The Role of Genomic Sequence in the Spatial and Temporal Propagation of Heterochromatin

by

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor of Philosophy in the University Program in Genetics and Genomics in the Graduate School
of Duke University

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ABSTRACT

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Abstract

Characterizing how genomic sequence interacts with trans-acting regulatory factors to implement a program of gene expression is critical to our understanding of genome function. One means by which patterns of gene expression are achieved is through the differential packaging of DNA into distinct types of chromatin. While chromatin state exerts a major influence on gene expression, the extent to which cis-acting DNA sequences contribute to the specification of chromatin state remains incompletely understood. To address this, we have used a fission yeast sequence element (L5), known to be sufficient to nucleate heterochromatin, to establish *de novo* heterochromatin domains in the *S. pombe* genome to address the role of DNA sequence in shaping the spatial and temporal propagation of heterochromatin. In this thesis, I describe a major effect of genomic sequences in determining spatial propagation of such *de novo* heterochromatin domains. I demonstrate that the sequence content of a genomic region plays a significant role in shaping its response to encroaching heterochromatin and suggest a role of DNA sequence in specifying chromatin state. Despite the role of DNA sequence in the spatial propagation of chromatin domains, I demonstrate that heterochromatin, once assembled, can propagate by an epigenetic signal, entirely independent of the original nucleating sequences. While the epigenetic signal is sufficient for maintenance and transmission of the heterochromatic state, it is insufficient for reestablishment of heterochromatin following its loss. Thus, these data demonstrate uncoupling of genomic and epigenetic signals necessary for the establishment, spatial propagation, and temporal propagation of chromatin states.

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1. Introduction

With the advent and implementation of genome sequencing, biologists have been able to uncover the complete and finite set of digital instructions contained within each cell. This information must, at the most fundamental level, underlie all biological processes, including the interpretation of the DNA instructions themselves. DNA encodes the proteins that will work to establish the correct patterns of gene expression that are required to make a functional organism. At a primary level, this can be achieved by the recruitment of activating and repressing factors to specific DNA sequences that regulate gene expression. However, adding to the complexity of this system, the static set of DNA instructions gives rise to dynamic states that underlie responses to changes in the environment and to the normal processes of development and aging (Blasco, 2007; Feinberg, 2007; Surani et al., 2007). Thus, identical genomes can give rise to very different cell fates, even within an individual organism, suggesting that not only does DNA encode the instructions for its own use, but that it has the flexibility to be interpreted in different ways without modifications to the underlying sequence. This flexibility cannot occur in a system in which patterns of gene expression are hardwired into the genetic code; rather, the system must allow for different patterns of expression even with identical DNA content. Viewed from this perspective, innumerable questions about details of particular systems in biology converge on two fundamental issues: what are the factors responsible for interpreting the code, and how do these proteins enact different, yet stable, interpretations of the same sequence?

If gene expression, at its simplest, is the effect of activators binding gene sequences, then the accessibility of DNA to those activators is paramount to gene regulation. A major impediment to the accessibility of DNA is its packaging into chromatin (Richmond and Davey,

2003). Thus, not only the gene regulatory sequences, but also the characteristics of the associated chromatin, referred to throughout this thesis as the chromatin state, impart constrains on gene expression. Furthermore, changes in chromatin state could contribute to changes in gene expression, without alteration in DNA sequence, as seen throughout development.

In addition to gene regulation on the level of individual genes, chromatin state, and thus gene expression, can also be regulated in a larger scale, such that domains consisting of multiple genes are influenced by common regulatory factors (reviewed in Dillon, 2006; Fourel et al., 2004; Grewal and Moazed, 2003; Lam et al., 2005; Straub and Becker, 2008). Two important examples of this type of regulation illustrate this effect on the expression of multiple genes within a chromosomal domain. One is the packaging of DNA into domains of heterochromatin, a chromatin state encompassing dozens to many thousands of kilobase pairs in different organisms that is generally refractive to transcription (reviewed in Grewal and Elgin, 2002). Similarly, dosage compensation to equalize gene expression on sex chromosomes between the sexes serves to control gene expression on the level of entire chromosomes (Lyon, 1961; Straub and Becker, 2007). In addition to these specific regulatory states, the expression of transgenes inserted throughout the genome reflects both the regulatory elements within the gene, as well as the average level of expression within the genomic region (or "neighborhood") into which the transgene has been integrated (Gierman et al., 2007). This type of regulation is often referred to as "sequence-independent" (Grewal and Jia, 2007), as gene expression is affected by neighborhood as opposed to inherent sequence-specific features of the gene and its regulatory elements. However, we find that this terminology fails to capture the interplay between genomic sequence and chromatin domains. At their core, these domains must integrate signals

