (a histone methyl transferase) (111) and several other chromatin modifying proteins (54,112). In fission yeast, *Schizosaccharomyces pombe*, Cdc20p, of DNA Polymerase-ε, preferentially enables the transcription of siRNAs in heterochromatin regions during leading strand synthesis to promote H3K9 methylation by Clr4p (an *S. pombe* homolog to the human SUV39h) of the same heterochromatin regions (113). From these examples, it is clear that there are a multitude of replication-coupled mechanisms for swift reestablishment of epigenetic marks on chromatin, which vary based on the organism and the specific PTMs involved.

**Pre-replication priming**

The third method for the inheritance of epigenetic marks occurs as a pre-replication boost of histone marks, which is diluted to normal levels during replication. However, this is clearly only viable for those marks whose normal levels are present on less than half of the nucleosomes in the region of epigenetic control (60). In the fruit fly *Drosophila*, it was shown that high levels of Polycomb group protein (PcG) binding and subsequent H3K27 trimethylation spiked at certain promoters (*AbdA*) as well as PcG regulatory elements (PREs) in early S-phase prior to replication (114,115). Similar pre-replication priming was observed for the active histone mark H3K4me3 in PREs (115), which acts as the second mark in the ‘bivalent’ target of PcGs (116). PcGs function through two main repressive complexes (PRC1 and PRC2), where PRC2 is an H3K27me3 methyl transferase and PRC1 recognizes this mark in order to catalyse transcriptional repression (117). Meanwhile, the opposing H3K4me3 marks, found within the H3K27me3 regions, seem to provide key developmental genes in embryonic stem cells with the ability to be silenced for continued pluripotency but also capable of switching to an active state as needed during differentiation (118). This interesting state that appears to prepare specific genes for epigenetic change may be connected to the position effect variegation (PEV) phenomenon, discussed below.

The combination of pre-, post-, and in concert-replication reestablishment of epigenetic marks allows for the faithful inheritance of the complex epigenetic profiles across the genomes of eukaryotic organisms. However, it brings into question how cells are able to specifically or globally alter the epigenetic states of genes during development and even through generations of single-celled organisms with epigenetic inheritance so tightly controlled.

### 1.3 Epigenetic Conversions: Gene Silencing and Position Effect Variegation

Whenever a particular epigenetic state is altered where the DNA has not changed, it is considered an epigenetic conversion if the new state is then inherited. This could mean that the correct PTMs simply failed to be transferred to the new histones once after a round of DNA
replication and the change was perpetuated from then on. It could also mean that a silenced gene was activated via a strict enzymatic pathway, which resulted in inheritable changes to the chromatin profile. In either case, if the change in gene expression was due to epigenetic effects then it would be an example of epigenetic change. How these epigenetic conversions are possible despite the mechanisms for inheritance of epigenetic marks and how they are controlled is key to our understanding of the epigenetic changes that bring about cancer as well as those that allow for cellular differentiation. Therefore, it is important to examine cases where epigenetic conversions are known to occur and to determine what is driving that ability.

1.3.1 Spreading of heterochromatin and gene silencing

It has been observed in several organisms that euchromatic genes are generally silenced if repositioned into heterochromatic regions (119-121). It is unknown if the euchromatic features are lost during this translocation or after the translocation. However, in these cases whether or not the translocated gene remains silenced depends on the spreading or leaking of heterochromatin modifications over the gene of interest (122). This spreading of silencing has been extensively studied in the mating-type loci, HMRa and HMLα, of S. cerevisiae.

**Spreading of silencing in the mating type loci of S. cerevisiae**

HMRa and HMLα are each flanked by E and I silencer elements. Remarkably, ARSs have been identified as essential elements in all four silencers of the mating type loci (123-125). For example, the HMRa-E silencer contains ARS317 as well as binding sites for Rap1p and Abf1p, whereas the HMRa-I silencer contains ARS318 and an Abf1p binding site (Fig. 1.3). Depending on the genomic context, both Abf1p and Rap1p bind to gene silencers or activator elements (126,127). Just as in replication origins, ORC binds to the bipartite ACS/B1 of the ARSs in the mating type loci silencers. However, instead of recruiting replication machinery, Orc1p recruits Sir1p, while Rap1p and Abf1p bind and recruit Sir3p and Sir4p. As shown in Fig. 1.3, Sir1p, Sir3p and Sir4p recruit Sir2p to establish a focal point of silencing and initiate the spreading of the SIR proteins. Similar events take place at ARS318 in the HMRa-I silencer (128). In wild type cells (where silencers are unmodified), any ordinarily active gene inserted within the silencing region between the pair of silencers is strictly and stably repressed.

It should be noted that the ARSs of the mating type loci are not substantially different from the replication initiation ARSs. Both types of ARSs bind to ORC in vivo and in vitro (129). If placed on a plasmid, the silencer ARSs act as perfectly good origins of DNA replication (40). The opposite is also true: replicator ARSs can acquire “silencer” activity when inserted in the mating type loci (130-132). However, since the silencer effects of these ARSs and the mating type loci within their boundaries may be replication-independent, as with other organisms (122,133,134), it brings to question whether it is possible for these boundaries to change and if so, how that would be controlled.
As part of the E silencer, ARS317 recruits ORC, which in turn recruits the Silent Information Regulator protein Sir1p. Both Rap1p and Abf1p bind to their respective sites and recruit Sir3p and Sir4p. The combined effects of Sir1p, Sir3p and Sir4p confer the nucleation of the silenced domain and recruits Sir2p. Sir2p deacetylates adjacent histone tails, which allows for further recruitment of the Sir3p and Sir4p. The spreading of SIR proteins continues (to the right) until it reaches SIR proteins spreading (leftwards) from the I silencer. The a1 and a2 genes are therefore maintained in a silenced state.

1.3.2 Position Effect Variegation (PEV) and Telomere Position Effect (TPE)

**Position effect variegation**

A particular epigenetic phenomenon occurs when a euchromatic gene is moved close to the boundary of a heterochromatin block. Often, it becomes fully silenced in some cells while fully expressed in a subset of others, displaying a phenotype called position effect variegation (PEV) (135,136). PEV is the result of rare epigenetic conversions. Although these effects may be seen as the result of DNA translocation, the actual sequence of the gene of interest and its promoter remains unchanged, as only its chromosomal location has been altered. Epigenetic changes alone determine whether a given cell will express the gene or repress it. This phenomenon was first described in *Drosophila* more than 80 years ago and is one of the first definitive examples of epigenetic effects (137). When the wild-type *white* allele in *Drosophila* is relocated by an inversion to the basal centromeric heterochromatin of the X-chromosome, it produces a “mottled-eye” phenotype, where a random group of the cells express the *white* gene (producing pink cells) while in the rest of the cells *white* is repressed (producing white cells) (137). This is a classic, visual representation of the PEV phenotype, which has since been observed in numerous organisms (122).
Telomere position effect
A similar phenotype to that seen in PEV is observed at the telomeres in *S. cerevisiae* and is referred to as Telomere Position Effect (TPE) (138,139). In fact, studies in *S. cerevisiae* and *S. pombe* indicate that PEV is a phenotype that occurs at numerous silencing regions and is not dependent on the translocation of a particular gene. For example, in both *S. cerevisiae* and *S. pombe*, PEV occurs naturally in the sub-telomeres and within the rRNA gene clusters (rDNA), as well as within the mating type loci if the silencers have been compromised by mutation. PEV also occurs near the centromeres in *S. pombe* (121). It has been proposed that PEV is actually a variable middle ground between silencing and expression as an alternative to reduced levels of expression (140). The key to PEV and TPE is the ability to swiftly alter the epigenetic state and then maintain the new state for a significant number of generations before reverting back. However, while the mechanisms for the spreading of silencing are well established, our understanding of these epigenetic conversions involved in PEV and TPE is still quite unclear.

1.3.3 Silencing from telomere repeats in *S. cerevisiae*

TPE in *S. cerevisiae* provides an opportunity to study the idea of a moveable heterochromatin/euchromatin boundary (Fig. 1.4). Here, ARSs were found to play a somewhat similar silencing role to their silencing activity in the mating type loci. However, the telomeric repeats act as the principle silencers while ARSs have a silencer-enhancing role (141). The telomeric TG1,3 repeats provide multiple binding sites for Rap1p. Similar to the mating type loci, Rap1p recruits Sir3p and Sir4p to establish the initiation point for the SIR protein spreading (125,141). ARSs and Sir1p are not required for the spreading stage. However, the absence of sub-telomeric ARSs or Sir1p significantly reduces the span of the silenced domain and its stability while the artificial tethering of Sir1p to the telomere boosts the silencing (142). Thus, sub-telomeric ARSs and their ability to recruit Sir1p through Orc1p play an important, yet a secondary role to the telomeric repeats in gene silencing. Even more, at other locations, isolated ARSs do not induce gene repression, but can boost the activity of an existing silencer. For this reason they were classified as proto-silencers (141).

The complexity of telomeric silencing does not end there. Besides ARSs, the middle repetitive Core X and Y’ elements in the sub-telomere also contain isolated Rap1p and Abf1p binding sites. All of these act as weak proto-silencers. In addition, the Core X and Y’ elements also harbour anti-silencer modules called sub-telomeric anti-silencing regions (STARs) (143,144). The whole assembly of proto-silencers and weak anti-silencers produce a multitude of variations in the strength, stability and spreading of telomeric silencing (144). Lastly, telomeres and sub-telomeres can fold to produce ternary structures and generate discontinuous silenced domains many kilobases away from the telomere (144,145). Chapter 4 of this thesis focuses on a project which delves into the dampening effects of these sub-telomeric elements on the extreme variations of gene silencing.
Figure 1.4 - Gene silencing at the telomeres.
Rap1p binds to the telomeric repeats and recruits Sir3p and Sir4p. The recruitment of Sir2p and the spreading of SIR proteins is as explained in the text. Sub-telomeric ARSs recruit ORC and Sir1p to enhance the spreading of SIR proteins and histone deacetylation away from the telomeres. This spreading is countered by Histone-Acetyl-Transferases and is limited by chromatin boundaries and insulators. Repressed chromatin acts to silence any genes wrapped within it while genes within de-repressed chromatin remain active.

1.4 Epigenetic Conversions: Mechanisms

So how is it possible for these well-established silencing regions to suddenly shift to an active epigenetic state? There are several competing and/or complementary mechanisms that have been proposed to answer this question (140).

1.4.1 Competition for silencing factors

The first mechanism for the control of epigenetic conversions is based off of the idea that there is a certain abundance of silencing factors available in the cell and that the silenced loci must compete for their share of these silencing factors. A specific example is seen in *S. cerevisiae*, where silencing is dependent on Sir2p at the mating type loci, the telomeres and rDNA (23).
Competition is shown as telomere silencing is abolished by deletion of telomeric Rap1p binding sites, which results in greater silencing at intrachromosomal sites (146) and is demonstrated again when deletion of ZDS1, whose protein product interacts with telomeric silencing factors, resulted in the expected loss of silencing at the telomeres but also increased silencing at the rDNA and mating type loci (147). In human cells, competition between small regulatory RNAs (siRNA and miRNA) has recently been observed for many of the components involved in post-transcriptional silencing, like Argonaute (Ago) (148). Furthermore, if Ago is abundant, miRNAs would then compete over target sites for silencing (149).

Regions of PEV, like the telomeres in *S. cerevisiae*, could be less competitive than areas of strict silencing, like the mating type loci. Therefore, if the total amount of gene silencing for the genome can be regulated by adjusting the abundance of silencing factors, reducing it could restrict PEV to the most competitive silencers and cause a loss of PEV at weaker silencers. Alternatively, if there is a more even distribution of silencing factors, one could expect to see more silencers become PEV loci (140). There appears to be more evidence of the latter. In replicative aging *S. cerevisiae* cells, Sir2p was found to decrease gradually, resulting in a loss of histones and gain of H4K16 acetylation in certain telomeres and hyper-recombination of rDNA loci (150) while inactivation of *SAS2* in telomerase mutants (*tlc1*) showed a loss of H4K16 acetylation at the telomeres and delayed senescence (151). The loss of silencing factors and gain of activation factors compromised the silencing of the telomeres, causing an increased number of epigenetic conversions from silent to active state. It is possible that this pressure on the competition for silencing is what allows for the multitude of epigenetic changes in differentiating embryonic stem cells (ESCs) (152). However, this does not explain the relative frequency of the epigenetic conversions seen in PEV loci and their ability to switch back to a silenced state several generations later.

The reversible nature of the PEV loci is explained by another aspect of the competition mechanism, which is the constant interplay implied between the silencing factors and the counteracting activators (140). Transcription factors have a natural competition with nucleosomes for binding to DNA (153,154) and are capable of recruiting chromatin remodelling complexes to help evict the nucleosomes (155), so the silencing machinery is constantly needed to keep the nucleosomes from being removed. Furthermore, other trans-activators are able to overcome telomeric silencing at certain points during the cell cycle, like directly after replication for PPR1 activation of *URA3* (156), or at certain distances away from the silencing elements where the reach of the silencing factors is gradually diminished (157). In fact, evidence suggests that maintaining the dynamics of silencing/activation factors as well as continuous turnover of new and old histones throughout the cell cycle is actually what allows for the maintenance of epigenetic memory while providing access to certain areas of DNA (158). However, we are still left with the question of how similar cells, with the same genetic landscape and a similar abundance of heterochromatin factors, are able to shift the epigenetic state at PEV loci (140).

### 1.4.2 Deregulation of heterochromatin boundaries

A second mechanism for the control of epigenetic conversions goes hand-in-hand with our understanding of the spreading of silencing and the idea of competition for silencing factors. As described earlier, it is possible for heterochromatin factors to spread along DNA to cover euchromatic genes (122,159). Furthermore, this spreading is able to affect long ranges and even
skip over areas of activity using chromosomal organization to its advantage by the looping of chromosomes (122), clustering of telomeres at the nuclear periphery (160) and bringing mating type loci close to the telomere for cooperation (161,162). When Sir2p deacetylates H4K16, it also hydrolyses NAD and produces an O-acetyl-ADP ribose (AAR). AAR stimulates the binding of multiple Sir3p proteins (163). This oligomerization of Sir3p may extend the reach of the SIR complex to allow the spreading of silencing across these long range chromosomal interaction loci (122), similar to the interactions at the metazoan chromocentre reservoirs (164,165).

However, eukaryotic genomes tend to have regions of euchromatin and heterochromatin that meet at sharp rather than gradual boundaries (166,167). These chromatin boundaries are often mediated by a variety of boundary element insulators and their interacting proteins, but the mechanisms of their control are not well understood (168). It has been suggested that the translocation of the white gene in Drosophila may have removed a chromatin boundary to initiate PEV (169), suggesting that PEV may be specific to loci surrounding compromised or poorly regulated chromatin boundaries. Indeed, unchecked heterochromatin results in the spreading of repression, leaving only competing transcription and activation factors to return expression to the region (170,171). In S. cerevisiae, the SIR complex, silencers and proto-silencers combine to work against HATs, STARs and possibly chromatin boundaries. However, these interactions are far from a simple repression/activation dichotomy as certain HATs actually seem to limit activation (31,172) and some HDACs seem to limit silencing (32). An example of this is in the deletion of RPD3, an HDAC whose deletion counter-intuitively increases the spreading of the SIR complex. The authors suggest that it replaces the deacetylation of H4K16 Sir2p, preventing Sir2p from creating the AAR which is coupled to its deacetylation activity. Without this bi-product, Sir3p would be unable to oligomerize and, consequently, unable to cause looping or long distance spreading of the SIR factors (32). Thus, areas with compromised heterochromatin boundaries could have far-reaching effects that shift the complex balance of competing factors throughout the life of a cell.

There are numerous suppressors of PEV and TPE which have been shown to expand or contract the reach of silencing from certain PEV loci (173) and mutations that show gains of expression in otherwise silenced reporters (174,175). However, it is difficult to determine if these effects are caused by generally weaker repression, a reduced number of repressed genes, or elevated rates of epigenetic conversions (140). There have been few cases which clearly show an effect on the actual mechanism of epigenetic conversions. Smith et al. demonstrated that specific mutations preventing the acetylation of lysines in the N-terminal tails of H3 and H4 were able to lock TPE into a silenced state (176) while Xu et al. showed that some of these mutations increased silencing in certain sub-telomeres, antagonized the incorporation of H2A.Z variants, or reduced the rate of decay of the silenced state (177). Still, there are some major questions which remain concerning the control of epigenetic conversions by competition of silencing factors and/or deregulation of chromatin boundaries. For example, if the conversions are random events, how do they happen so rarely without causing crippling epigenetic instability? Surprisingly, the answer to this may lie in the rapid turnover of new and old histones.

1.4.3 Dynamic chromatin and histone turnover

Chromatin is not static. Histones and other structural subunits are exchanged at both transcribed and non-transcribed regions throughout the cell cycle (98) with studies showing continuous H3-
H4 and H2A-H2B exchange in euchromatin and DNA replication-dependent exchange in heterochromatin (94) as well as genome wide exchange, with the highest frequency observed at active promoters (95). Earlier studies in *S. cerevisiae* showed that the eviction of nucleosomes was associated with the activation of transcription where histones were first hyperacetylated (178) and then unfolded and dissociated from the promoter for transcribed genes (179,180). In support of the idea that histone exchange helps to preserve the active epigenetic state (158), several histone chaperones (Asf1, FACT, Spt6) work with histone modifying enzymes in the disassembly and reassembly of nucleosomes during transcription elongation by RNA Polymerase II (181). Furthermore, HIRA and Vps75 may assist in the histone exchange at promoters but deletion mutations of these chaperones are mild (182).

Interestingly, a high H3 turnover was also present at several proposed chromatin boundaries (180), including certain tRNA genes (183), nuclear pore associated sites (184) and binding sites of Raplp (185) and Reblp (186). It is possible that this rapid turnover prevents the spreading of silencing at PEV/TPE loci (140). Thus, if a mistake was made during histone exchange at a chromatin boundary, it could provide the silencing factors with the shift in balance needed to compromise the activity of the boundary and allow the spreading of heterochromatin. Then, since histone exchange mostly only occurs during DNA replication for regions of heterochromatin, a second histone exchange mistake during DNA replication would be necessary to return the trans-activators and activation marks to the boundary domain. Evidence for this idea is provided by the pausing of replication forks and transcription machinery at these sites of high histone turnover in *S. cerevisiae*.

Pausing at sites of high turnover may lead to PEV by providing increased opportunities for the incorporation of incorrect histones (140). Replication fork pausing is prevalent in the *Core X* and *Y*’ elements of yeast sub-telomeres (187) and the rDNA (188-190) as well as several highly transcribed genes, including those for RNA polymerase II (191). This pausing was shown to be a result of interaction between a terminator protein (specifically Fob1 in *E. coli* and *Bacillus*) and the helicase (192), implying that a certain number of nucleosomes behind the helicase would be disproportionately available for histone exchange, particularly in heterochromatin where histone exchange is mostly dependent on replication. However, some earlier studies in *S. cerevisiae* have indicated that passage through replication is not necessary for the establishment of silencing (133,134), but rather, passage through S phase is the deciding factor (193). Both of these studies used an extra-chromosomal non-replicating DNA ring that was primed for silencing with either strong silencer elements (HMR) (133) or a tethered Sirlp (134). Thus, they show that DNA replication is not required for establishing silencing at strong silencers or for spreading of silencing factors short range, but they do not indicate whether DNA replication is required for silencing where trans-activators and transcription factors are prevalent. Additionally, numerous replication factors have been shown to affect silencing (86,128,194,195) providing a strong link between silencing and replication.