encoded in the genome to provide specificity to their localization. Here, I discuss the role of DNA sequence in three aspects of these domains: establishment, maintenance and spreading from the perspective of repressive chromatin in yeast and flies, dosage compensation in higher eukaryotes as well as paramutation in maize. Where appropriate, the emphasis will be on heterochromatin in fission yeast, which will serve as background to the topic of Chapters 2 and 3 of this thesis.

1.1 Domain architecture

Understanding the molecular and mechanistic features that characterize these chromatin domains is critical to our comprehension of gene regulation and genome organization. Within the general context of chromatin, these domains likely reflect the collective effects of nucleosome positioning, post-translational histone modifications, histone variants, DNA methylation, non-histone chromatin proteins and nuclear localization (Figure 1) (reviewed in Richards and Elgin, 2002; Schneider and Grosschedl, 2007). Their end function is to exclude or recruit factors that contribute to control of gene activation and transcription (Schneider and Grosschedl, 2007; Sugiyama et al., 2007). The mechanisms by which these features influence the function of entire domains are discussed below.

In eukaryotes, 146 base-pairs of DNA are wrapped around a nucleosome consisting of two copies of each histone protein, H2A, H2B, H3 and H4 (reviewed in Kornberg and Lorch, 1999). This wrapping, in addition to higher-order chromatin folding, fulfills a fundamental need for compaction of the DNA fiber, but also imparts accessibility constraints on the associated DNA (Richmond and Davey, 2003). This constraint is also an opportunity for regulation; if

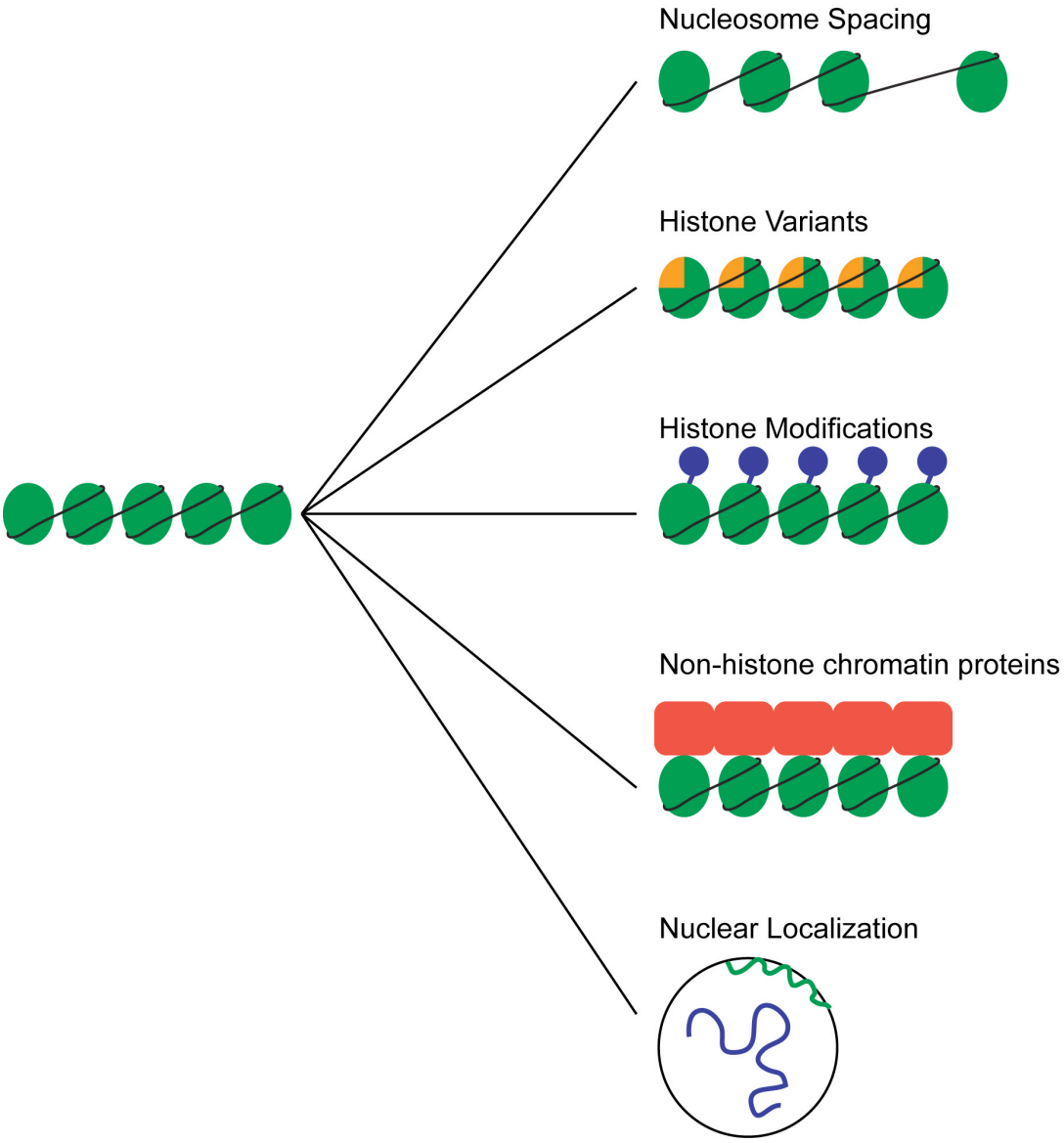


Figure 1: Model of factors that influence domain organization

Shown here are features that could contribute to the organization of genomes into domains. The spacing of nucleosomes within a domain affects accessibility of genomic sequence to trans-acting factors. Incorporation of histone variants can affect nucleosome behavior and interacting partners. Post-translational histone modifications can recruit specific trans-acting factors to enact domain-specific functions. Non-histone chromatin proteins can also shape the behavior and accessibility of the associated genomic DNA. Finally, nuclear localization can affect chromatin state and transcriptional output. While these factors are depicted as separate entities, they are largely interdependent.

positioned nucleosomes occlude a DNA target from access by its regulatory proteins, then regulation of nucleosome position could in turn regulate gene expression. Gene activation and repression have been shown to correlate with losses and gains of promoter nucleosomes (Lee et al., 2004; Schwabish and Struhl, 2004). Nucleosome positioning can regulate expression over a domain, as gene repression associated with the silenced chromatin at *S. cerevisiae* telomeres is lost upon nucleosome depletion, in a manner that is partially independent of other silencing components (Wyrick et al., 1999). Furthermore, in the human genome, gene-rich domains are associated with regions of open chromatin (Gilbert et al., 2004).

In 2000 Strahl and Allis proposed, based on the panoply of post-translational modifications observed within histones, that modified histones could serve as a "histone-code," storing information that could be read by proteins with specificity for particular modified residues (Strahl and Allis, 2000). Thus, in addition to the information contained within the genetic code, histone modifications would also provide a layer of regulatory information that could shape the behavior of the associated genomic sequences. The modified histones could recruit secondary, non-histone chromatin proteins, to carry out domain specific behaviors. For example, heterochromatin correlates with histone hypoacetylation and methylation of H3 lysine 9 (Nakayama et al., 2001b). In fission yeast, this pattern of histone modification serves to recruit additional chromatin proteins that affect both nucleosome spacing and recruitment of RNA Polymerase II (Pol II) (Motamedi et al., 2008; Sugiyama et al., 2007).

In addition to histone modifications, canonical histones can be replaced in the nucleosome by specific histone variants. Incorporation of these variants can affect the stability, post-translational modifications, and interacting partners of nucleosomes over whole domains (reviewed in Henikoff et al., 2004; Talbert and Henikoff, 2010). The focus in the following section

is on the histone H3 variants as a paradigm for the roles of histone variants in domain structure; however variant H2A molecules also function in domain specification (Table 1).