Another link to pausing is the enhanced silencing caused by the binding of LacI or TetR proteins nearby, which is enhanced by RRM3 (196), a protein responsible for the release of stalled forks (194). Furthermore, in ESCs, 12% of genes undergo epigenetic conversions (silent to active or active to silent) during the transition from pluripotency to mesoderm and a full 98% of these genes contain paused RNA polymerase II (197). If at stalled replication forks or paused...
transcription complexes, the histone chaperones continue to assemble histones using only the pool of new histones, old epigenetic marks will be lost, giving the cells a chance at site-specific epigenetic conversions (140). However, while methods for quantitatively assessing the rate of histone exchange have been developed (178,198) there is a distinct need for adaptable quantitative assays that measure the rate of epigenetic conversions to test this connection (140). Then, it would be possible to determine which factors are involved in PEV and to test the possible mechanisms of epigenetic conversions as a broad model for the control of epigenetic change.

1.5 Summary

In summary, the epigenetic control of gene expression is mediated by heterochromatin and euchromatin domains across the genomes of eukaryotes that are characterized by complex patterns of histone PTMs, histone variants, DNA methylation, nuclear organization and the binding of a multitude of DNA/histone regulatory factors. Upon DNA replication, chromatin is disassembled and reassembled with twice the material to account for the doubling of DNA. The retained PTMs of (H3-H4)$_2$ tetramers drive the reestablishment of the pre-replication epigenetic profile before replication begins again. However, mechanisms must be in place to allow for the controlled epigenetic changes that take place as cells differentiate in higher organisms. Epigenetic conversions have been observed in the spreading of heterochromatin over translocated euchromatic genes as well as at naturally variegating loci, such as the telomeres in *S. cerevisiae*. The control of these natural epigenetic conversions is still relatively uncharacterized though it appears to be a combination of competition between silencing and activating factors/elements, deregulation of chromatin boundaries, histone exchange and replication/transcription pausing.

1.6 Preliminary data leading to hypotheses

1.6.1 Preliminary data leading to Hypothesis #1

At the time of my joining the lab, there were a few intriguing clues which ultimately led to the development of my three hypotheses, given below. The first clue was in *S. cerevisiae* cells with a deleted *CAC1* gene (Δcac1), the largest subunit of the chromatin assembly complex CAF-1. A common assay for assessing silencing in telomeres involves the insertion of *URA3* into the left sub-telomeric region of chromosome VII. The active state of this gene can be selected using synthetic complete media lacking uracil (SC-Ura) and the silenced state can be selected using media containing 5-fluoro-orotic acid (SC/FOA), a drug which is toxic to cells expressing *URA3*. Thus, if a particular mutant strain is unable to grow on SC/FOA media, it would suggest that it has lost its telomeric silencing of *URA3*. However, in Δcac1 cells it was observed that after selection on SC/FOA, the cells were unable to grow on SC-Ura, suggesting a gain of silencing. Meanwhile, selection on SC-Ura precluded its ability to grow on SC/FOA, suggesting a loss of silencing. These seemingly contradictory effects led to Hypothesis #1.

1.6.2 Preliminary data leading to Hypothesis #2

As described in section 1.2.2, it is known that in human cells the replication initiation kinase DDK (Cdc7p-Dbf4p) phosphorylates p150 (Cac1p) *in vitro* and affects its association with
PCNA. I speculated that DDK, and perhaps other kinases, would have a similar effect in *S. cerevisiae*, leading to Hypothesis #2.

### 1.6.3 Preliminary data leading to Hypothesis #3

Many HATs counteract the silencing activity of Sir2p in *S. cerevisiae*, particularly Sas2p which directly acetylates H4K16. However, it has been observed that deletion of *SAS2* actually results in a loss of silencing at the telomeres, opposite to the expected effect. It is important to note that many of these experiments are conducted in truncated telomeres, where the sub-telomeric elements like *Core X* and *Y′*, described in section 1.3.3, have been removed as a result of the integration of reporter genes. We speculated that the loss of these sub-telomeric elements could be the key to these paradoxical results, leading to Hypothesis #3.
Hypotheses

Hypothesis #1
- CAF-1 is required for epigenetic conversions at the telomeres of *S. cerevisiae*.

Hypothesis #2
- The association of CAF-1 with PCNA and chromatin is regulated by protein kinases.

Hypothesis #3
- Sub-telomeric Core X and Y’ elements confer the effects of HATs on TPE.

Objectives

1. To establish an assay that quantitatively measures the frequency of epigenetic conversions.

2. To use this assay to test the effects of deletion of *CAC1* in TPE with a focus on epigenetic conversions.

3. To test the effects of Δ* cac1* on epigenetic conversions at an alternate locus – *HMRα*

4. To analyse the phosphorylation of Cac1p *in vitro* by DDK and other kinases.

5. To decipher the effects of this phosphorylation *in vivo* by using point mutations at the key phosphorylation sites.

6. To conduct TPE experiments in various HAT-deletion strains at recombinant telomeres that contain sub-telomeric Core X and Y’ elements.
2.1 Introduction

As discussed earlier, gene silencing is mediated by compact heterochromatin, which is re-established after each passage of the replication fork (125,175,199). At certain loci, rare conversions between the silenced and active states of genes confer a quasi-stable pattern of gene expression called PEV (200,201). However, the mechanisms of conversion that produce PEV are not well understood. In model organisms (D. melanogaster, S. cerevisiae and S. pombe) the expression of otherwise silenced genes can be increased by mutations in many other genes. In many cases it is not clear if these effects are caused by poor maintenance and leaky expression of the gene of interest or by elevated rates of epigenetic conversions or both (174,175). On the other hand, many suppressors of PEV expand or contract the heterochromatin domain, thus repositioning the variegated locus (164,173) with no clear evidence that the switching mechanism is affected. The main reason for this feeble grasp on the nature of epigenetic conversions is the rare use of direct quantitative assays.

Most tests assess variegation at the endpoint of a multi-generational process but not the dynamics of conversions itself. These tests include the calculation of the proportion of URA3-expressing cells in S. cerevisiae, or URA4 in S. pombe, after a certain period of growth in non-selective medium; the scoring of pink segmentation of yeast colonies caused by the variegated repression of ADE2 in S. cerevisiae (ADE4 in S. pombe) or the scoring of the patchy red coloring of the D. melanogaster eye caused by positional silencing of the white gene (202). In all these cases, the proportion of cells with active versus silenced genes has been recorded, but the process that led to the variegated state has not been directly studied. Only a few reports have closely looked at the dynamics of epigenetic switches. An early paper has extensively analysed the maintenance and “decay” of gene silencing at the mating HMLα locus of S. cerevisiae (203). The authors have used highly specialized mating assays, such as “shmoo” farming and alpha-factor confrontation, to determine the rates of conversion of epigenetic states and to provide a framework for future analyses (203). More recent studies of the mating type loci have utilized GFP-reporters to focus on the early events in the establishment and decay of gene silencing at a single cell resolution (204-206). Interestingly, the rates of epigenetic conversions measured by these short-term and long-term assays are considerably different. Finally, in certain histone mutants an enhanced stability of epigenetic state at the telomeres of S. cerevisiae has been observed (176,177), but the authors have not elaborated on the rates of conversion.

In Plasmodium falciparum, a sophisticated system of silencing and switching of 60 sub-telomeric var genes contributes to allelic exclusion phenotypes and immune evasion. These processes are believed to be responsible for the persistence of malaria infections and have attracted significant attention in recent years (207,208). Several studies have assessed the on- and
off-rates of var genes through a complex algorithm and the measurements of the abundance of different var RNAs or different surface antigens in cloned cultures (209-212).

Here I have generated a simple model for the quantifying of conversions at any PEV locus and used this model to predict patterns of variegated expression based on the frequency of active→silenced (A→S) and silenced→active (S→A) transitions of a gene. I have expanded and substantially modified two existing assays for the assessment of gene silencing at the telomeres (139) or at the mating type loci (213) of S. cerevisiae and have fit the data to theoretical curves based on this model.

2.2 Materials and Methods

2.2.1 Strains, growth conditions and integrating constructs

All strains used are listed in Table 1 in Appendix 1. Since many of the strains used were temperature sensitive, all cells were grown at 23°C in non-selective (YPD) or on synthetic complete (SC) media lacking Uracil (SC-ura) or Arginine (SC-arg) or SC containing 0.1% or 0.2% 5-Fluoro-Orotic Acid (SC/FOA), 10 mM Hydroxyurea (HU) or 60 µg/ml canavanine as indicated. FOA was from Toronto Research Chemicals Inc. HU and canavanine were from Sigma-Aldrich. The experiments with pol30 mutants were conducted in media without Tryptophan (SC-trp). These mutants lack the genomic copy of POL30 and their viability is maintained by plasmids (pRS, ARS1, CEN4, TRP1) carrying mutant or wild type copies of POL30 (86).

The integrating constructs ADH4-URA3-tel, VR-URA3-tel and ADH4-ADE2-URA3-tel were produced by digestion of pUCAIV or pUCAV with SalI and EcoRI as described (139) and cells were transformed by electroporation. Correct telomeric integration was confirmed by PCR and by growth on both SC-ura and SC/FOA plates.

The number of generations of each culture was calculated as follows: single colonies of freshly transformed cells were inoculated in 3 ml of YPD and grown to saturation. In this initial liquid culture the cells undergo between 8-12 doublings. Subsequently, the liquid cultures were routinely diluted 1000 times and re-grown to saturation. The OD_{600} of each saturated culture was measured and used to calculate the exact number of generations in each passage of the culture: number of generations = (log(Final OD_{600} / Initial OD_{600})) / log(2).

2.2.2 Telomere Position Effect (TPE) assays using URA3

Cells were transformed with the integrating constructs and selected for URA3 expression by two consecutive passages on SC-ura plates. The cells were then transferred to non-selective YPD medium to allow for unrestricted A→S transitions and silencing of URA3 and then spread on both SC-ura and SC/FOA plates. Three single colonies from each plate were inoculated in 3 ml of YPD media and grown for 10-40 generations. Aliquots from these cultures were serially diluted and spotted on YPD, SC-ura and SC/FOA plates. After four days of growth at 23°C, the colonies on the plates were counted and used to calculate the percent of cells growing on SC-ura (these represent cells with active URA3) and SC/FOA (these represent cells with silenced URA3). The average values and standard deviations from all experiments are shown in Table 2.
2.2.3 Assay for gene silencing at HMRa using GFP

Strains containing a green fluorescent protein (GFP) expression cassette in the HMRa locus (72) were grown in YPD and briefly sonicated to disperse cell aggregates. The culture was then diluted to 1 cell/ml and 200 µl aliquots were dispensed in several 96-well plates. The plates were incubated overnight at 23°C until a single cluster of cells could be seen at the bottom of the well. Under these conditions most of the mini-cultures originate from a single cell and go through about 15 generations. Each well containing a single cluster of cells was analyzed by fluorescence activated cell sorter (FACS) to determine the percent GFP-fluorescing cells.

2.3 Results and Discussion

2.3.1 Model and Rationale

Positional variegation is defined by infrequent A→S and S→A conversions of a gene. I recaptured this process through modeling and simulation. A diagram depicting the parameters I used is shown in Fig. 2.1 A. A distribution algorithm was applied to calculate the proportion of cells with silenced (Y₃) and active (Y₄) gene in a given generation (n-1, n, n+1...) based on the proportion of cells with silenced and active gene in the preceding generation and two coefficients of conversions (C₃→₄ and C₄→₃) (Fig. 2.1 A). The equations and related calculations are presented in Appendix 1. I used the formula Y₄(n)=Y₄(n-1)C₃→₄+(1-Y₄(n-1))C₄→₃ to simulate the frequency of conversions at any PEV locus. Y₄(n) is the proportion of cells with an active gene in any generation (n-1, n, n+1...), C₃→₄ is the coefficient of conversions from active to silent state and C₄→₃ is the coefficient of conversions from silent to active state. The analysis of this recurrence relation (Appendix 1) shows that the rates of conversion (C₃→₄ and C₄→₃) must be between 0 and 100% and that they are independent of the proportions of cells with silent (Y₃) and active (Y₄) gene. More importantly, they are independent of each other. This formula allows for the projection of experimental outcomes based on the C₃→₄ and C₄→₃ conversion rates and fixed initial values of Y₄(0). It also provides means for the calculation of the C₃→₄ and C₄→₃ rates based on experimental data. The calculated C₃→₄ and C₄→₃ values represent a direct measure of the “A→S” and “S→A” rates.

This model does not address the precise nature of conversions as shown by the “branching” diagrams in Fig. 2.1 B. For example, during DNA replication none, one or both of the duplicated genes can maintain the pre-existing state (“A” or “S”). My model does not distinguish if one or both progeny genes convert in a single step (Fig. 2.1 B, 2 and 3). In addition, my model does not distinguish between replication-coupled (Fig. 2.1 B, 2 and 3)) and replication-independent (Fig. 2.1 B, 4)) conversions.
2.3.2 Simulation of TPE

Most TPE assays in *S. cerevisiae* are conducted by the insertion of *URA3* next to the left telomere of chromosome VII (139). After selection on plates without uracil (SC-ura, this corresponds to $Y_{(A)}0$ value close to 100%) the cells are grown in non-selective media for 20 or more generations to allow for unrestricted $A \rightarrow S$ and $S \rightarrow A$ conversions. It is assumed, but rarely confirmed, that at this time point the culture has reached a dynamic equilibrium of cells with silenced ($Y_S$) and active ($Y_A$) *URA3*. Subsequently, aliquots of these cultures are applied to plates containing 5-FOA (SC/FOA) and to non-selective SC plates. 5-FOA (5-Fluoroorotic Acid) is a
non-toxic precursor of UMP synthesis, but is turned into highly toxic 5-FUMP by the *URA3*-encoded Orotidine-5'-phosphate-decarboxylase. Any cell expressing *URA3* at the time of plating will die on SC/FOA while cells with repressed *URA3* will produce a colony (202). So, the level of *URA3* silencing is assessed by the number of colonies on SC/FOA divided by the number of colonies on non-selective plates (%FOA$^R$).

In *wild type* cells, such TPE experiments produce equilibrium in the vicinity of 30% FOA$^R$/70% FOA$^S$ cells (128,195,214). If the initial value of $Y_{A0}$ is set at 100% (corresponding to selection on SC-ura plates) or at 0% (corresponding to selection on SC/FOA plates), this equilibrium will be reached in 20 generations with $C_{S\rightarrow A}=15\%$ and $C_{A\rightarrow S}=6\%$. Values of $C_{S\rightarrow A}=7\%$ and $C_{A\rightarrow S}=3\%$ will produce the same FOA$^R$/FOA$^S$ equilibrium in 40 generations (Fig. 2.2 A). Hence, TPE assays based solely on the assessment of the $Y_S/Y_A$ equilibrium give little information on the frequency of epigenetic conversions. Even more, the assessment of effects on gene silencing is credible only if evidence for dynamic equilibrium is produced, something that is rarely done.

I used the above considerations to empirically measure the frequency of *URA3* conversions at the *VIIl* telomere in *wild type* (W303) cells. The cells were selected in parallel on both SC/FOA and SC-ura plates and then transferred to non-selective YPD medium. Aliquots were taken out at known generation numbers after the removal of selection and the percent FOA$^R$ cells were measured and plotted (Fig. 2.2 B). Best fit algorithm produced values of $C_{S\rightarrow A}=8.0$ and $C_{A\rightarrow S}=6.3$.

### 2.3.3 Simulation of loss or gain of silencing

The reduction in the proportion of FOA$^R$ cells to 1% or below is widely used as key evidence for loss of gene silencing at the telomere (125,128,195). However, it is not clear if this shift of equilibrium is caused by increase of $C_{S\rightarrow A}$ at fixed $C_{A\rightarrow S}$ or if both parameters change. This uncertainty poses a technical challenge. If $C_{A\rightarrow S}$ is reduced to 0.01% and $C_{S\rightarrow A}$ increases to 15%, equilibrium will be reached in less than 30 generations. However, if $C_{S\rightarrow A}$ remains at 8%, the equilibrium will be reached after 50 generations (Fig. 2.2 C). Similar dynamics can be projected for previously observed situations of gain of silencing, where FOA$^R=85\%$ or higher (214-216) (Fig. 2.2 D).
Figure 2.2 - Simulation of PEV and calculation of $C_{A\to S}$ and $C_{S\to A}$.

A) Simulation of the conversions of $URA3$ after selection of SC/FOA plates (grey diamonds or triangles) or SC-ura (black diamonds or triangles) at $C_{S\to A}=15\%$ and $C_{A\to S}=6\%$ (grey and black diamonds), or $C_{S\to A}=7\%$ and $C_{A\to S}=3\%$ (grey and black triangles). B) Best fit analysis of the conversion rates in wild type (W303) cells. Cells were selected on SC/FOA (large grey squares) and SC-ura plates (large black squares) and transferred to YPD medium. Aliquots were taken out at known generation numbers and the percent FOA$^B$ cells were measured and plotted. Best fit algorithm (small diamonds) produced values of $C_{S\to A}=8.0\%$ and $C_{A\to S}=6.3\%$. C) Simulation of loss of gene silencing. Simulations are similar to those in A). The following values were used: $C_{S\to A}=15\%$ and $C_{A\to S}=0.01\%$ (grey and black diamonds) or $C_{S\to A}=7\%$ and $C_{A\to S}=0.01\%$ (grey triangles). D) Simulation of gain of gene silencing. Simulations are similar to those in A). The following values were used: $C_{S\to A}=3\%$ and $C_{A\to S}=15\%$ (grey and black diamonds) or $C_{S\to A}=3\%$ and $C_{A\to S}=7\%$ (black triangles).

2.3.4 Simulation of loss or gain of epigenetic stability

In the preceding section, I have assumed that $C_{A\to S}$ increases or remains constant while $C_{S\to A}$ decreases, or vice versa. However, my model allows for independent variations of these two parameters (See Appendix 2). I therefore simulated situations where both parameters increase or decrease.
If \( C_{A \rightarrow S} \) and \( C_{S \rightarrow A} \) both increase to 15\%, equilibrium will be reached within 10 generations (Fig. 2.3 A). This situation corresponds to epigenetic instability that exceeds any observed PEV phenomenon and would be difficult to detect by currently existing assays. In the case of URA3-based TPE analyses, this situation will be accompanied by slow growth of the cells on both SC-ura and SC/FOA media.

\[ \text{Figure 2.3 - Simulation of gain and loss of epigenetic stability.} \]

**A)** Loss of epigenetic stability. Simulations similar to Fig. 2.2 A with \( C_{S \rightarrow A} = 15\% \) and \( C_{A \rightarrow S} = 15\% \) (grey and black diamonds).

**B)** Gain of epigenetic stability. Simulations similar to Fig. 2.2 A with \( C_{S \rightarrow A} = 1\% \) and \( C_{A \rightarrow S} = 1\% \) (grey and black diamonds).

If \( C_{A \rightarrow S} \) and \( C_{S \rightarrow A} \) both decrease to 1\%, the dynamic equilibrium will be reached after 100 or more generations (Fig. 2.3 B). Such a scenario defines a substantial gain in epigenetic stability. Assessment of the percent of FOA\(^R\) will strongly depend on the initial (SC-ura or SC/FOA) selection used. Importantly, if selection has been performed only on SC-ura plates (as in many studies), the data will serve as evidence for loss of gene silencing, not for gain of epigenetic stability.
In summary, parallel experiments with selection for the active and silent state of the gene (SC/FOA and SC-ura in the case of URA3) are essential when significant differences between the A→S and S→A rates are expected or if these rates are unknown. In practice, a single time point measurement of \( Y_A \) and \( Y_S \) at the twentieth generation can serve as evidence for equilibrium (or lack of equilibrium) and as an indicator, but not evidence for substantial changes in the frequency of epigenetic conversions.

2.3.5 CAC1 regulates the frequency of epigenetic conversions at telomeres

I used the above considerations to search for genes that affect the frequency of epigenetic conversions. URA3 was inserted in the VIII telomere of several strains with previously reported deviations in gene silencing (Fig. 2.4 A)(86,195,214,217). The transformed cells were then selected on SC-ura and SC/FOA plates, transferred to liquid non-selective medium and grown for 20 generations. Aliquots of these cultures were then serially diluted and plated in parallel onto non-selective, SC-ura and SC/FOA plates. The proportions of URA\(^{+} \) and FOA\(^{R} \) cells (measured as the number of colonies that survived on selective media divided by the number of colonies on the non-selective plates) were then calculated (Table 2) and plotted (Fig. 2.4 and Fig. 2.5).

Relative to the wild type counterpart, most of these strains showed an increase or decrease in the proportion of FOA\(^{R} \) cells regardless of the initial selection on SC/FOA or SC-ura plates. For example, RIF1 counteracts the association of Sir proteins with the telomere-bound Rap1 and has general anti-silencing activity (218,219). As expected, its deletion led to an increase in the \%FOA\(^{R} \) cells (Fig. 2.4 A). On the other hand, mutations in SAS2 and SAS3 are known to reduce silencing at the telomere. As expected, their deletion reduced the \%FOA\(^{R} \) values. The other mutants in Fig. 2.4 A have shown loss of silencing in many earlier studies, but the mechanisms of action for some of them are not completely understood (195,214). Importantly, the similar \%FOA\(^{R} \) and \%FOA\(^{S} \) values at the 20\(^{th} \) generation after selection indicated that all these cultures were approaching or had already reached the point of dynamic equilibrium. Hence, all these strains displayed loss of silencing consistent with the simulations presented in Fig. 2.2 C and D, respectively. I did not attempt to precisely measure the frequency of conversions.

The \( \Delta cac1 \) strain presented a notable exception. Its \%FOA\(^{R} \) was strongly dependent on the initial selection (Fig. 2.4 A). This phenotype was consistent with the scenario depicted in Fig. 2.3 B and suggested lower rates of URA3 conversions and gain of epigenetic stability at the VIIl telomere. I followed up by measuring the \%FOA\(^{R} \) cells at multiple time points after the removal of selection. Best fit analyses of the data from the first 50 generations indicated that for this period \( C_{S\rightarrow A}=0.44\% \) and \( C_{A\rightarrow S}=0.06\% \) (Fig. 2.4 B). An 18-fold reduction for \( C_{S\rightarrow A} \) and a 105-fold reduction for \( C_{A\rightarrow S} \) as compared to wild type. Surprisingly, the continuing culturing of the cells in non-selective medium showed a sharp decline in the proportion of \%FOA\(^{R} \) cells after the 60\(^{th} \) generation. This decline was observed in cells selected on both SC/FOA and SC-ura (Fig. 2.4 B). By the 100\(^{th} \) generation FOA\(^{R} \) cells appear at less than 0.01\% regardless of the initial selection.
Figure 2.4 - Assessment of the frequency of conversions at the VIIL and VR telomeres.

A) Frequency of conversions at the VIIL telomere. *URA3* was inserted in the VIIL telomeres of the strains shown below the graphs. Cells were selected on SC/FOA (upper graph) or SC-ura (lower graph) and single colonies were transferred to YPD medium and grown for about 15-20 generations at 23°C. The cultures were serially (1:10) diluted and spotted on SC, SC-ura and SC/FOA plates. The colonies were counted and the percent of *URA3*+ (white columns) and FOA*R* (black columns) cells were calculated for at least 6 independent cultures and plotted. *See next page...*
Fig. 2.4 continued... Error bars show standard deviations. Data is from Table 2.

B) Long-term rates of conversion in Δcac1 cells. Cells were selected as in A) and grown for 160 generations. Aliquots of the cultures were removed at known generations and the percent FOA<sup>R</sup> cells was measured and plotted. Best fit algorithm of the conversion rates for the first 50 generations produced values of $C_{S\rightarrow A}=0.44\%$ and $C_{A\rightarrow S}=0.06\%$. Conversion rates in later generations were not measured. One of two independent long-term experiments is shown. C) Frequency of conversions at the VR telomere. URA3 was inserted in the VR telomeres of BY4742 and Δcac1 strains. Analyses were performed as in A). D) Frequency of conversions at the VIIL telomere in single and double histone chaperone mutants. Analyses were performed as in A).

In contrast, all other freshly transformed mutant strains (Fig. 2.4 A, Fig. 2.5 A) showed low %FOA<sup>R</sup> in less than 20 generations after removal of selection. While I do not completely understand this rapid loss of telomeric repression in later Δcac1 passages, I suspect that it could be related to the reduced level of Sir2p in aging cells as observed earlier by Dang et al. (2009) and Kozak et al. (2010) (150,151).

I also tested if the observed suppression of epigenetic conversions in the early passages after selection applies to another sub-telomeric locus. I inserted URA3 in the right arm of chromosome V as in (139) and conducted analyses as in Fig. 2.4 A. Again, Δcac1 cells that were selected on SC-ura produced very low %FOA<sup>R</sup>, while cells selected on SC/FOA showed the opposite trend (Fig. 2.4 C). In conclusion, I have acquired evidence for enhanced epigenetic stability at the VIIL and the VR telomeres in Δcac1 cells in non-aging cultures.

CAC1 encodes a component of the histone chaperone CAF-1 and plays an important role in the replication-coupled assembly of nucleosomes (220). I considered the possibility that any general perturbation of histone exchange and/or nucleosome assembly could have an inhibitory effect on TPE. To address this question, I tested the effect on epigenetic stability at the VIIL telomere in other histone chaperone mutants. Experiments were performed exactly as in Figure 2.4 A. I show that the deletion of ASF1 and HIR1 only slightly altered TPE (Fig. 2.4 D). Interestingly, the Δrtt106 mutant had a minor gain of epigenetic stability, but not as strong as the Δcac1 mutant. I also considered that the observed effects are indirect and that in the absence of CAC1 some other chaperone plays a key role in reducing the frequency of epigenetic conversions. In Fig. 2.4 D, I show that double deletion of chaperone-encoding genes Δcac1Δrtt106, Δcac1Δasf1 and Δcac1Δhir1 produced phenotypes similar to that of Δcac1, while Δhir1Δasf1 had little effect on epigenetic conversions. Hence, loss of variegation in Δcac1 cells cannot be restored by deletion of other chaperones. These observations imply that CAC1 specifically contributes to position-effect variegation at the telomeres.
Figure 2.5 - Effects of POL30 (PCNA) and HU on epigenetic conversions.

**A)** Frequency of conversions at the VIIL telomere in pol30 (PCNA) mutants. Analyses were performed as in Fig. 2.4 A. In the pol30 mutants the genomic copy of POL30 is deleted and viability is restored and maintained by ARS/CEN/TRP1 plasmids containing wild type (pol30-0) and mutant (pol30-6, pol30-8, pol30-79) alleles. Wild type (BY4742) and Δcac1 cells are shown for comparison. **B)** Effect of HU on conversions at the VIIL telomere. Δcac1 and isogenic wild type BY4742 cells containing URA3 in the VIIL telomere were selected on SC/FOA plates and then transferred to SC-ura and SC/FOA plates containing 10 mM HU or to control SC-ura and SC/FOA plates as indicated. The Δcac1 cells on the left-hand side of the wild type cells have been grown for about 10 generations in non-selective medium. The Δcac1 cells on the right-hand side of the wild type cells were re-streaked directly from SC/FOA plates.

### 2.3.6 POL30 (PCNA) and Ribonucleotide Reductase (RNR) effects on epigenetic conversions

As described in Chapter 1, it is believed that CAF-1 is recruited to replication forks via interactions with Pol30p (PCNA) (220). POL30 is involved in multiple transactions at the replication forks (221). In certain POL30 mutants a correlation between gene silencing and impaired binding to CAF-1 has been observed (80,86). Specifically, the pol30-6, pol30-8 and pol30-79 mutants all display reduced telomeric silencing, while in biochemical assays Pol30-8p and Pol30-79p poorly associate with Cac1p (86). I tested if these POL30 mutants display a phenotype similar to Δcac1. The experiments in Figure 2.5 A revealed that Δcac1 and the pol30 mutants share the loss of silencing. However, only Δcac1 suppressed variegation at the VIIL telomere. While these results do not exclude the possibility that the interaction of CAF-1 with Pol30p is important for TPE, they clearly show that only the loss of CAF-1 contributes to variegation. Hence, there is no complete overlap between the TPE phenotypes of pol30 and cac1 mutants.
Recent studies have suggested that the toxicity of 5-FOA, while being primarily caused by the URA3-encoded Orotidine-5'-Phosphate-Decarboxylase, could be enhanced by the stimulation of RNR (202,222,223). Even more, suppression of RNR by low concentration of Hydroxyurea (HU) could reverse the sensitivity of dot1, pol30 and Δcac1 mutants to 5-FOA (223-225). I considered the possibility that RNR and HU could have a role in the frequency of epigenetic conversions in Δcac1 cells and performed experiments similar to the ones in (223,224). First, I confirmed that under my experimental conditions the sensitivity to 5-FOA strictly depends on the expression of URA3 (Fig. 2.6). Next, I streaked Δcac1 and isogenic BY4742 cells on SC/FOA plates with and without 10 mM HU and grew them for 3 days at 23°C (Fig. 2.5 B). At this concentration HU moderately reduces the dNTP pools, but does not induce S-phase arrest (223,224,226,227). My data indicates that HU did not suppress or reverse the gain of epigenetic stability of URA3 (Fig. 2.5 B). Similar data were obtained with cells that were initially selected on SC-ura plates (not shown). Therefore, it seems unlikely that RNR contributes to the reduced rate of epigenetic conversions in Δcac1 cells.

### 2.3.7 Toxicity of 5-FOA depends on the expression of URA3 and is not reversed by Hydroxyurea

It has been shown that Hydroxyurea (HU) has different effects on DNA replication at different temperatures (226) and that HU can modulate the sensitivity to 5-FOA (223,224). It is possible that under certain conditions (different temperatures, different concentrations of Hydroxyurea and 5-FOA) URA3-expressing cells can survive in the presence of 5-FOA. I tested if under my experimental conditions toxicity to 5-FOA depends on the expression of URA3 (Fig. 2.6). The variegating URA3 at the VIIL telomere of W303 cells confers growth on medium without uracil and on medium containing 2 μg/ml 5-FOA. In contrast, the plasmid-borne URA3 confers growth on SC-ura. However, its loss renders the cells non-viable because of the concomitant loss of POL30. Wild type wine yeast cells are also non-viable indicating complete sensitivity to 5-FOA. The addition of 10 mM Hydroxyurea does not reverse the toxicity of 5-FOA after 6 days of incubation. I conclude that at the conditions used the toxicity of 5-FOA strictly depends on the expression of URA3 and not on the activity of RNR.

**Figure 2.6 - Toxicity of 5-FOA to URA3-expressing cells in the presence of HU.**

Wild type wine yeast (URA3), W303 (ura3-52 URA3::VIIItel) and W303Δpol30 cells carrying POL30 on a plasmid (pBL230-POL30 URA3) were streaked on SC, SC-ura and SC/FOA plates supplemented with 10 mM Hydroxyurea and grown at 23°C for 2 days on SC and SC-ura plates and for 6 days on SC/FOA plates.
2.3.8 Epigenetic conversions in wild type and Δcac1 strains are not due to spontaneous mutations

I also tested if 5-FOA and HU increase the mutation rates in Δcac1 cells and if these can alter the resistance to 5-FOA and mimic epigenetic stability. I used a routine assay for the accumulation of forward mutations in CAN1 (78,228,229). CAN1 encodes an arginine transporter and is not related to RNR or URA3. I found that the mutation rates in Δcac1 cells do not exceed 1x10⁻⁶ (Fig. 2.7). Such rates can have only negligible contribution to the generation of 5-FOA-resistance of Δcac1 cells with telomeric URA3, which occur at frequencies of 10⁻² to 10⁻³. Even more, these 5-FOA-resistant cells revert to expression of URA3 and to sensitivity to 5-FOA, albeit at significantly lower rates compared to wild type cells (Fig. 2.4 B). I also found that 5-FOA, HU or the combination of both does not increase the yield of mutations (Fig. 2.7).

**Figure 2.7 - Spontaneous mutation rates in BY4742 and Δcac1 cells in the presence of 5-FOA and HU.**

Liquid cultures of Δcac1 or isogenic BY4742 cells with or without URA3 at the VIII telomere, respectively, were grown in YPD and washed once in sterile water. 2-3 x10⁷ cells were spread on SC-arg plates containing 60 μg/ml canavanine (+Can), 2 μg/ml 5-FOA or 10 mM HU as indicated. Plates were grown for 4 days and photographed.
Furthermore, I tested if the mutation rates change depending on whether the cells contain telomeric URA3 in the presence of 5-FOA and/or HU. As demonstrated earlier (78,229), there is a moderate increase in the forward CAN1 mutations in Δcac1 cells (Fig. 2.7 see SC+Can plates). Such spontaneous mutation rates (less than 10^(-6)) could not account for the incidence of FOA-resistant cells (Fig 2.7, see SC/FOA plates) in Δcac1 with telomeric URA3 (10^2 to 10^3). I also show that 5-FOA, HU or the combination of both did not increase the mutation yields in cells without URA3 and that the mutation rates in the presence of the drugs were not enhanced by the insertion of URA3 in the telomeres. Hence, 5-FOA derivatives that are produced by the URA3-encoded Orotidine-5'-phosphate-decarboxylase do not increase mutation rates. I do not exclude that at significantly lower concentration of 5-FOA such derivatives could show mutagenic activity. However, in TPE assays the toxicity of 5-FOA seems to significantly exceed its potential mutagenicity.

### 2.3.9 Resistance to 5-FOA is not caused by a loss of URA3 reporter

Earlier reports have shown significant, but not complete de-repression of telomeric URA3 in Δcac1 mutants (80,86). Similar observations have been made in Δsas2 and Δsas3 cells (172,214,230-232). On the other hand, I have observed stable resistance to 5-FOA in Δcac1 cells after selection on SC/FOA. I tested if loss of the URA3 reporter could account for the resistance to 5-FOA in Δcac1, Δsas2 and Δsas3 cells. I picked up colonies from YPD, SC-ura and SC/FOA plates and analysed them by PCR with primers specific for URA3 at the recombinant VIIL telomere. These assays showed that all cells continue to harbor URA3 when grown on SC/FOA plates (Fig. 2.8). I conclude that my observations could not be attributed to the loss of the reporter.

![Figure 2.8 - PCR confirmation for the presence of URA3 at the VIIL telomeres of ADH4-URA3-tel transformed cells after selection on SC-ura and SC/FOA.](image)

Δsas2, Δsas3, and Δcac1 were transformed with the ADH4-URA3-tel construct and selected on SC-ura. A colony from each strain was grown in 3ml YPD and the cells spread on SC-ura, SC/FOA and YPD. Genomic DNA was isolated from individual colonies and PCR was performed with the primers as shown in the diagram. All colonies displayed the expected 1.5 kb PCR fragment regardless of selection on SC-ura or SC/FOA. We conclude that URA3 was not lost upon selection on SC/FOA.
2.3.10 Different frequencies of conversion at \textit{HMRa}

I asked if variations in the frequency of epigenetic conversions can be observed at another extensively studied locus, \textit{HMRa}. I made use of a modified \textit{HMRa} that contains a GFP reporter under the control of the \textit{URA3} promoter (213). In wild type cells the genes in \textit{HMRa} (including this GFP construct) are permanently silenced. However, mutations in many genes lead to a variegated mode of expression (125,203,213). Such strains provide the means to measure gene silencing in the absence of 5-FOA and to alleviate the risk of its potential side effects (202,223), but no means for drug-based selection of the silenced state. For this reason, I diluted and dispensed such strains in 96 well-plates to produce mini-cultures, which originate from a single cell, and then grew them for about 15 generations. Depending on the state of the GFP reporter in the seeding cell, the initial value of the \(Y_{(A)0}\) is set at 100% or 0%. I simulated this process for 50 parallel mini-cultures (see Appendix 2) and plotted the produced \(Y_{(A)15}\) values in Fig. 2.9 A and B. Note that because the cultures start from a single cell, the first S→A or A→S transition can occur anywhere in generation 1-6 thus producing significant variations in \(Y_{(A)15}\). With assumed rates of \(C_{S\rightarrow A}\) and \(C_{A\rightarrow S}\) of 7% these values distribute linearly between 36% and 61% (Fig. 2.9 A) while reduction of the \(C_{S\rightarrow A}\) and \(C_{A\rightarrow S}\) conversions rates to 1% produces a distinct two-phase curve (Fig. 2.9 B).

Next, I performed such experiments in \(\Delta cac1\), \(\Delta cac1\Delta rtt106\), \(\Delta cac1\Delta asf1\) and \(\Delta cac1\Delta hir1\) cells harboring the \textit{HMRa::URA3p::GFP} reporter (Fig. 2.9 C-E). Previous studies have shown that a single deletion of any of these chaperone genes, including \textit{CAC1}, does not de-repress the \textit{HMRa::URA3p::GFP} locus (72,213). However, in double deletion mutants the locus acquires a variegated mode of expression (213). In agreement, of the analyzed 226 \(\Delta cac1\) \textit{HMRa::URA3p::GFP} clones, I detected 176 clones with no GFP\(^+\) cells (Fig. 2.9 C and not shown). In the remaining 50 clones the proportion of GFP\(^+\) cells varied between 0.2% and 0.9% (Fig. 2.9 C). This low incidence of GFP\(^+\) cells points to robust silencing with no conversions at all or to a very low S→A rate and precludes further analyses.

In the double deletion mutants I observed curves that suggest substantial differences in the conversion rates. Similar to the simulation in Fig. 2.9 B, the \(\Delta cac1\Delta asf1\) mutant displayed low conversion rates of about 1% per generation. The \(\Delta cac1\Delta hir1\) and \(\Delta cac1\Delta rtt106\) mutants showed profiles of conversions rates that are in-between the scenarios in Fig. 2.9 A and B. These results did not distinguish if \textit{CAC1} or other chaperones play a primary role in the execution of the conversions. However, they clearly demonstrated that the epigenetic transitions at this locus proceed at different rates in different strains. In addition, these experiments also indicated that different sets of genes could be involved in the control of epigenetic conversions at \textit{HMRa} and at telomeres.
Figure 2.9 - Assessment of the frequency of conversions at the HMRa::URA3p::GFP locus.

Cells were seeded in 96 well plates at less than 1 cell per well and grown for 15-18 generations at 23°C. Wells with a single cluster of cells were identified and the proportion of GFP+ cells was assessed by FACS. The %GFP cells from each clone were individually entered in a spreadsheet, the values were sorted and plotted as separate columns. About 45-50 individual clones were analysed in D), E) and F) and 226 clones were analyzed in C).

A) Simulation of the process at conversions rates of $C_{S\rightarrow A}=7\%$ and $C_{A\rightarrow S}=7\%$.  
B) Simulation of the process at conversions rates of $C_{S\rightarrow A}=1\%$ and $C_{A\rightarrow S}=1\%$.  
C), D), E), F) Experiments were performed with the strains listed on the top of the graphs.

2.3.11 Rates of conversions in Asir1 strains

SIR1 is one of the few genes that have been implicated in the control of epigenetic conversions. Its deletion de-represses HMLa and HMRa and produces higher S→A than A→S conversion rates at both of these loci. However, the precise calculated rates differ depending on the assays used (203,204,206). I tested the validity of my model using strains with HMRa loci, which contain a URA3 reporter and a destroyed Abf1p site in the E silencer (hmr-a1Δ::URA3) (Fig. 2.10) (204). The hmr-a1Δ::URA3 locus remains almost completely repressed in wild type and Δdot1 cells, but is de-repressed in Asir1 cells (204). Separately, I produced a Δsir1 strain with an
integrated URA3 in the VIIIL telomere exactly as in all other strains used in Figure 2.4 A. Because this construct does not contain ARS (ARS binds ORC and is critical for the recruitment of Sir1p (125)), this strain is not expected to display SIR1-dependent effects on URA3.