There are there major forms of H3 in metazoans, including H3, H3.3 and cenH3 (reviewed in Henikoff et al., 2004). H3 and H3.3 (H3.2 in plants) differ in protein sequence by only four amino acids. However, their pattern of deposition is distinct (Ahmad and Henikoff, 2002). The manner of deposition is coupled to the chaperone complex that specifically associates with each variant. H3 associates with the CAF-1 histone chaperone complex that interacts with the replication fork to deposit H3 on replicated DNA, whereas H3.3 is deposited by the HIRA or ATRX chaperone complexes in a replication-independent fashion (Ahmad and Henikoff, 2002; Goldberg et al., 2010; Janicki et al., 2004; Tagami et al., 2004). Thus, the distinguishing feature between these two variants appears to be their choice of chaperone complex.

In addition to associating with different chaperone complexes, the H3.3 variant is more enriched in active chromatin modifications and depleted for H3K9me2 relative to H3 (McKittrick et al., 2004). Whether these modifications are cause or consequence of the association of H3.3 with active transcription is unknown. However, it is possible that the manner of deposition, or other features specific to the variant histones, alters the post-translational modification profile. For example, the replication fork is known associated with various chromatin modifiers (reviewed in Moldovan et al., 2007) and could alter the behavior of newly deposited H3.1 relative to H3.3.

The other major H3 variant, cenH3, is the primary determinant of centromere localization and thus is paramount for appropriate chromosome segregation (reviewed in Black and Bassett, 2008). The structure of cenH3-containing nucleosomes is controversial, and

Table 1: Histone variants and their roles in *S. pombe*

Adapted from (Henikoff and Ahmad, 2005)

	Found	**Role in *S. pombe***
H2A, H2B, H3, H4	Canonical histones	Canonical histones
H2AZ	Throughout eukaryotes. Activates transcription in *S. cerevisiae* and associates with heterochromatin in mammals	Suppression of antisense transcripts (Zofall et al., 2009). Role in chromosome stability (Kim et al., 2009).
MacroH2A	Vertebrate-specific. Enriched on the inactive X chromosome	
H2A-Bbd	Vertebrate-specific. Depleted on inactive X chromosome	
H2AX	Phosphorylated at sites of double strand breaks	*S. pombe* lacks a H2AX variant. However, *S. pombe* H2A can be phosphorylated at sites of double strand breaks (Nakamura et al., 2004).
cenH3	Marks active centromeres	Marks active centromeres (Takahashi et al., 2000)
H3.3	Deposited in a replication-independent fashion. Enriched in regions of active transcription. Replaces H3	*S. pombe* lacks a H3.3 variant. However, *S. pombe* H3 can be deposited in both a replication-coupled and replication-independent fashion (Choi et al., 2005).

studies in different organisms support distinct conclusions (Camahort et al., 2009; Dalal et al., 2007; Foltz et al., 2006; Mizuguchi et al., 2007). However, inclusion of cenH3 within a nucleosome imparts distinct features on the nucleosome and the associated centromeric DNA (Black et al., 2007; Black et al., 2004; Furuyama and Henikoff, 2009). Thus, inclusion of the histone variant cenH3 affects the behavior of the associated genomic domain and illustrates the defining importance of histone variants therein.

In addition to domains of chromatin variants and modifications, the genome can also be divided into domains based on nuclear localization. In the human genome, distinct and reproducible regions of the genome can be found within distinct territories (Barr and Bertram, 1949; Bobrow and Heritage, 1980; Cremer et al., 1982; Guelen et al., 2008; Manuelidis, 1985). For example, genomic regions associated with the nuclear lamina have lower levels of gene expression than non-associated regions (Guelen et al., 2008). Furthermore, inducible tethering of a locus to the nuclear periphery imparts transcriptional repression (Andrulis et al., 1998; Reddy et al., 2008). These data suggest that nuclear localization is sufficient to establish domain-wide regulation and thus could serve as an additional mechanism of large-scale regulation.

Furthermore, development of chromosome conformation capture (3C) and derivative technologies, which allows the identification of genomic regions that are in close proximity (Dekker et al., 2002), have allowed mapping of DNA-DNA interactions within the nucleus. Applying this methodology to all the possible pair-wise sequence combinations has demonstrated that sequences are located within one of two compartments in human cells (Lieberman-Aiden et al., 2009). Interestingly, these compartments correlated with regions of closed versus open chromatin.