Figure 2.10 - Frequency of conversions in Δsir1 cells.
Δsir1, Δcac1 and wild type cells harboring URA3 at the VIIIL telomere and Δsir1, Δdot1 and wild type cells harboring hmr-a1Δ::URA3 were selected on SC-ura and SC/FOA plates, grown in YPD medium for 20 generations, and analyzed as in Figure 2.4 A. A) Analysis of cells with URA3-tel. B) Analysis of cells with hmr-a1Δ::URA3. C) Best fit analysis of the conversion rates in Δsir1 hmr-a1Δ::URA3 cells. Cells were selected on SC/FOA (large grey squares) and SC-ura plates (large black squares) and transferred to YPD medium. Aliquots were taken out at known generation numbers and the percent FOA<sup>R</sup> cells were measured and plotted. Best fit algorithm (small diamonds) produced values of $C_{S\rightarrow A}=5.66\%$ and $C_{A\rightarrow S}=0.17\%$. 
As predicted, experiments in cells with telomeric URA3 showed no effect of SIR1 and the characteristic low rates of conversion in Δcac1 cells (Fig. 2.10 A). In Δsir1 hmr-a1Δ::URA3 cells the initial selection on SC-ura produced predominantly de-repressed URA3 while the cells selected on SC/FOA produced a mixed population of cells with active and silent URA3 (Fig. 2.10 B). A time-course experiment with Δsir1 cells revealed S→A rates of 5.7% and A→S rates of 0.17% (Fig. 2.10 C). This result is in good agreement with the higher S→A than A→S rates reported in (203,206) and with the overall reduced silencing of hmr-a1Δ::URA3 reported in (204). Again in agreement with (204), Δdot1 and wild type cells generated only rare colonies on SC-ura plates and these did not maintain the de-repressed state (Fig. 2.10 B). Hence, my experimental system has faithfully recaptured earlier observations and has confirmed the validity of my findings in Δcac1 cells.

2.4 Summary and Conclusions

In this chapter, I have addressed an important aspect of position-effect variegation: the frequency of conversions at PEV loci. In essence, position-effect phenotypes are determined by rare S→A and A→S conversions. In turn, the acquired states of the gene are epigenetically maintained through multiple cell divisions (201). In metazoans, such conversions could represent the point of tissue-specific epigenetic repression/activation of a gene. The acquired state could persist throughout the life of the organism. Untimely conversions (meaning to subject the locus to an S→A or A→S switch) lead to cancer and to a variety of genetic and psychiatric disorders (233-237). In contrast, stem cells maintain their functional plasticity via maintaining the ability to convert the epigenetic state of numerous genes (197,238). Given the significance of epigenetic conversions, it is surprising how little we know about them. In this chapter, I have contributed to this specific field of genetics.

2.4.1 Methodology

One of the important aspects of this chapter is methodological. The loose grip on the mechanisms of epigenetic conversions is in part due to the limited use of assays which directly test the dynamics of these processes. For example, a few earlier reports have analysed epigenetic switches at the mating type loci of S. cerevisiae (203-206). These have used specialized mating assays (“shmoo” farming and “alpha-factor confrontation”) or alternative assays with GFP and YFP reporters. Interestingly, the conversion rates measured by these approaches seem considerably different and may lead to different interpretations regarding the role of SIR1. Studies in another organism, P. falciparum, have extensively documented on- and off- switching rates of the sub-telomeric var genes (209,211,212). However, their methodology accounts for the substantially more complex situation of multiple var loci and monoallelic exclusion and is not applicable to simpler cases (207,239,240).

Here, I introduced a comprehensive model for PEV and project various scenarios based on independent rates of conversions at a single locus. This model represents an extension of the concepts outlined in (203,206,209). The model presented here (Fig. 2.1) is applicable to any PEV locus and any organism. The projections based on this model provide solid definitions for the frequently used terms “loss” and “gain” of gene silencing (Fig. 2.2). More importantly, I have also introduced the terms “loss” and “gain” of epigenetic stability (Fig. 2.3) and have defined them graphically and through calculations of the coefficients of conversions $C_{A→S}$ and $C_{S→A}$. 
Through simulations I have shown how “loss of silencing” and “gain of epigenetic stability” can be confused if the assays do not specifically address the frequency of epigenetic switches. Finally, I introduce solid quantitative criteria and methodology for the precise measurement of the frequency of A→S and S→A conversions at any PEV locus (Fig. 2.2 and Fig. 2.3). This methodology can be used for the deciphering of the mechanisms of epigenetic switches in simple model systems and then be applied to more complex scenarios.

An example of such applications is provided by revisiting the earlier experiments at the mating type loci of *S. cerevisiae* in Δsir1 cells (203-206). “Shmoo” farming and “α-factor confrontation” had indicated that equilibrium of silencing at the *HMLα* locus was approached after about 60 generations (203). A similar study with GFP and YFP had determined S→A rates of 13% and A→S rates of 8% when fluorescence was measured by FACS or 35% and 15%, respectively, when fluorescence was measured for three generations by microscopy (206). According to my simulations, the first set of conversion rates will produce an S/A equilibrium in about 20 generations (Fig. 2.2 A) and is consistent with loss of gene silencing. However, the set of short-term rates suggests that equilibrium will be reached in less than 10 generations (Fig. 2.3 B) and is consistent with loss of epigenetic stability. Yet, the long-term “α-factor confrontation” assays clearly show extended epigenetic stability. Ultimately, these results indicate that short-term experiments depict a process that contributes to, but is not identical with a long-term epigenetic conversion. Similar conclusions have been reached by (204,205,241).

Using this model, I have substantially modified two existing assays for gene silencing in *S. cerevisiae* to exclusively focus on the rate of conversions from repressed to active state and *vice versa*. These assays have shown that I can reliably detect “gain of epigenetic stability” in this model organism and that the histone chaperone CAF-1 is involved in the regulation of epigenetic switches at the telomeres (Fig. 2.4). Hence, my theory and methodology can be used for the identification of genes specifically involved in epigenetic conversions. The analysis of the mating type *HMRα* locus does not provide the same level of confidence on the involvement of *CAC1*. Indeed, previous studies have indicated that histone chaperones can play different roles at the mating type loci and at the telomeres (80,213). Still, the analyses in Fig. 2.9 provide evidence for different conversion rates at a non-telomeric locus by a completely independent assay.

Many earlier studies have reported suppression of variegation because of contraction of a heterochromatin domain or because of repositioning of a hypothetical chromatin boundary (164,173). Such phenomena should not be linked to the switching mechanism. On the other hand, many studies have shown increased levels of expression of otherwise silenced reporters (reviewed in (174,175)). These observations have often been attributed to “poor maintenance” of gene silencing, meaning an elevated rate of S→A switches, or to incomplete repression of the gene in the PEV locus. As I have shown in Fig. 2.3 A, “poor maintenance” is hard to detect, leaving these alternatives open to interpretation. A decrease in the rate of switching is a more reliable criterion for deregulation of positional variegation (Fig. 2.3 B). To date, only two papers have reported “enhanced memory for heritable transmission” (176,177). These studies have shown that mutations in Histone H4 increase the stability of both the repressed and the transcribed states of telomeric reporters. Here I show that the deletion of *CAC1* has similar effects (Fig. 2.4). In this vein, it is important to mention that the principle role of CAF-1 is to reassemble Histones H4 and H3 after the transition of a replication fork (220).
2.4.2 Role of CAF-1 in epigenetic conversions

In theory, any perturbation of DNA replication and especially of fork integrity could lead to aberrant reassembly of chromatin (242). However, such events do not necessarily lead to enhanced or reduced epigenetic stability. For example, many of the strains in Fig. 2.4 A and Fig. 2.5 A harbor mutations in core replication factors with well-established defects in DNA replication, but none of them showed the gain in epigenetic stability that I see in Δcac1 mutants. The same reasoning applies to nucleosome assembly factors. The deletions of rtt106, asf1 and hir1 had little effect on TPE when compared to Δcac1 (Fig. 2.4 D). Hence, the gain of epigenetic stability at telomeres is specific to CAC1 and is not related to defects in DNA replication or nucleosome assembly in general.

Recent studies have raised the possibility that the loss of silencing in pol30 and cac1 mutants, which has been observed via telomeric insertion of URA3 and the use of 5-FOA, could be produced by elevated RNR activity and unbalanced pools of dNTPs (223,225). So, could the gain in epigenetic stability in Δcac1 be linked to elevated RNR? Several arguments suggest the opposite. Firstly, if RNR is important for the observed effects, it should induce sensitivity to 5-FOA regardless of the prior selection of the cells. However, I see no sensitivity but robust resistance to 5-FOA in cells that have been initially selected on 5-FOA. This resistance could not be attributed to elevated RNR. Secondly, RNR activity can be reversed by low concentrations of Hydroxyurea (223,224,243). I saw no effect of HU on the frequency of epigenetic conversions in cells with wild type CAC1 (BY4742) or in Δcac1 cells (Fig. 2.5). Thirdly, RNR is stimulated by a variety of mutations that cause replication stress (222,226,227) including most of the mutants I used in this study (Fig. 2.4). None of these mutations caused reduction of telomeric variegation (Fig. 2.4). I do not exclude the possibility that the absence of CAC1 in combination with RNR stimulation has a strong impact on telomeric silencing (202). However, I favor the idea that CAF-1 rather than RNR is the key player in epigenetic conversions.

Many reports have shown that the deletion of CAC1 reduces gene silencing at telomeres (80,86,213,223,224,244,245). However, they have not assessed the stability of the repressed state of URA3 after selection on SC/FOA plates. My experiments agree with the findings in these papers, but also add the analyses of the repressed state. Consequently, according to my criteria (Fig. 2.2 and Fig. 2.3) I observe a gain of epigenetic stability rather than a loss of silencing. On the other hand, short-term (1-3 generations) experiments in Δcac1 cells have revealed extremely high rates of telomeric S→A conversions (40%) (246). Such rates would preclude the detection of 5-FOA-resistance in Δcac1 cells, yet I and many others can readily establish FOA	extsuperscript{R} colonies. I suspect that, similarly to HMLα in Δsir1 (204,206), short term experiments in Δcac1 cells capture an intermediate silencing state that is not the same as a stable epigenetic switch.

One very interesting aspect of the epigenetic stability in Δcac1 cells is the loss of telomeric silencing after the 60th generation (Fig. 2.4 B). At this point I do not entirely understand the basis of this phenomenon. Earlier reports have shown a reduction in the abundance of Sir2p and concomitant decline in telomeric gene repression in aging cells (150,151). The authors have implicated replicative lifespan and the histone acetyl transferase SAS2 as key players in this process (150). While Δsas2 and Δcac1 cells show little similarity in terms of epigenetic stability in early cultures (Fig. 2.4 A), the possibility that aging has a major impact in Δcac1 cells remains
open for future studies.

2.4.3 Mechanism of epigenetic stability in Δcac1 cells

At present, I cannot address the mechanism of gain in epigenetic stability in Δcac1 cells. CAF-1 is thought to play a central role in the re-assembly of nucleosomes behind the replication forks and in the transmission of epigenetic states (80,165,220). Some preliminary data (R.O., D.J., B.W.) point to the possible involvement of pausing of replication forks in the conversion of epigenetic state. Interestingly, it is also known that replication forks tend to pause in the sub-telomeric regions (187,194). It is tempting to speculate that when replication forks pause, CAF-1 acts as a “lax” chaperone and allows lenient assembly of nucleosomes. This situation provokes poor transmission of the pre-existing epigenetic marks and predisposes the locus to epigenetic conversions. The absence of CAF-1 at such paused forks can explain the reduced rate of epigenetic conversions at the telomeres. More focused mechanistic studies need to address these issues.
Chapter 3 - CDC28 regulates the association of Chromatin Assembly Factor I with chromatin


3.1 Introduction

Current studies suggest that CAF-1 is recruited to replication forks via contacts with PCNA (POL30), the replication sliding clamp (80,82,86,247). Mutations in POL30 or CAC1 (which encodes the largest subunit of CAF-1) that cripple their interaction in vitro also impair the assembly of chromatin in cell extracts (247,248) and show gene silencing defects in vivo (244,246,248,249). However, the mechanisms that regulate the association of CAF-1 with PCNA and with chromatin are poorly understood.

Many PCNA-interacting proteins share a PCNA interaction peptide (PIP) consensus. Two PIPs are present in the largest CAF-1 subunit in humans, but only one in S. cerevisiae (247). The S. cerevisiae PIP in Cac1p is required for the interaction with PCNA (248). Interestingly, the low-affinity PIP (but not the high-affinity one) of human PCNA is required for replication-coupled nucleosome assembly. An additional putative PIP is found in the second largest subunit of CAF-1 in both species, but this PIP alone does not confer binding to PCNA (247). Nevertheless, the phosphorylation of this subunit in human CAF-1 by Cdk2 kinases stimulates the assembly of chromatin in vitro (250). Others have reported that the phosphorylation of the largest subunit of human CAF-1 (p150) by Cdc7-Dbf4 promoted its binding to PCNA and that the recruitment of CAF-1 to chromatin was reduced in Cdc7-depleted extracts (82). Cdk2-CycA also phosphorylated p150 in vitro, but this kinase had no effect on CAF-1 binding to PCNA or chromatin. Thus, human Cdc7, but not Cdk2, has been implicated in the coordination between DNA replication and CAF-1 functions during S phase (82).

Two kinases, Cdc7p and Cdc28p (a homolog of Cdk2), play distinct critical roles in S. cerevisiae during S phase (221). Both kinases regulate key events at the onset of DNA replication, which hypothetically coincides with the loading of CAF-1 on replication forks (251). Both kinases also phosphorylate other substrates that are not directly involved in DNA replication. Recent proteome-wide mass spectrometry analyses in S. cerevisiae have identified several sites of phosphorylation on Cac1p (http://phosphopep.org)(252). None of these sites are found in the Cac1p-PIP. No phosphorylation site has been reported on Cac2p and only one site has been observed in Cac3p (252). In this chapter, I embark on a detailed investigation of the phosphorylation of Cac1p by the Cdc7p and Cdc28p kinases.
3.2 Materials and Methods

3.2.1 Strains and growth conditions

The strains used in this chapter are listed in Table 3 in Appendix 1. The experiments with W303 and the isogenic cdc7-1 or cdc28-1 strains were routinely conducted in YPD at 23°C, unless otherwise indicated. For temperature shift assays, cell cultures were grown to OD\textsubscript{600}=1.0, split in halves. Cells were centrifuged and resuspended into pre-warmed YPD medium for the lengths of time indicated in the figure legends. The cultures then received 0.1% NaN\textsubscript{3} to prevent cell growth. The Δcac1 cells (BY4742 background) carrying plasmids for the expression of Cac1p-FLAG point mutants were routinely grown at 30°C in synthetic complete media lacking Leucine (SC-Leu).

3.2.2 Plasmids

The plasmids for the expression of GST-Cac1p and GST-Rtt106p were generated by amplifying the open reading frames of CAC1 and Rtt106 from W303 genomic DNA and cloning them in pGEX2T. The plasmid for E. coli expression of Cac1p\textsubscript{226-607}-His6 was created by inserting a PCR fragment between the NcoI and NotI sites of pET28b. The plasmids for ectopic expression of Cac1p were created by amplifying a 2.1 kb fragment containing the CAC1 promoter and the open reading frame fused to three FLAG epitopes and cloning it in the low copy pRS315(ARS CEN6 LEU2) plasmid. The sequences of the primers are available upon request. The cac1-FLAG3 (Serine to Alanine) mutant plasmids were generated using the QuickChange site-directed mutagenesis kit (Stratagene). All plasmids were sequenced to verify that there were no PCR-induced mutations.

3.2.3 PhosTag\textsuperscript{TM} gel retardation assays

The experiments in Fig. 3.1 A were conducted by Immunoprecipitation (IP) and Phosphatase treatment of extracts from W303 cells. Briefly, 50 ml cultures were grown to OD\textsubscript{600}=1.0. Cells were washed and disrupted with 0.5 mm glass beads in buffer containing 20 mM Hepes pH 7.6, 50 mM KAc, 5 mM Mg(COO\textsuperscript{-})\textsubscript{2}, 0.1 M sorbitol, 0.1 % Triton-X 100, 2 mM DTT and protease inhibitors (Sigma). The extract was immunoprecipitated with 15 µl Dynabeads-protein G coated with anti-MYC (mouse) antibody at 4°C for 1.5 hours. Beads were washed with phosphatase buffer (NEB) and one third was incubated with 2 units/µl Lambda Protein Phosphatase (NEB) for 30 min at 30°C, while the rest of the sample was incubated with buffer alone. Beads were then boiled in 4% SDS/8M Urea and run on 7.5% SDS-polyacrylamide gels containing 60 µM PhosTag\textsuperscript{TM} and 120 µM MnCl\textsubscript{2} or 120 µM ZnCl\textsubscript{2} as described (253). The proteins were then transferred to PVDF membranes and analyzed by Western blotting with anti-MYC (rabbit) antibodies. For the experiments in Figure 3.1 B and C and Figure 3.4 A, cell pellets were directly boiled in 4% SDS/8M Urea and disrupted with glass beads before being analyzed by the PhosTag\textsuperscript{TM} gel retardation assay.

3.2.4 Kinase assay

The GST-Cac1p and GST-Rtt106p substrates were expressed from pGEX vectors in BL21(DE)\textsubscript{LysS} cells and the proteins were purified on Glutathione-Sepharose 4B columns (GE
The Cac1<sub>226-606</sub>-His<sub>6</sub> and Hst3-His<sub>6</sub> substrates were expressed from pET28 vectors in Arctic Express (DE3)RIL cells and purified on Ni-NTA Agarose (Qiagen). The kinase complexes (GST-Cdc28p/Clb5p and His<sub>5</sub>-Cdc7p/Dbf4p) were co-expressed in Sf9 cells co-infected with recombinant baculoviruses expressing each polypeptide subunit. The kinases were purified with Glutathione Sepharose or Ni-NTA Agarose columns, respectively. Kinase reactions (25 μl) were conducted in 40 mM Hepes-KOH (pH7.6), 0.5 mM EDTA, 0.5 mM EGTA, 1 mM β-glycerophosphate, 1 mM NaF, 2 mM DTT, 8 mM Mg(COO)<sub>2</sub>, and 0.1 mM ATP with 1 μCi of [γ-<sup>32</sup>P] ATP. Substrates were 0.5 μg of affinity-purified GST-Cac1p or 1 μg of GST-Rtt106p. 50 ng of affinity-purified Cdc7-Dbf4 complex or 100 ng of affinity-purified Cdc28p-Clb5p complex were added to the substrates and incubation was for 45 min at 30°C. The kinase reactions were resolved on 5-20% gradient SDS-polyacrylamide gels, dried and exposed to Phosphorimager® screen. Equal loading of the substrate proteins was confirmed by Coomassie staining of the gel prior to drying.

### 3.2.5 LC-MS/MS analyses

The kinase assays for the MS analysis of Cac1p phosphorylation by Cdk7p-Dbf4p (Appendix 3) were performed with 50μM cold ATP for 30 min at 37°C. The reaction products were denatured with 1% SDS and ammonium bicarbonate was added to 50 mM. Proteins were then reduced with 0.5 mM TCEP for 20 min at 37°C, alkylated with 50 mM iodoacetamide for 20 min at 37°C, and quenched with 5 mM DTT. 50 mM ammonium bicarbonate was added to dilute SDS to 0.1% and the mixture was subjected to trypsin digestion for 4 h at 37°C. After proteolysis, the digest was acidified with TFA and evaporated in a Speed-Vac. Phosphopeptide enrichment was in TiO<sub>2</sub> micro-columns (GL Sciences, Japan). The eluate was acidified with 1 μl of TFA prior to MS analysis. LC-MS/MS analyses were conducted with a LTQ-Orbitrap XL hybrid mass spectrometer equipped with a nanoelectrospray ion source (Thermo Fisher Scientific) and coupled to a nano-flow LC system (Eksigent). The chromatographic separation was performed using a trapping column (4 mm length, 360 μm i.d.) and an analytical column (10 cm length, 150 μm i.d.) packed in house with 3 μm C18 particles (Jupiter 300Å, Phenomenex). Peptides were analyzed in data-dependent mode. Product ions corresponding to a neutral loss of 80 Da from the precursor peptide were subsequently selected for MS3 fragmentation. For all experiments, an internal mass lock (protonated (Si(CH<sub>3</sub>)<sub>2</sub>O))<sub>6</sub> with m/z 445.120025) or an external calibration mixture (Caffeine, MRFA and Ultramark) were used for calibration and provided mass accuracy within 5 ppm. MS data were analyzed using Xcalibur software (version 2.0 SR1) and Mascot distiller software (version 2.1.1, Matrix science).