The effect of nuclear localization on gene expression could work through a chromatin-dependent or independent mechanism. For example, in many organisms heterochromatin ectopic silencing is more robust when near the telomeres or centromeres (Dorer and Henikoff, 1997; Haynes et al., 2006; Maillet et al., 1996; Wakimoto and Hearn, 1990). In 1990 Wakimoto and Hearn proposed that that phenomenon was the result of enhanced silencing within certain regions of the nucleus (Dorer and Henikoff, 1997; Wakimoto and Hearn, 1990), such that proximity to a silent domain would increase the chances of being included within a heterochromatin compartment in the nucleus. These studies demonstrate the connection between nuclear localization and chromatin state.

1.2 General models of establishment, maintenance and spreading of chromatin states

Establishment

Establishment is the process whereby the specific factors required for formation of the domain are recruited to the appropriate genomic loci. As the features that distinguish domains are distinct, we imagine the mechanisms that establish them are similarly diverse. However, I would like to point out here that establishment necessitates genomic sequence. Chromatin modifying enzymes, for example, have no inherent sequence specificity in vitro (Ptashne, 2007). Thus, the specificity observed in vivo (that is, the restriction of specific domains to specific loci) must be regulated at least at the level of recruitment, and thus by DNA sequence. Throughout, I will refer to sequences that establish specific chromatin states as “nucleating sequences” and will describe examples of these sequences in the following sections.

Spreading

Chromatin states, including those implicated in both heterochromatin and dosage compensation, have the capacity to propagate along the chromosome fiber (reviewed in Talbert and Henikoff, 2006). Juxtaposition of euchromatic sequences with these domains results in the spreading of gene silencing and dosage compensation into these regions (Ercan et al., 2009; Hall et al., 2002b; Muller, 1930; Partridge et al., 2000). The molecular mechanism through which this happens will be discussed in detail in subsequent sections for heterochromatin in the fission yeast, *S. pombe*. However, generally, heterochromatin is thought to propagate in a sequential fashion whereby recruitment of a chromatin modifier creates a binding site for a chromodomain protein, proteins that specifically bind to modified histone tails, that then recruits additional modifiers (Bannister et al., 2001). At least in its simplest form, this model, referred to as "oozing," would predict heterochromatin spreading to be linear, which, while sometimes true (Renauld et al., 1993), is not always the case (Talbert and Henikoff, 2000).

DNA sequences could shape the spreading of chromatin states in either a positive or negative fashion by enhancing or restricting their spread. For example, heterochromatin barriers are specific DNA sequences that stop the spread of heterochromatin into euchromatic domains (Donze and Kamakaka, 2002). The best studied of these barriers, the β-globin HS4 element in chicken erythrocytes, works to prevent heterochromatin spreading through the recruitment of active chromatin modifications (West et al., 2004). Conversely, protosilencers are DNA sequences that help to propagate the silenced state and have been characterized in *S. cerevisiae* as well as implicated in the spread of X inactivation in mammals. The extent to which these types of sequences exist in the genomes of *S. pombe* and other organisms will be discussed further in this introduction and Chapter 2 of this thesis.