### 3.2.6 Chromatin fractionation

50 ml cell cultures were grown to OD<sub>600</sub>=1.0, harvested and treated with 250 μM zymolyase for 25 min at 30°C in 50 mM Tris pH 7.5, 10 mM β-mercaptoethanol, 5 mM MgCl<sub>2</sub> and 0.8 M sorbitol. Normally, more than 95% spheroplasting was visible when cells were removed from sorbitol. Spheroplasts were washed twice with ice-cold 50 mM Tris pH 7.5, 80 mM KCl, 2 mM EDTA and 0.8 M sorbitol and then lysed in 150 μl of Extraction Buffer (EB: 50 mM Tris pH 7.5, 80 mM KCl, 2 mM EDTA, 0.2 % Triton-X 100, 5 mM NaF, 5 mM β-glycerophosphate, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>) supplemented with protease inhibitors (Sigma) and 50 μg/ml PMSF. Samples were spun at 12000 rpm for 10 min and supernatants were taken as cytosolic fractions. Pellets were resuspended in another 150 μl EB and cushions of 50 μl EB containing 30% sucrose were
pipetted underneath. The samples were spun again at 12000 rpm for 10 min and the supernatants were discarded. The pellets and aliquots of the cytosolic fractions were then boiled in Laemmli loading buffer and analyzed in SDS-10% polyacrylamide gels.

### 3.2.7 Co-immunoprecipitation

Cells were harvested in the presence of 0.1% NaN$_3$ and disrupted with 0.5 mm glass beads in ice-cold IP Buffer (IPB: 50 mM Tris pH 7.5, 80 mM NaCl, 2 mM EDTA, 5 mM NaF, 5 mM β-glycerophosphate, 0.1 mM Na$_3$VO$_4$) supplemented with protease inhibitors (Sigma) and 50 µg/ml PMSF. The lysates were spun at 12000 rpm for 10 min and the supernatants were immunoprecipitated overnight with 15 µl of EZview-anti-MYC beads (Sigma). The beads were washed three times in IPB plus 0.2% Triton-X 100, 0.03% Deoxycholic acid, once with IPB plus 420 mM NaCl, and once with IPB. Samples were boiled in Laemmli loading buffer and analyzed in SDS-10% polyacrylamide gels. Cell extracts containing Cac1p-FLAG proteins were similarly prepared and immunoprecipitated with 15 µl EZview-Protein G beads (Sigma) coated with anti-FLAG (mouse) antibody.

### 3.3 Results and Discussion

#### 3.3.1 In vivo phosphorylation of Cac1p by Cdc28p

To address the roles of Cdc7p and Cdc28p in the regulation of CAF-1, I analysed the phosphorylation of Cac1p in wild type, cdc7-1 and cdc28-1 mutant strains, which harbor a MYC-tagged genomic copy of CAC1. Extracts were prepared by boiling the cells in 8M Urea/4% SDS and the phosphorylation state of Cac1p-MYC was assessed by a modified PhosTag™ gel retardation assay (253,254). Briefly, the PhosTag™ ligand associates with phosphorylated serines, threonines and tyrosines in the presence of Mn$^{++}$ or Zn$^{++}$ and retards the mobility of phosphorylated proteins in SDS-polyacrylamide gels (253). In Fig. 3.1 A, I show an apparent retardation of Cac1p-MYC in the presence of Mn$^{++}$ and PhosTag™. Pre-treatment of the extract with phosphatase abolished this retardation. The PhosTag™ assays with extracts from wild type (W303), cdc7-1 and cdc28-1 cells showed no substantial differences between these strains at the permissive temperature of 23°C (Fig. 3.1 B, lanes 1-4). When cells were shifted to 37°C for 1 hour, the low-mobility Cac1p band disappeared in cdc28-1 cells, but remained unchanged in the cdc7-1 and wild type cells (Fig. 3.1 B, lanes 5-8). While these results shed some doubt on whether Cdc7p phosphorylates Cac1p in vivo, they clearly suggested that Cdc28p could be such a kinase.

It is known that the exposure of both the cdc7-1 and cdc28-1 strains to 37°C leads to accumulation of cells in G1/S phase (255,256). Hence, the lack of Cac1p phosphorylation in cdc28-1 could reflect the G1 phase arrest and not necessarily the loss of the CDK activity. Conversely, the lack of effect in cdc7-1 cells could be due to slow progression through S phase (255), which could be past the point of Cac1p phosphorylation by Cdc7p/Dbf4p (DDK). To address these issues, I synchronized W303, cdc7-1 and cdc28-1 cells in G1 with alpha-factor for 3 hours, released them towards S phase and then shifted half of the cultures to 37°C for 30 min. This treatment is expected to inactivate the kinases Cdc7p and Cdc28p in S phase. Aliquots of the cultures were collected and analyzed by FACS (Fig. 3.1 C) and by the PhosTag™ retardation assays (Fig. 3.1 D).
**Figure 3.1 - Phosphorylation of Cac1p-MYC in cdc7-1 and cdc28-1 mutants.**

A) Cell extract from wild type (W303) CAC1-MYC::KanMX was immunoprecipitated with Dynabeads-protein G and anti-MYC antibody. Half the sample was treated with mock solution (lanes 1 and 2) and half with lambda protein phosphatase (lane 3) for 30 min at 30°C. These were run with 50 µM PhosTag™ in SDS- 6.5% PAGE with or without 100 µM MnCl₂ as indicated. Western blotting was performed with anti-MYC antibody.

B) Cells were grown at 23°C (lanes 1-4) and then shifted to 37°C for one hour (lanes 5-8) before extracts were prepared by boiling the cells in loading buffer. Samples were run in SDS-PAGE gels containing PhosTag™ and analyzed as in A). One of three independent experiments with similar results is shown.

C) Cell cultures were arrested with alpha-factor for 3 hours at 23°C, transferred to fresh YPD medium for 20 min and then split and grown for 30 min at 23°C and 37°C, respectively. Samples from the cultures were taken out at the indicated times, stained with propidium iodide and analyzed by FACS. Unsynchronized cells were analysed to show the normal 1c/2c distribution in each strain (unsync). Cells were arrested in M-phase with Nocodazole (M) to show 2c. Numbers 1-12 indicate the samples analyzed and correspond to the lanes in D. Left-pointing arrows highlight the 23°C and 37°C 30 min samples for comparison.

D) Samples of the cultures were taken out at the indicated time points after release from alpha-factor arrest and analysed in SDS-7.5% polyacrylamide gels containing 60 µM PhosTag™ and 120 µM ZnCl₂. Proteins were transferred to membranes and analyzed by Western blotting with anti-MYC antibodies. Densitometry graphs of lanes 3, 4, 7, 8, 11, 12 were acquired with the ImageJ software and are shown underneath the lanes. One of two independent experiments with similar results is shown.
The samples from the cells that were synchronized in G1 and the samples collected 20 min after the release displayed mostly unphosphorylated Cac1p (Fig. 3.1 D). The samples that were incubated at 23°C for an additional 30 min displayed significant and comparable levels of Cac1p phosphorylation in all three strains (Fig. 3.1 D, lanes 3, 7, 11). These results showed that Cac1p is not phosphorylated in G1 and that under permissive conditions the cdc7-1 and cdc28-1 alleles do not preclude its phosphorylation in S phase. Importantly, at 37°C there was a noticeable decrease in the phosphorylation of Cac1p in cdc28-1 as compared to wild type and cdc7-1 cells (Fig. 3.1 D, lanes 4, 8, 12). It is unlikely that this decline in Cac1p phosphorylation in cdc28-1 cells at 37°C is caused by cell cycle effects because there is a very similar cell cycle distribution of the cdc28-1 cultures at 23°C and 37°C (Fig. 3.1 D, lanes 11-12). I concluded that the loss of Cac1p phosphorylation in cdc28-1 cells is caused by the inactivation of Cdc28p rather than an arrest in G1 phase.

3.3.2 Identification of CAF-1 phosphopeptides

It has been demonstrated that the human homolog of Cac1p (p150) is a substrate of Cdc7/Dbf4 (82). The persistence of Cac1p phosphorylation in cdc7-1 cells (Fig. 3.1 B and D) raised the possibility that, unlike its human counterpart, budding yeast Cac1p is not phosphorylated by Cdc7p. Moreover, it is not known if yeast Cac1p is directly phosphorylated by Cdc28p. In order to resolve these issues, our lab established a collaboration with Marlene Gharib, Pierre Thibault and Alain Verreault at the Institute for Research in Immunology and Cancer, Succursale Centre-Ville, Montréal. They provided mass-spec data showing in vivo phosphorylation of six CAF-1 phosphopeptides (Appendix 3 Fig. 1), using affinity-purified S. cerevisiae CAF-1 as previously described (257). The three subunits of CAF-1 were all present in the purified complex and of the six phosphopeptides, five were located in the Cac1p subunit and one was located in the Cac3p subunit (Appendix 3 Fig. 1). Some of these sites have been identified in proteome-wide studies (http://phosphopep.org), suggesting that they are not low abundance phosphopeptides. Unexpectedly, no phosphorylation site was found in Cac2p (Appendix 3 Fig. 1 and http://phosphopep.org), the homolog of CAF-1 p60 that is extensively phosphorylated in human cells (250,258). Interestingly, four of the Cac1p phosphorylation sites identified in vivo conform to either CDK (SP) or DDK sites (SD).

3.3.3 In vitro phosphorylation of Cac1p by Cdc7p and Cdc28p

To provide evidence that Cac1p is directly phosphorylated by those kinases, I performed in vitro assays in collaboration with Naoko Kakusho, Zhiying You and Hisao Masai at the Genome Dynamics Project, Department of Genome Medicine, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan. They provided me with recombinant DDK and Cdc28p-Clb5p (hereafter referred to as CDK) to be used with purified GST-Cac1p. I chose Cdc28p-Clb5p, as opposed to Cdc28p associated with other cyclins, because the form of CDK containing Clb5p phosphorylates several proteins during S-phase (259). As a control, I used GST-Rtt106p, which the Masai lab has recently identified as a Cdc7p substrate. These assays showed that both kinases phosphorylate GST-Cac1p (Fig. 3.2, lanes 2 and 4). The two kinases did not cooperate in the phosphorylation of this substrate in vitro (Fig. 3.2, lane 3) as previously shown for other substrates of DDK and CDK (260,261). Importantly, the two kinases exhibited distinct phosphorylation patterns on GST-Cac1p. My preparations contained full length GST-Cac1p and several shorter peptides, which were presumably generated by C-terminal truncation and were
pulled out by the GST tag attached to the N-terminus of Cac1p (Fig. 3.2, lanes 5-8).

CDK efficiently phosphorylated many of these shorter peptides presumably because they harbor the Cdc28p target sites. Consistent with this, one CDK site phosphorylated in vivo was S94. In contrast to CDK, DDK clearly preferred the full-length protein. None of the shorter bands was a product of auto-phosphorylation as they were not present in the kinase reactions lacking the substrate (Fig. 3.2, lanes 9-11). GST-Rtt106p was phosphorylated by DDK but to a far lesser extent by CDK (Fig. 3.2, lanes 16 and 18) further strengthening the idea that the observed activity of Cdc28p-Clb5p is not caused by non-specific contaminating kinases. Hence, it is apparent that Cdc28p-Clb5p phosphorylates Cac1p in its N-terminal segment, but other Cdc28p target sites closer to the C-terminus could not be ruled out. On the other hand, it seems that DDK phosphorylates Cac1p at a position(s) closer to its C-terminus.

We revisited this issue by performing kinase assays with an N-terminally truncated Cac1p$_{226-606}$-His$_6$ substrate, in collaboration with the Verreault lab. As shown in Appendix 3 (Fig. 2 A, lanes 2 and 5), this Cac1p fragment retains the DDK phosphorylation site(s). After repeating the kinase reactions with cold ATP, the products were analysed by mass spectrometry. Consistent with the in vivo mass-spec data (Appendix 3 Fig. 1), a phosphopeptide corresponding to phosphorylation of Cac1p-S503 was detected (Appendix 3 Fig. 2 B).

![Figure 3.2 - In vitro phosphorylation of GST-Cac1p by Cdc7p/Dbf4p and Cdc28p/Clb5p kinases.](image)

GST-Cac1p or GST-Rtt106p were mixed with Cdc7p/Dbf4p, Cdc28p/Clb5p or both (as indicated above the lanes) in the presence of $^{32}$P-$\gamma$ATP. The reaction products were resolved through 4-20% polyacrylamide gradient gels and exposed to Phosphorimager screens. The Coomassie-stained gels are also shown. The positions of full length GST-Cac1p and GST-Rtt106p are shown by arrows. One of two independent experiments with similar results is shown.
3.3.4 Phosphorylation of Cac1p-FLAG S94A and S515A point mutations

I tested the *in vivo* significance of DDK and CDK phosphorylation sites. Cac1p-S94 and S515 are phosphorylated *in vivo* and match the S/TP consensus for Cdc28p targets. In addition, Cac1p-S501 and S503 are also phosphorylated *in vivo* and conform to DDK consensus sites (S/T residues in proximity of D) (262-264). I also mutated S238, which resembles a DDK target and is located just after the PIP (residues 227-234) (248) FLAG-tagged Serine to Alanine point mutants at these positions were expressed from low copy pRS315 plasmids in Δcac1 cells.

First, I employed the PhosTag™ retardation assay to assess the contribution of these serines to the phosphorylation of Cac1p *in vivo*. The mobility of the FLAG-tagged proteins was compared to that of Cac1p-MYC in alpha-factor synchronized cells (unphosphorylated Cac1p) and to the slower moving forms of Cac1p observed in non-synchronised cells (Fig. 3.3 A). Similarly to Cac1p in cdc28-1 cells at 37°C (Fig. 3.1 B), slowly migrating forms of Cac1p were substantially depleted in cells expressing either Cac1p-S94A or Cac1p-S515A (Fig. 3.3 A). It is interesting that a mutation at either of these sites was sufficient to preclude the accumulation of the slower migrating bands. This observation suggests that the inability to phosphorylate one of these residues precludes the phosphorylation of other sites. Alternatively, the phosphorylation of both S94 and S515 may be necessary for the retardation by PhosTag™. This interpretation suggests that the PhosTag™ retardation assay might miss single phosphorylation events and that the fast migrating bands in Fig. 3.1 and Fig. 3.3 A may retain some degree of phosphorylation. Nevertheless, this does not alter my main conclusion: the S94A and S515A mutations recapitulate the effect of Cdc28p inactivation. In contrast, the S238A, S501A or S503A mutations did not abolish the presence of the slow migrating forms of Cac1p *in vivo*.

3.3.5 Association of Cac1p-FLAG point mutants with chromatin

I tested the role of these residues in the association of Cac1p with chromatin using a previously published procedure (265,266). Briefly, spheroplasts from cells expressing Cac1p-FLAG mutants were gently lysed and spun through a cushion of 30% sucrose. The supernatant was designated as “cytosol”. The pellet was washed, re-spun and designated “chromatin”. The abundance of Cac1p-FLAG in these fractions was assessed by Western blotting. These assays showed that, in contrast to wild type Cac1p, S501A and S503A, the S94A and S515A mutants poorly associate with chromatin (Fig. 3.3 B). Hence, mutations that preclude its phosphorylation at the Cdc28p consensus sites strongly reduce the association of Cac1p with chromatin. The fact that the S503A, S501A or S238A mutations did not impair Cac1p binding to chromatin raises an important question. At present, Cac1p-S503 is the only confirmed direct target of DDK. On the other hand, in human cell extracts Cdc7-Dbf4 promotes the association of p150 (Cac1p) with PCNA (82). One explanation for my negative results is that the employed assays cannot detect transient *in vivo* effects mediated by DDK. Alternatively, the prevention of phosphorylation at S503 may not be sufficient to impair Cac1p binding to chromatin or PCNA. Hence, other unknown serines could be targeted by DDK. Finally, I cannot rule out the possibility that yeast DDK may not be involved in the regulation of CAF-1.
Figure 3.3 - Analysis of Cac1p-FLAG Serine to Alanine mutants.
A) Cells with a MYC-tagged genomic copy of CAC1 (lanes 1-3) and Δcac1 cells harboring plasmids that express FLAG-tagged copies of Cac1p with no mutation (CAC1) or the indicated point mutations (lanes 4-9) were analyzed by the PhosTag™-retardation assay as in Fig. 3.1. A sample of Δcac1 cells without a Cac1p-FLAG expression plasmid is shown in lane 10. One of three independent experiments is shown. The anti-FLAG Western blot from normal SDS-PAGE (without PhosTag) is shown below.

B) Spheroplasts from Δcac1 cells harboring plasmids for the expression of FLAG-tagged Cac1p were lysed and spun to obtain the cytosol fractions (lanes 1-5). The pellets were resuspended and spun through a 30% sucrose cushion, then boiled in Laemmli buffer and loaded in lanes 6-10. One of three experiments with similar results is shown.

3.3.6 Association of Cac1p-MYC with chromatin and PCNA in cdc7-1 and cdc28-1 mutants

I addressed these uncertainties by testing the binding of Cac1p to chromatin and PCNA in conditional cdc7-1 and cdc28-1 mutants. The strains were grown at 23°C and then shifted for 15 or 30 min to 37°C. Cytosol and chromatin fractions were prepared as in Fig. 3.3 B and the abundance of Cac1p-MYC relative to PCNA was assessed by Western blotting (Fig. 3.4 A) and densitometry analysis (See Appendix 4).
Figure 3.4 - Association of Cac1p-MYC with chromatin and PCNA in *cdc7-1* and *cdc28-1* mutants.

**A)** Cell cultures were grown at 23°C and then shifted to 37°C for 15 min or 30 min. Cytosol (lanes 1-9) and chromatin (lanes 10-18) fractions were prepared as in Figure 3.4 B. One of four independent experiments is shown. **B)** Cell cultures were grown as in A) and disrupted with glass beads to obtain cell extracts (lanes 1-9) that were immunoprecipitated with anti-MYC antibodies (lanes 10-18). One of three independent experiments with similar results is shown. All samples in A) and B) were analyzed by Western blotting with anti-MYC and anti-PCNA antibodies as indicated. Equal loading was confirmed by staining the membranes with India ink.
After 15 min at 37°C both cdc7-1 and cdc28-1 cells displayed amounts of chromatin-associated Cac1p that were comparable to the amounts in wild type cells (Fig. 3.4 A, lanes 13-15). However, after 30 min at 37°C an apparent loss of chromatin-associated Cac1p and corresponding increase in the cytosol fraction were seen in the cdc28-1 cells (Fig. 3.4 A, lanes 8 and 17). In contrast, in the cdc7-1 cells incubated at 37°C for 30 min there was a small increase in chromatin-associated Cac1p. I have shown that short exposures to restrictive temperature do not cause major cell cycle redistribution in these mutants (Fig. 3.1 C). Therefore, it seems unlikely that cell cycle effects cause the specific loss of chromatin-bound Cac1p in cdc28-1 cells.

For the analysis of the Cac1p-PCNA interaction, cells were grown at 23°C and 37°C, disrupted with glass beads and spun to obtain total cell extracts. Co-immunoprecipitation experiments reproducibly showed a 2-fold decrease (relative to wild type) in PCNA bound to Cac1p in the cdc7-1 strain at both temperatures (Fig. 3.4 B; Appendix 4: Table 8 and Fig. 2). In the cdc28-1 strain Cac1p-bound PCNA decreased about 1.4 to 1.6 fold regardless of temperature (Fig. 3.4 B; Appendix 4: Table 8 and Fig. 2). My results show that mutations in Cdc7p and Cdc28p affect the interaction between Cac1p and PCNA. However, the results shown in Figure 3.3 B raise the question of whether this regulation is direct or indirect. Importantly, the data in Figures 3.3 and 3.4 show little correlation in the association of Cac1p with chromatin and the association of Cac1p with PCNA, which supports the possibility that the recruitment of CAF-1 to chromatin is at least partially independent of its binding to PCNA.

3.3.7 cdc7-1, cdc28-1, and Cac1p-FLAG point mutations do not exhibit loss of TPE epigenetic conversions

Earlier studies have demonstrated that the deletion of the CAC1 gene causes significant loss of telomeric gene silencing (80,86) and a decrease in the frequency of epigenetic conversions at telomeres (Chapter 2) (267). In Fig. 3.5, I show that these phenotypes are reversed by the expression of Cac1p-FLAG in Δcac1 cells. However, none of the point mutations phenocopy the loss of CAC1. Hence, the phosphorylation of Cac1p by Cdc28p or Cdc7p does not seem necessary for telomere position effects. Interestingly, other CAC1 mutations that affect its binding to PCNA (cac1-13 and cac1-20) also do not disturb telomere position effects (248). Furthermore, the cdc7-1 and cdc28-1 kinase mutants did not show a decrease in the frequency of epigenetic conversions either (Fig. 3.5), though this was not unexpected since the experiments had to be performed at permissive temperature to allow mitotic growth. Together, these observations show that the point mutations in Cac1p do not recapture the complete loss of CAC1 and that there is a level of complexity in the control of telomere position effects by CAF-1 that remains unclear.
3.4 Summary and Conclusions

In summary, I have demonstrated that Cdc28p phosphorylates Cac1p-S94 and S515. These phosphorylation events take place during the G1/S transition and/or S-phase and regulate the association of Cac1p, and presumably CAF-1, with chromatin. Although this timing coincides with the activation of early origins of DNA replication, the association of PCNA with Cac1p does not appear to correlate with its phosphorylation, suggesting that phosphorylation of S94 and S515 may not directly regulate the interaction between CAF-1 and PCNA and, presumably, with replication forks. I postulate that there is a second mechanism by which CAF-1 is recruited to chromatin during S phase. The nature of this second mechanism is currently unknown. In tune with this notion, I and others have reported incomplete overlap in the \textit{in vivo} effects of mutations.
in PCNA (pol30) that impair its interaction with Cac1p, and the deletion of CAC1 itself (Chapter 2) (86,248,267).

While this study highlights the role of CDC28 as a direct regulator of CAF-1, it sheds some uncertainty on the role of CDC7 in S. cerevisiae. In human cells, the Cdc7-Dbf4 kinase acts as a regulator of the PCNA-CAFI association (82). I did not obtain strong support for this model in S. cerevisiae. I have confirmed that DDK phosphorylates Cac1p in vitro and my collaborators (Gharib and Verreault) have identified S503 as a target of this kinase, but I have failed to show a functional consequence of this phosphorylation. As speculated earlier, DDK could phosphorylate multiple serines on Cac1p. Indeed, there are 13 reported sites of phosphorylation on this peptide (252). My data certainly do not argue against the model presented in (82). However, I believe that it only partially depicts the regulation of CAF-1 by DDK. In addition to the largest subunit of CAF-1, DDK and its homologs in other species phosphorylate many other substrates. These are involved in the regulation of the cell cycle, replication fork stalling, DNA damage and meiosis (221,261,268-272). At least some of these processes could directly or indirectly affect the activity, the association partners or the turnover of CAF-1. Hence, DDK could regulate the PCNA-Cac1p interaction, but also other functions of CAF-1. In support of this notion, in Fig. 3.6, I show preliminary data pointing to a degradation of Cac1p in chromatin. This degradation is reduced in the cdc7-1 strain. I have not fully developed these observations to present a compelling case. However, they fuel the notion that more than one aspect of CAF-1 regulation is affected in the cdc7-1 mutant and that the links between DDK and CAF-1 are not completely understood.

**Figure 3.6 - Suppressed degradation of Cac1p in cdc7-1 and cdc28-1 mutants.**

Wild type (W303) and isogenic cdc7-1 cells harboring a MYC-tagged genomic copy of CAC1 were grown at 23°C and spheroplasts were prepared and lysed by osmotic shock to obtain whole cell extracts (WCE, lanes 1, 6). The extracts were spun through a 30% sucrose cushion to separate the cytosol (lanes 2, 5) and chromatin (lanes 3, 4) fractions. Aliquots of all samples were separated in 7.5% polyacrylamide gels and analysed by Western blotting with anti-MYC antibodies. Higher mobility anti-MYC interacting bands (about 90 and 70 kDa, depicted by asterisks) were detected in the chromatin fractions of wild type, but not cdc7-1 cells. One of two independent experiments with similar results is shown.
In conclusion, my study points to a direct role of *CDC28* in the loading of CAF-1 to chromatin and, as a result, the reassembly of chromatin during DNA replication. This novel role for CDK broadens the scope of the effects for this master-regulator of the cell cycle and presents the possibility of a DDK/PCNA-independent mechanism for the association of CAF-1 with chromatin.
Chapter 4 - Sub-telomeric Core X and Y’ elements in S. cerevisiae suppress extreme variations in gene silencing

* denotes equal contribution

4.1 Introduction

Telomere Position Effect (TPE) is governed by strong repression signals emitted by the telomere (125). As described in Chapter 1, these signals are relayed by weaker proto-silencers (Rap1p and Abf1p binding sites and ACSs), which are positioned in the sub-telomeric Core X and Y’ elements (141,143,145,161,273). The sub-telomeres also contain sequences which display anti-silencing properties and are referred to as STARs (Sub-telomeric Anti-silencing Regions) (144). The antagonizing silencing and anti-silencing activities emitted by these elements confer the peculiar quasi-stable mode of sub-telomeric gene silencing/expression that is TPE in S. cerevisiae.

The HDAC Sir2p, plays a central role in the establishment and maintenance of silencing at all repressed loci in yeast. At telomeres there are two means of engaging Sir2p. The telomeric TG1,3 repeats bind Rap1p, which in turn recruits Sir3p and Sir4p to eventually recruit Sir2p (23). Two proteins, Rif1p and Rif2p, interfere with the interaction between Rap1p and Sir3/Sir4 thus acting as anti-silencing factors (219,274,275). At the same time the sub-telomeric ACS proto-silencers bind ORC (Origin Recognition Complex). ACS-bound Orc1p associates with Sir1p to independently recruit Sir2p to these positions (23). Consequently, Sir2p deacetylates the nearby nucleosome and spreads over the neighboring ones with the aid of Sir3p and Sir4p (Chapter 1). The spreading of histone deacetylation by Sir2p is counteracted by Histone Acetyl Transferases (HATs), but the mode of their action is not understood to the extent of the SIR genes.

HATs acetylate lysines of core histones to generate events which culminate in chromatin decondensation. To date, nine HATs have been described in S. cerevisiae (276). Several studies have pointed to SAS2 as the principal SIR2-counteracting HAT at telomeres (32,150,151,277,278). Sas2p is responsible for the acetylation of H4-K16 in vivo, while Sir2p deacetylates this position, thus portraying SAS2 as an anti-silencing factor (277,278), with the two opposing enzymes generating a dynamic chromatin boundary at sub-telomeres. Paradoxically, deletion of SAS2 moderately increases the silencing of natural sub-telomeric genes (32,150,151,277,278), but dramatically reduces silencing at synthetic telomeres (172,230-232). This stark discrepancy has not been adequately explained. On the other hand, many other lysines in H3 and H4 are hypo-acetylated in sub-telomeric chromatin (279) suggesting that other HATs are also directly involved in anti-silencing.

For this chapter, I collaborated with Patricia Power and Muhammad Attiq Rehman to address these questions. We have characterized the roles of five HATs (HAT1, GCN5, SAS2, SAS3, Rtt109), as well as RIF1 and CDC6 on several recombinant telomeres made up of Core X, Y’ and STARs. I performed experiments with Rtt109 and RIF1 and contributed to the experiments completed with SAS2, SAS3, GCN5 and HAT1. I have included the combined data of all
collaborators as they are necessary for the coherency of the chapter, with the clear message that only the Rtt109 and RIF1 data and portions of the SAS2, SAS3, GCN5 and HAT1 data are produced by me.

4.2 Materials and Methods

4.2.1 Strains and growth conditions

Deletions of HAT1, GCN5, SAS2, SAS3, YNG1, Rtt109 and RIF1 are derivatives of BY4742 and were obtained from ATCC. All other mutants are derivatives of W303. All strains used in this study are listed and referenced in Table 5 in Appendix 1. Growth conditions were as described (Section 2.2.1).

4.2.2 Telomeric constructs

All constructs are flanked by a portion of ADH4 and telomeric TG_{1,3} repeats (see Fig. 4.1 A) and are designed for targeted integration in the left telomere of chromosome VII. URA3-tel (139), GF2, GF3, GF6, GF9, GF44, GF46 and GF61 were previously described (143). GF6ΔSTAR, GF6ΔACS and GF44ΔACS were produced by excision of the STAR element in GF6 or by site directed mutagenesis of ACS in the Core X elements, respectively. All integrating constructs were produced by restriction digestion of the corresponding plasmids as described (Section 2.2.1).

4.2.3 Telomeric integration and analysis of gene silencing

Cells were transformed with integrating constructs and three single colonies were selected from SC-ura plates. To warrant for the loss of un-integrated constructs (linear DNAs lacking CEN elements), transformants were restreaked on Sc-ura and again a single colony from this SC-ura plate was streaked on both SC-ura and SC/FOA. By the third re-streaking, the transformed cells have been grown for about 60 generations. This procedure uniformly produces cells that have integrated the test constructs (Fig. 4.1) in the VII telomere when analyzed by PCR. Finally, a single colony was taken from the third SC-ura plate and grown for about 30 generations in non-selective (YPD) medium. Serial 1:10 dilutions were prepared for each culture and 5 μl aliquots were spotted on SC and SC/FOA plates. Colonies in two consecutive spots with less than 50 colonies (these correspond to two consecutive dilutions) were counted. The %FOA^R for each independent culture was acquired as the number of colonies on SC/FOA plates divided by the number of colonies on SC plates. Finally, the average %FOA^R of the counts in three independent cultures ± standard deviation were calculated and are shown Table 6 in Appendix 1. Average values and the ratios between %FOA^R in different strains and/or constructs were calculated and plotted in Microsoft Excel.

4.3 Results and Discussion

4.3.1 Core X and Y’ curtail variations in TPE caused by deletion of HAT genes

We used the set of telomeric reporters shown in Fig. 4.1 A to analyze the role of several non-essential HATs in TPE. These reporters contain URA3 and different combinations of sub-telomeric Core X, Y’ and STAR elements (Fig. 4.1 A).
Figure 4.1 - Analysis of Telomere Position Effect in Histone-Acetyl-Transferase Mutants.

A) Telomeric reporters used in this study. Maps (not to scale) of the used constructs are shown. The positions of core X element from the IIR telomere and the Y’ element from the XII-L telomere (black rectangles), the STARs from the same telomeres (grey rectangles), URA3, ADH4 and the telomeric TG1-3 repeats (black triangles) are as indicated. The position of the destroyed ACS (ARS Consensus Sequence) is depicted by an open diamond. The 5’→3’ direction of URA3 transcription is indicated in the URA3-tel construct (top) and is the same for all constructs shown. The insertions between URA3 and the telomeric repeat add 145-900 base pairs as compared to URA3-tel. Fig. 4.1 B on next page...
Fig. 4.1 continued... B) Percentage of FOA<sup>R</sup> cells in different strains and constructs. The reporter constructs shown along the vertical axis were integrated in the strains shown on the left. Percentage of FOA<sup>R</sup> cells was measured in at least three independent experiments. Average %FOA<sup>R</sup> ± std. dev. were calculated and plotted. Data is from Table 6 (Appendix 1). Fig. 4.1 C on next page...
Fig. 4.1 continued... C) URA3-tel recapitulates silencing effects in mutant strains. The URA3-tel construct was integrated in the strains shown along the vertical axis. The ratios of %FOAR in the mutant strains versus the %FOAR in the isogenic wild type strain were calculated and plotted. The effects of Δsas2, Δsas3, Δyng1, Δrtt109, Δhat1, Δgcn5 and Δrif1 were assessed using BY4742 as the wild type strain (Table 6, Appendix 1). The effects of orc2-1, orc5-1, mcm5-461, cdc6-1, cdc45-1 and cdc7-1(sas1) were assessed using W303 as the wild type strain (data not shown). There is little difference in the levels of telomeric silencing between BY4742 and W303. The arrows underneath the exponential graph indicate increase or decrease of silencing.

The ADH4-URA3-tel construct (139) is one of the most frequently used telomeric reporters and serves as a direct cross-reference between other studies and the current one. GF2 and GF3 contain STARs derived from the Core X-IIR or Y'-XIIL elements, respectively. GF6 and GF9 contain the same STARs, but also the Core X and Y' from the same telomeres as GF2 and GF3, respectively. In GF44 and GF46 the Core X and the Y' are positioned distal to the telomere beyond URA3. In GF61 URA3 is away from the telomere beyond two STARs, Core X and TRP1. In addition, ACS and STAR were destroyed in GF6 and GF44 as indicated. The insertions between URA3 and the telomeric repeat add 145-900 base pairs in different constructs as compared to URA3-tel. Several studies have shown that the telomeric silencing for these and other constructs does not directly correlate to the distance from the telomeres (128,141,143,145,195). Instead, silencing is discontinuous and is strongly influenced by the nature and the positions of different regulatory elements (280,281). Therefore, the variety of elements in these constructs allows for broad assessment of TPE in different strains.

All constructs were integrated in the left telomere of chromosome VII in BY4742 and its derivatives Δsas2, Δsas3, Δyng1, Δrtt109, Δhat1 and Δgcn5 and selected on SC-ura plates. Colonies were then streaked on SC/FOA plates, which render the URA3-expressing cells sensitive to the drug while the cells with repressed URA3 form FOAR colonies. After confirming the variegated mode of expression of the integrated reporters, three colonies were grown in non-
selective medium for 30 generations to allow for the re-establishment of the silenced/active equilibrium of URA3 in these cultures. The percentage of FOA\textsuperscript{R} was calculated as the number of colonies on SC/FOA plates divided by the number of colonies on SC plates. The average values ± standard deviations were calculated (Table 6 in Appendix 1) and are plotted in Figure 4.1 B.

Next, we cross-referenced the acquired data to available data in earlier publications. URA3-tel, GF2, GF3, GF6, GF9, GF44, GF46, GF61, GF6ΔSTAR, GF6ΔACS and GF44ΔACS showed very similar levels of %FOA\textsuperscript{R} in BY4742 cells as compared to the previously used W303 strain (128,143,195). In addition, the prototype URA3-tel construct recapitulated the silencing defects observed in sas2, sas3, orc2-1, orc5-1, mcm5-461, cdc6-1, cdc45-1 and cdc7-1(sas1) (Fig. 4.1 C) (172,230,282,283). Finally, we compared the magnitude of SAS2-dependent de-repression of URA3-tel in BY4742 and W303. The deletion of SAS2 in W303 had decreased repression in the range of 10-50 fold (172,232), while in BY4742 we observed a reduction of 14 fold. Thus, our data is in close agreement with all earlier studies. We used the values in Table 6 in Appendix 1 to calculate the ratios of %FOA\textsuperscript{R} in the mutant strains versus the %FOA\textsuperscript{R} in the isogenic wild type BY4742 strain. These ratios provide quantitative assessment of the effect of each gene on the silencing of URA3 in each individual construct.

The deletion of SAS2 and SAS3 caused 10-100 fold de-repression in URA3-tel, GF2 and GF3, whereas the deletion of YNG1 (a modulator of SAS3 activity in the NuA3 complex (284)) and Rtt109 caused 5-50 fold decrease of repression (Fig. 4.2 B). In contrast, the deletion of HAT1 and GCN5 moderately (2-10 fold) increased repression (Fig. 4.2 B). The gain in silencing in ∆hat1 and ∆gcn5 cells is comparable to the effect of the deletion of RIF1 (Fig. 4.2 B), a key telomeric anti-silencing factor. We do not understand the mechanisms that lead to these somewhat surprising effects for HAT genes. However, the similarity in the magnitude of effects in ∆hat1, ∆gcn5 and ∆rif1 cells indicates that the increase in repression in ∆hat1 and ∆gcn5 is significant.

Hence, at telomeres lacking core X or Y' elements, different HATs operate by different mechanisms and can produce both positive and negative effects on TPE. As expected, the addition of STARs in GF2 and GF3 further reduced the level of silencing in Δsas2, Δsas3, rtt109 and Δyng1 cells. Surprisingly, the calculations for ∆hat1 and ∆gcn5 cells showed that the addition of STARs generated modest, but consistent, increases in telomeric silencing. It is conceivable that STAR activity is diminished in these mutants. Alternatively, the overall increase of telomeric silencing in them may over-compensate the anti-silencing effect of STARs. We deal with this ambiguity in Fig. 4.6 later in this chapter.

The calculations of %FOA\textsuperscript{R} in the mutant strains versus %FOA\textsuperscript{R} in the wild type strain in GF6, GF9, GF44, GF46 and GF61 revealed that the silencing of these reporters was marginally influenced by the deletions of individual HAT genes (Fig. 4.3 B). All these reporters contain a single copy of Core X or Y' (black rectangles in the graphs shown on top of Figure 4.3). Hence, the strong repression or anti-repression effects which were observed in URA3-tel, GF2 and GF3 (Fig. 4.2 B) were dramatically reduced by the addition of Core X or Y' regardless of the position of these elements relative to URA3 and the telomere. The consistent decrease of silencing aberrations in all mutants and constructs strongly suggests that the sub-telomeric Core X and Y' elements curtail variations in TPE and maintain the epigenetic plasticity of these loci.
Figure 4.2 - Alterations of TPE in constructs lacking core X or Y’ elements.
The URA3-tel, GF2 and GF3 constructs (shown on top) were integrated in the strains shown on the left and the level of URA3 silencing was calculated as %FOA° cells. A) Levels of URA3 silencing (%FOA°). The 0-10% range is spread out to properly show differences at very low levels of silencing. B) Ratios of %FOA° in the mutant strains versus the %FOA° in the wild type (BY4742) strain. Data is from Table 6 (Appendix 1). The arrows underneath the exponential graph indicate increase or decrease of silencing.
Figure 4.3 - Core X or Y’ restrain alterations in TPE.
The GF6, GF9, GF44, GF46 and GF61 constructs (shown on top) were integrated in the strains shown on the left and the level of URA3 silencing was calculated as %FOA<sup>R</sup> cells. A) Levels of URA3 silencing (%FOA<sup>R</sup>). B) Ratios of %FOA<sup>R</sup> in the mutant strains versus the %FOA<sup>R</sup> in the wild type (BY4742) strain. Data is from Table 6 (Appendix 1). The arrows underneath the exponential graph indicate increase or decrease of silencing.
4.3.2 Core X and Y’ curtail variations in TPE in cdc6-1 and Δrif1 cells

We tested if the observed “cushioning” behavior of X and Y’ is similar in non-HAT mutants. For these analyses we selected cdc6-1 and Δrif1 cells. Rif1p counteracts the association of Sir3p/Sir4p with the telomere-bound Rap1p (274,275). Consequently, the deletion of Rif1p boosts telomeric silencing (280). On the other hand, the cdc6-1 mutation dramatically reduces telomeric silencing independently of the ACS proto-silencers that are positioned in the Core X and Y’ elements (195). Hence, these two mutations provide two opposing effects on TPE that are not directly mediated by Core X and Y’. In Fig. 4.4 B we show the analysis of telomeric silencing in these two mutants. As expected, cdc6-1 and Δrif1 significantly decreased or increased the silencing of URA3 in the constructs lacking Core X and Y’ (URA3-tel, GF2, GF3). These effects were not seen in the constructs with Core X and Y’ (GF6, GF9, GF46). In conclusion, we observed that Core X and Y’ can curtail both positive and negative effects on TPE in diverse mutants.

4.3.3 ACSs and STARs confer opposing activities upon deletion of GCN5 and Rtt109

Sub-telomeric ACSs function as weak silencers (141), which relay the silencing signals emitted by the telomere. In an earlier study, Rehman and Yankulov demonstrated that in several strains which harbor mutations in replication factor genes, ACSs convert to weak anti-silencers (285). Is it then possible that the cushioning effect of Core X and Y’ is linked to similar conversions of these ACSs? We tested this possibility by destroying the ACS in two of the constructs to produce GF6ΔACS and GF44ΔACS. We introduced these constructs in HAT-deletion mutants and then calculated the ratios %FOA$_{GF6ΔACS}$/%FOA$_{GF6}$ and %FOA$_{GF44ΔACS}$/%FOA$_{GF44}$. The results are shown in Fig. 4.5. The deletion of the ACS in both GF6 and GF44 reduced the silencing in BY4742, Δsas2, Δsas3, Δyng1 and Δhat1 cells. In contrast, the destruction of ACS had very little effect in Δgen5 and Δrtt109 cells. This observation suggests that GCN5 and Rtt109 directly or indirectly stimulate the silencing activity of sub-telomeric ACSs. At this point we cannot explain the mechanism of their actions. We also noticed that the deletions of SAS2, SAS3, YNG1 and HAT1 did not alter the ACS-dependent silencing in GF6 relative to wild type cells, while in GF44 there was about two-fold reduction in these mutants. The differences between GF6 and GF44 are clearly caused by the different position of Core X, but at present we cannot explain the nature of this specific effect.

Another set of experiments was conducted to directly assess the effects of STARs within the mutant strains by comparing the levels of silencing in STAR-less (URA3-tel and GF6ΔSTAR) and STAR containing (GF2 and GF6) constructs. Our calculations showed that the STAR in GF2 was 2-3 fold more efficient in Δsas2, Δsas3 and Δyng1 cells relative to wild type cells, but 4-6 fold less efficient in Δrtt109, Δhat1 and Δgen5 cells. The STAR in the Core X-containing GF6 operates at marginal efficiency. These observations demonstrated that Core X can dominantly suppress the contribution of STARs to the overall level of gene silencing and that STARs probably function through the joint activity of Rtt109, Hat1 and GCN5. More importantly, the deletions of Rtt109 and GCN5, which have reduced the anti-silencing activity of the tested STAR (Fig. 4.6 B) have also reduced the silencing activity of the ACSs proto-silencers in Core X (Fig. 4.5 B). These observations provide a plausible mechanism for the chromatin modulating activity of Core X and Y’.
Figure 4.4 - Effects of Core X and Y’ in Δrif1 and cdc6-1 cells.
The URA3-tel, GF2, GF3, GF6, GF9 and GF46 constructs (shown on top) were integrated in the strains shown on the left and the level of URA3 silencing was calculated as %FOA<sup>R</sup> cells. A) Levels of URA3 silencing (%FOA<sup>R</sup>). The 0-10% range is spread out to properly show differences at very low levels of silencing. B) Ratios of %FOA<sup>R</sup> in the mutant strains versus the %FOA<sup>R</sup> in the wild type strain. Wild type depicts BY474 for Δrif1 and W303 (not shown) for cdc6-1. The arrows underneath the exponential graph indicate increase or decrease of silencing.

4.4 Summary and Conclusions

The comparison of eight recombinant telomeres in eight mutant strains has clearly demonstrated that Core X and Y’ elements curtail extreme changes in TPE. We show that telomeres without Core X and Y’ elements are subject to significant shifts towards de-repression or repression upon deletion of HAT genes (Fig. 4.2 and Fig. 4.4). In contrast, TPE remains largely undisturbed in Core X and Y’ containing telomeres (Fig. 4.3 and Fig. 4.4). In an earlier study, Rehman et al. also observed that the anti-silencing caused by mutations in DNA replication factors is also reduced by Core X and Y’ (195). Whereas the precise mechanism of the effects of each individual HAT or replication factor mutation remains unknown, it is apparent that Core X and Y’ moderate all these effects. We also need to point out that the synthetic Core X and Y’ containing telomeres display moderate deviations in TPE that compare, in magnitude, to the effects observed at natural telomeres (32, 150, 151, 277, 278).
Figure 4.5 - Effects of ACS proto-silencers in HAT deletion mutants.
GF6, GF6ΔACS, GF44 and GF6ΔACS constructs (shown on the left) were integrated in the strains shown along the vertical axis and the level of URA3 silencing was calculated as %FOA<sup>R</sup> cells. A) Levels of URA3 silencing (%FOA<sup>R</sup>). The levels of silencing of ACS-containing (black bars) and ACS-less (grey bars) constructs are shown side by side. B) Ratios of %FOA<sup>R</sup> in ACS-less versus ACS-containing constructs. The ratios %FOA<sup>R</sup><sub>GF6ΔACS/FOA<sup>R</sup>GF6</sub> and %FOA<sup>R</sup><sub>GF44ΔACS/FOA<sup>R</sup>GF44</sub> were calculated and plotted. The arrows underneath the exponential graph indicate increase or decrease of silencing.
Figure 4.6 - Effects of STAR in HAT deletion mutants.

The URA3-tel, GF2, GF6 and GF6ΔACS constructs (shown on top) were integrated in the strains shown along the vertical axis and the level of URA3 silencing was calculated as %FOA<sup>R</sup> cells. A) Levels of URA3 silencing (%FOA<sup>R</sup>). The 0-10% range in the upper graph is spread out to properly show differences at very low levels of silencing. The levels of silencing of STAR-containing (grey bars) and STAR-less (black bars) constructs are shown side by side. B) Ratios of %FOA<sup>R</sup> in STAR-less versus STAR-containing constructs. The ratios %FOA<sup>R</sup>_<sub>URA3-tel</sub>/%FOA<sup>R</sup>_<sub>GF2</sub> and %FOA<sup>R</sup>_<sub>GF6ΔSTAR</sub>/ %FOA<sup>R</sup>_<sub>GF6</sub> were calculated and plotted. The arrows underneath the exponential graph indicate increase or decrease of silencing.

It has been previously shown that Core X and Y’ contribute to gene repression and that sub-telomeres contain anti-silencing modules, such as the STARs (128,143,145,195). The opposing signals emitted by these elements have been implicated in the variegated nature of sub-telomeric gene expression (144). An important feature of TPE at individual telomeres is that despite the seemingly random conversion between active and repressed state, the proportion of cells with active/repressed genes remains stable. The mechanisms that sustain this meta-stable balance are not so well understood. Here we propose the sub-telomeric Core X and Y’ could play a significant and unexpected role in the dynamic meta-stability of telomeric gene expression. Previous studies have provided extensive evidence in support of their ability to reconstitute telomeric gene repression when silencing is decreased (141,143,273,281). For this reason, Core X and Y’ are generally viewed as proto-silencers. Our data show that these elements can also reduce telomeric gene repression when silencing increases.

We propose that these elements contain not only individual proto-silencers such as ACS and binding sites for Rap1p and Abf1p (141), but also some unidentified anti-silencers. These anti-
silencers are independent of the previously characterized STARs. Ultimately, the multiplicity of individual weak proto-silencers and anti-silencers in *Core X* and *Y’* build up “buffering” cis-elements, which suppress extreme variations in TPE. Such individual elements can acquire opposing activities upon changes of environment or in different genetic contexts. Indeed, we show that the deletion of *GCN5* or *Rtt109* reduces both the anti-silencing activity of a STAR and the silencing activity of an ACS (Fig. 4.4). Consequently, the net effect of the deletions of these two genes on the tested *Core X* and *Y’* containing telomeres is minimal.

### 4.4.1 What are the STARs?

STARs have been characterized as anti-silencing modules residing in proximity of *Core X* and *Y’* elements (143). Independently of the *Core X* and *Y’,* STARs reduce silencing when introduced in a modified *HMRa* mating type locus (143). The mechanism of action of STARs is largely unknown. They contain binding sites for Tbf1p and Reb1p thus implicating these two proteins in STAR activity (143, 286), but additional details are missing. Here, we show that *GCN5, RTT109* and *HAT1* affect the strength of STAR activity (Fig. 4.6 B). It is therefore possible that Tbf1p and Reb1p promote the activity of these HATs. Finally, STARs significantly reduce the silencing only at telomeres, which do not contain *Core X* or *Y’* (Fig. 4.4). Hence, *Core X* and *Y’* activity seems dominant relative to STARs.

### 4.4.2 Technical issues in studies on TPE

Several earlier studies have pointed out significant discrepancies in the silencing at natural telomeres and at synthetic telomeres on truncated chromosomes. For example, the deletion of *SAS2* had caused 10-50 fold reduction of silencing of the simple truncated *URA3-tel* reporter (172, 232). Yet, RT-PCR or microarray analyses of natural sub-telomeric genes had shown only moderate (two fold) alteration in expression in Δsas2 cells (277, 278, 287).

In this study we show that synthetic telomeres, which contain *Core X* and *Y’* elements, closely recapitulate the modest effects of the deletion of *SAS2* at natural telomeres. The same moderate effects apply for all other HATs tested. Hence, analyses of telomeric reporters, which contain *Core X* and *Y’* elements, present a solid alternative to the analyses at natural telomeres.

On the other hand, “complex” synthetic telomeres can muffle weak effects on TPE. For example, studies on *SAS3* have been said to be hampered by the lack of readily detectable phenotypes (31). Here we demonstrate a readily detectable effect of the deletion of *SAS3*. Indeed, the deletion of *SAS3* reduces telomeric silencing as strongly as the deletion of *SAS2* (Fig. 4.2). Therefore, “simple” synthetic telomeres need to be used for the analysis of weak silencing effects.

### 4.4.3 Role of different HATs in TPE

This study was initiated as a screen for the effects of different HATs on TPE before it was refocused on the consistent effects of *Core X* and *Y’.* Consequently, we provide abundant data on the effects of HAT deletions on TPE. Whereas none of these effects is guaranteed to be direct, two points of potential significance need to be raised.
The first point is the modest but consistent reduction in the efficiency of STARs in $\Delta rt109$, $\Delta hat1$ and $\Delta gcn5$ (Fig. 4.4). As mentioned, very little is known about the mode of operation of these cis-elements. It is premature to suggest that STARs recruit these HATs. The weak effects of $Rtt109$, $HAT1$ and $GCN5$ corroborate this notion. It is more likely that these sub-telomeric regions somehow confer access to HATs, which can passively act to disrupt the spreading of heterochromatin. This hypothesis should be tested by focused mechanistic studies in single and double mutants in these genes.

The other point of discussion is the similarity in the effects of $SAS2$, $SAS3$, $YNG1$ and $Rtt109$ on simple telomeres. $SAS2$ counteracts the deacetylation of H4-K16 by Sir2p (150,151,277,278). Hence, in these meticulous studies, $SAS2$ is acting as an anti-silencing factor. However, at simple telomeres or modified mating type loci the deletion of $SAS2$ causes dramatic loss of repression, portraying $SAS2$ as a silencing factor (Fig. 4.2 and (172,230-232)). It is possible that loss of boundary activity and/or the redistribution of a limiting silencing factor such as Sir3p (147,288,289) could indirectly produce these effects. If so, $SAS3$ and $Rtt109$ could also act to limit the indiscriminate association of silencing factors to chromatin away from the telomere as is the case with $SAS2$ (277,278). The possible role of these HATs in boundary formation should also be considered. In summary, this chapter provides clues for the possible roles of HATs in TPE. The actual mechanism of their action will be addressed in future studies.
Chapter 5 - General Discussion and Future Directions

In this thesis, I have addressed six main research objectives that revolve around the fundamental control of epigenetic conversions in \textit{S. cerevisiae}. Chapter 2 focussed on the development of an assay that could quantitatively measure the frequency of epigenetic conversions of TPE (Objective 1) and using this assay I discovered that \textit{CAC1} is key to the control of these epigenetic conversions (Objective 2). I also developed a mathematical model for the switching of epigenetic states and tested it at the \textit{HMRa} locus as well as the telomeres (Objective 3). In Chapter 3, I analysed the control of Cac1p and its recruitment to chromatin by \textit{in vitro} (Objective 4) and \textit{in vivo} (Objective 5) phosphorylation from DDK and CDK to show that CDK is a major regulator of Cac1p and its association with chromatin. Finally, in Chapter 4, I teamed up with P. Power and M.A. Rehman to answer the question of why certain HAT mutations appear to cause a loss of silencing at truncated telomeres (Objective 6) and established the role of sub-telomeric elements as important regulators of TPE silencing. In the following discussion, I amalgamate my main findings from the three chapters, suggest future directions for this research and discuss the implications and significance of my conclusions.

5.1 How are epigenetic conversions controlled in \textit{S. cerevisiae}?

The fundamental question that I set out to answer was how epigenetic changes are controlled. My approach was to examine an area of relatively frequent epigenetic changes in \textit{S. cerevisiae} and determine which genes were needed for these changes to occur. Firstly, the precarious balance of silencing and anti-silencing factors that is regulated by the sub-telomeric elements in \textit{S. cerevisiae} (Chapter 4) provides the power for the spreading of either heterochromatin or euchromatin beyond the chromatin boundary domains. With both counteracting factors present in abundance, all that is required for a major shift in epigenetic state is a small change in the histone modifications to tip the balance in one direction or the other. In Chapter 2, I show that \textit{CAC1} is one of the genes necessary for this epigenetic shift at telomeres.

I suspected that the role of Cac1p in TPE would be accomplished during the process of chromatin assembly at the replication fork and would therefore be mediated by its interaction with chromatin. To test this, I examined the phosphorylation control of Cac1p by the cell cycle kinases DDK and CDK, as phosphorylation of p150 (Cac1p) was implicated in the recruitment of CAF-1 to the replication fork via PCNA in humans (82). From these experiments, I was able to determine that Cac1p is phosphorylated by CDK at S94 and S515 to enable its association with chromatin at wild type levels, but these phosphorylation events were not essential for the role of Cac1p in allowing epigenetic conversions (Fig. 3.6) and its association with chromatin did not directly correlate with its interaction with PCNA (Fig. 3.1, Fig. 3.4 and Appendix 4). However, the results provided a novel role for CDK in the control of chromatin assembly factor regulation as well as implications for a PCNA-independent pathway for chromatin association of Cac1p. It would be interesting to determine if other histone chaperones are regulated in a similar fashion, further connecting cell cycle and chromatin assembly.

My data does not preclude the possibility of a DDK-driven PCNA-Cac1p interaction in \textit{S. cerevisiae}. In fact, if CAF-1 is able to be recruited to chromatin by both a PCNA-dependent and PCNA-independent mechanism, it would explain why mutations that prevent Cac1p-PCNA...
interaction do not confer the same TPE effects as complete deletion of CAC1 (Fig. 2.5), since it could still associate with chromatin utilizing the CDK-driven pathway. Meanwhile, inhibition of the CDK-driven pathway, as demonstrated in the S94A and S515A point mutations (Fig. 3.6), would also not confer the same effects as CAC1 deletion, since the CDK-driven pathway would still be available. Using my assay for assessing the frequency of epigenetic conversions, it would be possible to test this hypothesis by combining the pol30-8 or pol30-79 mutations with the cac1 S94A and/or S515A point mutations in a URA3-tel strain. One could then determine if the loss of epigenetic conversions seen in Δ cac1 is recapitulated by these paired mutations. However, whether or not there is one mechanism or two, the tight regulation for recruiting Cac1p to chromatin during S phase (Fig. 3.1), hints at the importance of this cell cycle connection (54,70).

It is also possible that DDK plays a post-replication initiation role in the control of CAF-1 and acts to maintain the association of Cac1p to the replication fork, rather than or in addition to recruiting it. This would be especially important during replication fork pausing when a multitude of potentially competing factors would be given an opportunity to displace Cac1p (290). In particular, Rrm3p, which interacts with PCNA by its PIP box (291) (similar to the PIP box on Cac1p) and is necessary for the resolution of replication fork barriers (RFBs), may compete with Cac1p for association with PCNA. In fact, preliminary yeast two-hybrid experiments by B. Wyse and H. Rowlands in our lab indicate that deletion of RRM3 enhances the association of Cac1p with PCNA. Thus, there is compelling evidence for competition between these two factors, though these experiments require further investigation to confirm these interactions. Furthermore, preliminary data from R. Oshidari suggests that deletion of RRMD also shows a reduction in the frequency of epigenetic conversions in the telomeres of S. cerevisiae, similar to Δ cac1, which provides another link between epigenetic conversions and replication fork pausing. Using these preliminary findings, the results of Chapters 2-4 and conclusions from current literature, I propose a mechanism for how epigenetic conversions are able to occur.

5.2 Proposed mechanism for the control of epigenetic conversions at sub-telomeres in S. cerevisiae

CAF-1 is recruited to chromatin during replication initiation by CDK phosphorylation of the Cac1p subunit on S94 and S515 (Chapter 3). As the replication fork progresses, CAF-1 reassembles nucleosomes onto the two new DNA daughter strands (73,76). When the replication fork reaches a boundary of heterochromatin and euchromatin in the sub-telomere, occasional replication fork pausing occurs (187). During this time, CAF-1 stops receiving old histones from FACT and Asf1p as the helicase is paused (192), precluding the disassembly of nucleosomes ahead of the fork. However, the influx of new histones is unhindered. CAF-1 could then assemble these new histones into nucleosomes onto the DNA strands without their corresponding old histones (80). Eventually, Rrm3p is recruited to the replication fork, whereby Cac1p is temporarily displaced, and Rrm3p utilizes its 5’ to 3’ helicase activity to resolve the replication fork barrier (194,291). This allows the progression of the replication fork and the nucleosomes made up of entirely new histones present the small change in histone modifications necessary to shift the balance of silencing and anti-silencing factors present at the sub-telomeric chromatin boundary. A schematic diagram of this model is provided in Figure 5.1.
According to my model, a change in the epigenetic state would therefore depend upon two variable events, which would explain the apparent randomness and quasi-stability of these epigenetic changes (6.3 to 8.0% epigenetic switching per generation in wild type cells, Fig. 2.2 B). The first event would be the amount of time available to CAF-1 for the assembly of nucleosomes using only new histones before it is displaced by Rrm3p. The amount of time given to CAF-1 for this purpose would determine if CAF-1 is able to assemble any all-new nucleosomes within the physical space available, likely no more than one or two nucleosomes, without incorporating the old histone PTMs. Only if CAF-1 is not swiftly displaced by competition with Rrm3p would it be able to incorporate the new histones and cause a shift in the balance between the competing silencing and anti-silencing factors. As discussed above, this maintenance of the association and competitiveness of CAF-1 may be regulated by the phosphorylation of Cac1p by DDK (Chapter 3) to maintain its interaction with PCNA (82,83).

Figure 5.1 - Model for epigenetic conversions at the telomeres of S. cerevisiae. 1) Within the sub-telomere, HDACs and HATs maintain a precarious balance of silencing and anti-silencing PTMs. 2) Occasionally, replication fork pausing will occur in these regions (black rectangle). 3) This prevents the disassembly of nucleosomes ahead of the replication fork, so CAF-1 does not have access to any old histones (dark grey circles). 4) New histones accumulate (light grey circles) and 5) CAF-1 assembles nucleosomes onto the two new strands of DNA using only the new histones. This results in one or two nucleosomes that do not carry the correct PTMs. This small change allows a shift in the balance of silencing and anti-silencing at the locus. Rrm3p is later recruited to resolve the replication fork pause.
Alternatively, this could be determined by the varying frequency and strength of replication fork pausing at the different chromatin boundaries and telomeres (187). It is possible that only very strong replication fork barriers provide the means for epigenetic conversions. In support of this, evidence suggests that there are two pools of Rrm3p, one that travels along with the replication machinery via interaction with Pol2 (of DNA polymerase ε) to resolve weak barriers (292) and another which is recruited at high levels when the replication fork encounters strong RFBs (293-295).

The second of the variable events would be the relative abundance of either silencing or anti-silencing histone modifying enzymes (HDACs, HATs etc.) at the specific site where all-new nucleosomes have been assembled. This would determine if the new histones were converted to a new epigenetic state or simply returned to their original state.

This mechanism also takes into account the various TPE phenotypes associated with the mutations of each of the factors involved. In the case of CAC1 deletion, CAF-1 would not be present to assemble the new histones and nucleosome reassembly would be carried out by other histone chaperones, such as Rtt106p or Asf1p. These would either be less efficient at assembling nucleosomes or less competitive with Rrm3p for association with the replication fork, thereby assembling fewer unmodified nucleosomes at paused forks. Alternatively, they could be more stringent and only assemble nucleosomes when the availability of old histones returns. In this way, epigenetic conversions would be greatly reduced in the Δcac1 cells, as I show in Chapter 2. RRM3 deletion is less straightforward. Intuitively, one would suspect that if a deletion of RRM3 results in more replication fork pausing and longer periods of time for CAF-1 to assemble all-new nucleosomes, then there should be an increase in epigenetic conversions. However, though CAF-1 may assemble a large number of all-new nucleosomes at these unresolved replication fork barriers, the lack of Rrm3p results in replication fork collapse and the need for subsequent double strand break (DSB) repair (296). Both the homologous recombination (HR) and non-homologous end joining (NHEJ) processes of DSB repair would provide the opportunity for CAF-1 and other histone chaperones to reassemble the nucleosomes according to the epigenetic marks of the template chromatin (297), once again preventing epigenetic conversions from occurring. Meanwhile, the deletions of other histone chaperones, mutations in POL30 and even mutations in certain replication factors would not hinder this CAF-1/Rrm3p controlled process.

5.3 Recommendations for testing the mechanism of epigenetic conversions

One unanswered question from my proposed mechanism is if CAF-1 continues to assemble nucleosomes using only new histones during replication fork pausing. It has been shown that CAF-1 can continue to assemble nucleosomes even if replication progression is inhibited (80,298). However, it is not known whether this occurs with exclusively new histones while paused at a replication fork barrier. To test this, an RFB sequence, which uni-directionally blocks the replication fork when bound by Fob1p (299), could be used to create a tightly controlled, synthetic site of strong replication fork stalling on a linear yeast artificial chromosome (YAC). With HA- or T7-tagged histone H3 expressed by an inducible Gal1,10 promoter, it would be possible to perform a simple chromatin IP of the tagged histones to determine the effects of Δcac1 and Δrrm3 on the incorporation of new histones at the site of replication fork pausing. Briefly, cells could be grown in the presence of galactose and nocodazole to simultaneously...
express the tagged histone and arrest the cells after one passage through S-phase in G2/M. DNA would be crosslinked to its associated proteins with formaldehyde, chromatin sheared by sonication and then immunoprecipitation of the tagged histones (198). Quantitative PCR of the DNA adjacent to the RFB would indicate the levels of new histone incorporation at the paused site in the mutant strains compared to wild type.

5.4 Significance and General Conclusion

If these efforts were to confirm the model for epigenetic conversions I presented here, it would open up numerous possibilities for investigation outside of *S. cerevisiae*. As described in the general introduction, we know that unregulated epigenetic changes, and by definition epigenetic conversions, are important in many forms of cancer. My model suggests that these changes could be a result of the increased proliferation of tumour cells, simply because of the frequent passages of the replication forks. Replication fork pausing would increase along with susceptibility to epigenetic change. From there, genetic drift could gradually shift the cell transcriptome towards the progression of cancer. The current process for the development of epigenetic anti-cancer therapies is to attempt to reverse the epigenetic changes associated with certain cancers. For example, one might observe that numerous types of cancer cells have a gain of DNA methylation at the promoters of several key tumour suppressor genes (TSGs), silencing those genes and correlating with apoptotic failure, unregulated cell proliferation and continual accumulation of mutations (4-6). So, to prevent or reverse the hypermethylation of these genes, a drug was developed that knocks down DNA methyltransferases (DNMTs). However, while most tumour cells expressed TSGs and underwent apoptosis, a subset of surviving cells began expressing pro-metastatic genes and actually increased tumour invasiveness (300). My model suggests that in order to combat cancer by epigenetic means, it may first be necessary to suppress the proliferation of the tumour to cause a lasting effect, a much more daunting task.

Our understanding for how epigenetic conversions are controlled also has links to the pluripotency of stem cells. Genes that undergo epigenetic changes during differentiation are correlated with paused transcription complexes (197). The pausing of transcription is much more frequent in higher eukaryotes than it is in *S. cerevisiae* and the interplay between replication and transcription in higher eukaryotes could provide the means for a more complex system of pausing and epigenetic conversions to account for the more complex epigenetic systems of these organisms. With this in mind, it would be interesting to examine the effects of replication fork and transcription pausing on the ability of a stem cell to differentiate or maintain pluripotency. In this way we could assess if the mechanism for epigenetic changes in differentiation is connected to the mechanisms of PEV and determine just how conserved this process is.

Finally, epigenetic change also plays a key role in the immune evasion of several major human pathogens, including *Plasmodium falciparum*, the protozoan parasite which is primarily responsible for the most severe forms of malaria (211,301). It is estimated that there were more than 200,000,000 severe cases of malaria worldwide in 2012 and the number of deaths is estimated at over 600,000 (302). These single-celled eukaryotes exhibit rare epigenetic conversions of the var genes, located adjacent to the telomeres, which encode different forms of PfEMP1, an immunodominant surface molecule of *P. falciparum* (erythrocyte membrane protein 1). By silencing all but one of the 60 var genes with only an occasional switch of the active gene, the parasite is able to continuously evade the host’s immune response (207,303). If the
mechanism for control of epigenetic conversions is conserved in these organisms, elimination of CAC1 in these parasites may eliminate their key immune evasion technique, finally allowing the immune system of the host to recognize and eliminate the pathogens.

In summary, replication fork and transcription pausing in the presence of competing silencing and anti-silencing factors may provide the means for epigenetic conversions in eukaryotes. This plays a significant role in the adaptation of many single celled eukaryotes (incl. pathogens like *Plasmodium*) to the changing environment (164). In metazoans, epigenetic stability is critical for cell differentiation and development, while untimely epigenetic conversions have been linked to cancer and to various genetic and psychiatric disorders (233-237). By using the telomeres of *S. cerevisiae* to examine the fundamental control of epigenetic conversions, I have provided the first steps towards developing an understanding of a potentially conserved process involved in carcinogenesis and cellular differentiation. Furthermore, I may also have revealed a clue for the development of a new line of anti-pathogen drugs against some of the world’s most deadly parasites.
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of histones H3/H4 and mutually exclusive interactions with Asf1 guide H3/H4 transitions
among histone chaperones and DNA. *Nucleic acids research*, **40**, 11229-11239.


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Appendix 1

Strains and tables used in this thesis.

**Table 1 - Strains used in Chapter 2**

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Table 2 - Measurements of the %FOA$^R$ and %URA$^+$ cells after selection on SC/FOA and SC-ura plates from Chapter 2.

Details on the measurements and the calculations are provided in the text.

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|            | | | |
| **VR telomere** | | | |
| wt (BY4742) | 64% 15% (n=3) | 22% 8% (n=3) | 51% 9% (n=3) | 28% 4% (n=3) |
| Δsir1 | 0.21% 0.04% (n=3) | 109% 23% (n=3) | 89% 12% (n=3) | 0.06% 0.03% (n=3) |
| Δdot1 | 0.003% 0.002% (n=3) | 78% 17% (n=3) | 0.2% 0.3% (n=3) | 86% 5% (n=3) |

|            | | | |
| **hmr-a1Δ::URA3** | | | |
| wt (W303) | 0.002% 0.002% (n=3) | 91% 23% (n=3) | 0.005% 0.001% (n=3) | 80% 12% (n=3) |
| Δsir1 | 62% 16% (n=3) | 53% 14% (n=3) | 97% 10% (n=3) | 1% 0.1% (n=3) |
| Δdot1 | 0.003% 0.002% (n=3) | 78% 17% (n=3) | 0.2% 0.3% (n=3) | 86% 5% (n=3) |
### Table 3 - Strains used in Chapter 3

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### Table 4 - Telomere Position Effect assays in CAC1 point mutants and in cdc7-1 and cdc28-1 mutant strains for Chapter 3, Figure 3.6.

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### Table 5 - Strains used in Chapter 4

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Table 6 - Levels of gene silencing in different mutants from Chapter 4

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|         | GF3       | 0.4  | 0.4  | %  | (n=3)   | GF3       | 19.7 | 2.7  |
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Appendix 2

Chapter 2: Calculations on a recurrence relation of the type

\[ Y(A)_n = Y(A)_{n-1} - Y(A)_{n-1} C(A\rightarrow S) + (1 - Y(A)_{n-1}) C(S\rightarrow A) \]

where \( Y(A)_{n-1} \) is the proportion of cells with Active gene at generation \( n \) (\( n_0 \ldots n-1, n, n+1 \ldots \) etc), \( C(A\rightarrow S) \) is the coefficient of conversions from Active to Silent state and \( C(S\rightarrow A) \) is the coefficient of conversions from Silent to Active, or simply presented as

\[ Y_n = Y_{n-1}(1 - C(A\rightarrow S)) + (1 - Y_{n-1}) C(S\rightarrow A) \]

I. The basic problem is to solve a first order recurrence relation,

\[ X_n = aX_{n-1} + b \text{ for } n \geq 1 \quad (1) \]

Where \( X_0, a \) and \( b \) are parameters that we specify in the model.

\[ a = 1 - C(A\rightarrow S) - C(S\rightarrow A) \]

\[ b = C(S\rightarrow A) \]

In the initial condition (growth on selection medium) \( X_0 \) could be \( X_0 = 1 \) or \( X_0 = 0 \).

A second order recurrence relation would be \( X_n = aX_{n-1} + bX_{n-2} + c \). We will not be concerned with second order recurrence relations other than to say that the method we describe will apply to these and higher order recursions as well. We then apply the method of telescoping with the idea to write down the system of equations that the \( \{X_n\} \)‘s satisfy, and then to multiply each equation by \( \{I, r, r^2, \ldots\} \) in succession. The value of \( r \) is then chosen so that the variables \( \{X_n\} \) cancel from the system.

As a first step, we write down the system of equations for the \( \{X_n\} \):

\[ X_n = aX_{n-1} + b \]

\[ X_{n-1} = aX_{n-2} + b \]

\[ \vdots \]

\[ X_2 = aX_1 + b \]

\[ X_1 = aX_0 + b \]

II. We keep the first equation, we multiply the second equation through by \( r \), the third equation through by \( r^2 \) and so on, so that the last equation is multiplied through by \( r^{n-1} \). The system of equations now looks this way:

\[ r^{n-1}X_n = r^{n-1}(aX_{n-1} + b) \]

\[ r^{n-2}X_{n-1} = r^{n-2}(aX_{n-2} + b) \]

\[ \vdots \]

\[ r^2X_3 = r^2(aX_2 + b) \]

\[ rX_2 = r(aX_1 + b) \]

\[ X_1 = aX_0 + b \]

\( X_1 \) appears once on the left hand side of the system, and once, as \( rX_1 \), on the right hand side of the system. If we want \( X_1 \) to cancel from the system, we must choose \( r \) such that \( 1 = ra \Rightarrow r = \frac{1}{a} \).

\( X_2 \) appears once on the left hand side of the system as \( rX_2 \), and once on the RHS of the system as
$r^2 a X_2$. If we also want $X_2$ to cancel from the system, we must choose $r$ such that $r = r^2a$. We may cancel $r$ from both sides of this equation, and we obtain $1 = ra \Rightarrow r = \frac{1}{a}$. This pattern repeats itself. Thus, the value of $r = \frac{1}{a}$ will cause all the variables \{ $X_{n-1}, X_{n-2}, \ldots, X_1$ \} to cancel from the system. We put $r = \frac{1}{a}$, and add all the above equations in (3), and we are left with:

$$r^{n+1}X_n = aX_0 + b(r^n + r^{n-2} + \cdots + r + 1)$$ \hspace{1cm} (4)

where all the intermediate variables have cancelled from the system (this is called telescoping). Now $1 + r + \cdots + r^{n-2} + r^{n-1}$ is called a geometric series, and the formula for summing a geometric series gives us

$$1 + r + \cdots + r^{n-2} + r^{n-1} = \frac{1-r^n}{1-r} = \frac{a^n - 1}{a^n - a^{n-1}}$$

The previous equation follows from putting $r = \frac{1}{a}$, and then multiplying both numerator and denominator through by $a^n$. Using this equation to sum the geometric series in (4), and then multiplying through both sides of (4) by $a^{n-1}$, we obtain the solution for $X_n$:

$$X_n = a^n X_0 + b \left( \frac{a^n - 1}{a^{n-1}} \right)$$ \hspace{1cm} (5)

III. With (5) in hand, we return to our initial recursion:

$$Y_n = Y_{n-1} - Y_{n-1} C_{(A\rightarrow S)} + (1-Y_{n-1}) C_{(S\rightarrow A)} = (1-C_{(A\rightarrow S)} - C_{(S\rightarrow A)}) Y_{n-1} + C_{(S\rightarrow A)}$$

It is clear that we should choose $a = 1 - C_{(A\rightarrow S)} - C_{(S\rightarrow A)}$ and $b = C_{(S\rightarrow A)}$. Putting these values in (5), we obtain

$$X_n = \left( 1 - C_{(A\rightarrow S)} - C_{(S\rightarrow A)} \right)^n + C_{(S\rightarrow A)} \left( \frac{\left( 1 - C_{(A\rightarrow S)} - C_{(S\rightarrow A)} \right)^n - 1}{\left( 1 - C_{(A\rightarrow S)} - C_{(S\rightarrow A)} \right) - 1} \right)$$

$$X_n = \left( 1 - C_{(A\rightarrow S)} - C_{(S\rightarrow A)} \right)^n + C_{(S\rightarrow A)} \left( \frac{1 - \left( 1 - C_{(A\rightarrow S)} - C_{(S\rightarrow A)} \right)^n}{C_{(A\rightarrow S)} + C_{(S\rightarrow A)}} \right)$$ \hspace{1cm} (6)

The last item to consider is what happens for large values of $n$, i.e., what is the convergence behavior as $n \rightarrow \infty$. A limit will only exist if

$$-1 < 1 - C_{(A\rightarrow S)} - C_{(S\rightarrow A)} < 1$$

which simplifies to give

$$0 < C_{(A\rightarrow S)} + C_{(S\rightarrow A)} < 2$$

As long as we have this condition, we may take $\lim_{n \rightarrow \infty}$ in (6), and we obtain a ratio of parameters:

$$\lim_{n \rightarrow \infty} X_n = 0 + \frac{C_{(S\rightarrow A)}}{C_{(A\rightarrow S)} + C_{(S\rightarrow A)}} (1-0) = \frac{C_{(S\rightarrow A)}}{C_{(A\rightarrow S)} + C_{(S\rightarrow A)}}$$ \hspace{1cm} (7)
Appendix 3

Collection of Chapter 3 data provided by Marlene Gharib, Alain Verreault and Pierre Thibault.

Appendix 3 Figure 1 A – Mass spectrometry analysis showing six in vivo phosphopeptides of CAF-1.

Appendix 3 Figure 1 B - LC-MS/MS analyses of Cac1p phosphopeptides as described in section 3.2.5. A-E) Mass spectra histograms (relative abundance (Y axis) by mass-to-charge ratio (m/z)) of the five Cac1p phosphopeptides identified in Appendix 3 Figure 1 A, shown in the top right of each section. S94, S238, S501, S503 and S515 indicate the phosphorylated amino acid residue of Cac1p.
In vitro phosphorylation of Cac1p by Cdc7p/Dbf4p and Cdc28p/Clb5p kinases.

**A)** Cac1<sub>226-606</sub>-His<sub>6</sub> is phosphorylated by Cdc7p/Dbf4p. Cac1<sub>226-606</sub>-His<sub>6</sub> or Hst3-His<sub>6</sub> as a negative control were incubated with Cdc7p/Dbf4p in the presence of <sup>32</sup>P-γATP. The reaction products were resolved through an SDS-12% polyacrylamide gel and visualized by autoradiography. The position of Cac1<sub>226-606</sub>-His<sub>6</sub> is shown by the arrow. The two radiolabeled bands in lane 2 (a kinase reaction performed in the absence of substrate) are generated by auto-phosphorylation of Cdc7p/Dbf4p.

**B)** Cac1<sub>226-606</sub>-His<sub>6</sub> was incubated with Cdc7p/Dbf4p and cold ATP. Reaction products were resolved by SDS-PAGE and stained with Coomassie. In-gel digestion of the Cac1<sub>226-606</sub>-His<sub>6</sub> band was performed with trypsin. Extracted phosphopeptides were enriched with a TiO<sub>2</sub> column and analyzed by LC-MS/MS. The experimental mass of the non-fragmented peptide (M<sub>exp</sub>) was within 2.4ppm of its theoretical mass (M<sub>theo</sub>). Although several Ser and Thr are present in this peptide, the y<sub>17</sub>²⁺ + PO4 fragment at m/z = 957.4 demonstrates the presence of Cac1p phosphorylation at S503.
Appendix 4

Calculations for the abundance of Cac1p and PCNA in the cytosol, chromatin and IP fractions in Chapter 3, Figure 3.4.

Abundance of Cac1p and PCNA in chromatin relative to cytosol (data from Figure 3.4 A). Cytosol fractions (1.6% of the total) and chromatin fractions (11% of the total) were loaded on gels and analysed. Signals from the Western blots were acquired by ImageJ software. Briefly, the below image was converted to 8 bit (grayscale), background was subtracted using rolling disk = 50, image was inverted, and signal was analyzed as mean gray value of the Western blot relative to the India Ink stain (with wild type of each temperature set as 1). The cytosol signals were amplified by 6.875 to reflect the lower proportion of extract loaded. The proportion of Cac1p and PCNA in the chromatin fractions were calculated from these corrected values. These calculations show that 15-17% of the total Cac1p and 7-11% of total PCNA are associated with chromatin at 23°C, with only significant differences between mutants and wild type strains. There were also no major differences in the results from 37°C (15min), except a slight decrease in WT and cdc28Δ cells and a small increase in cdc7-1 for %Cac1p in chromatin. However, at 37°C (30min), we observe a significant decline in the % of Cac1p in the chromatin of cdc28Δ cells (7% versus 17%) and an increase in the%Cac1p in chromatin for cdc7-1 (29% versus 15%). WT showed only a small increase in %Cac1p and %PCNA in chromatin. %PCNA in chromatin rose slightly in all strains at 37°C (30min).

Appendix 4 Figure 1 - Comparable Western blotting exposure for Fig. 3.4 A
Image used for ImageJ densitometry.
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Association of Cac1p and PCNA in whole cell extracts (data from Fig. 3.4 B). Signals from the cell extract immunoprecipitated samples in Figure 3.4 B have been acquired by ImageJ. The ratio of PCNA/Cac1p in wild type cells is set at 1 for each temperature. "Fold decrease" is the calculated decrease of PCNA in the immunoprecipitate relative to wild type cells. The calculations show 1.6-2.5 fold decrease in the Cac1p-associated PCNA in the cdc7-1 and cdc28-1 mutants that does not appear to be a result of temperature shift.

Table 8 - Appendix 3 table for calculations of PCNA/Cac1p ratio in whole cell extracts for Chapter 3.

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Appendix 4 Figure 2 - Comparable Western blotting exposure for Fig. 3.4 B
Image used for ImageJ densitometry.