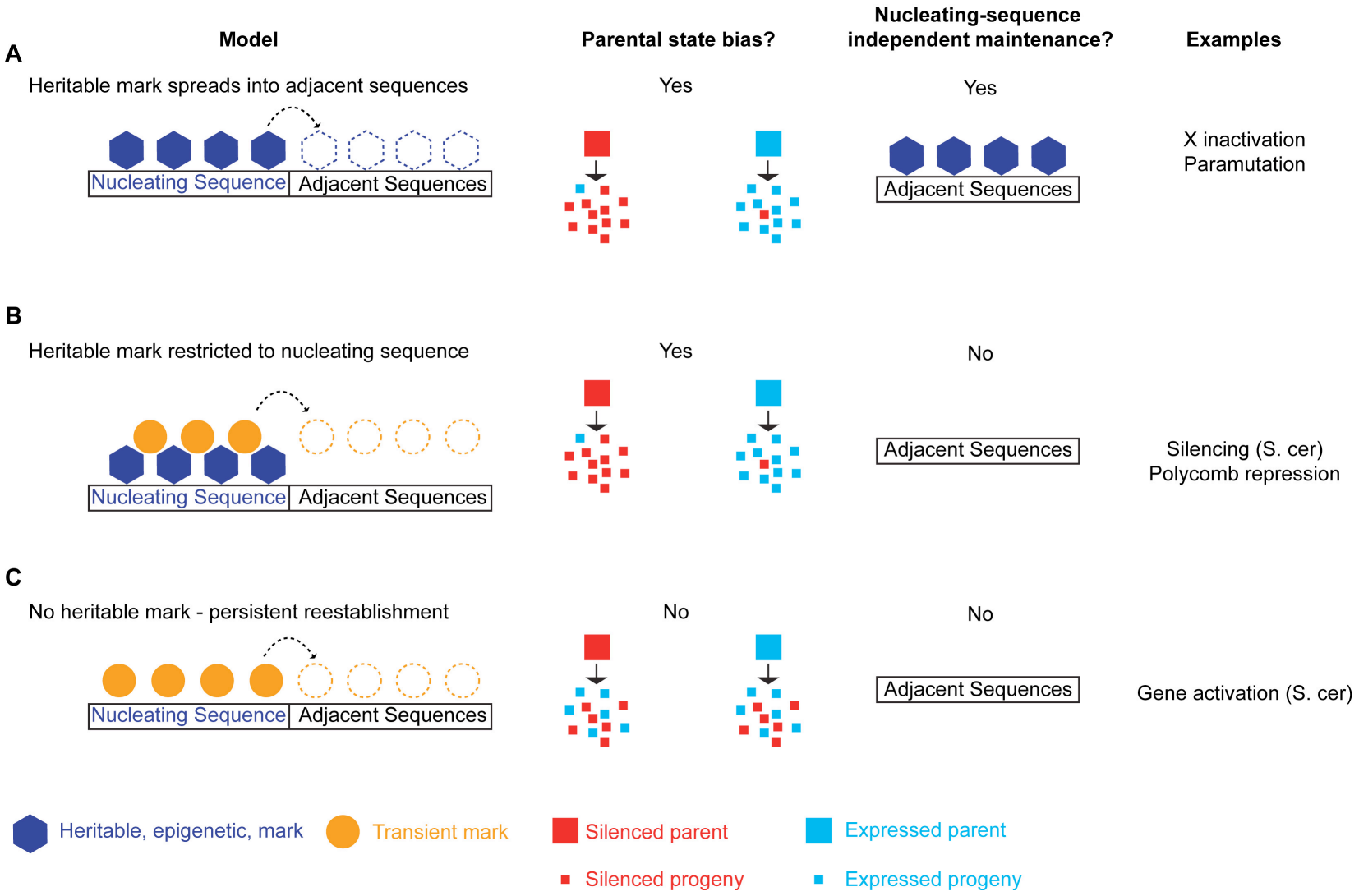
Maintenance

One of the underlying hypotheses of epigenetics and chromatin states is that the packaging of identical genomic regions into distinct types of chromatin results in the differential phenotypic outcomes required for differentiation and development (Buszczak and Spradling, 2006; Feinberg, 2007; Surani et al., 2007). While early chromatin modifications do indeed correlate with transcriptional outcome later in development (Mikkelsen et al., 2007), the extent to which chromatin states are maintained and the mechanism by which this occurs are largely unknown. The majority of nucleosomes, if not all, are replicated in a conservative fashion (Leffak et al., 1977) such that old nucleosomes are randomly partitioned onto the daughter strands. To maintain correct nucleosome density, gaps are then filled in with new nucleosomes (reviewed in Probst et al., 2009).

Thus, domains, including modified and variant histones and non-histone chromatin proteins, would require a mechanism to reestablish these marks on the newly deposited histones. One model suggests that this "fill-in" could occur by the same mechanism that allows heterochromatin and other epigenetic states to spread (Figure 2A). The maintenance mark could be features other than, or in addition to, histone modifications. An attractive candidate in some organisms would be DNA methylation, because specific machinery exists to copy this modification onto newly synthesized DNA (Bestor and Ingram, 1983; Holliday and Pugh, 1975; Riggs, 1975) (Hermann et al., 2004). However, many organisms that exhibit maintenance of

Figure 2: Models of chromatin state maintenance

(A) In this model, the heritable mark (siRNAs, DNA methylation, histone modifications) spreads into adjacent sequences. This system gives rise to parental state bias, whereby the phenotype of the parent influences the phenotype distribution in the progeny. Unique to this type of maintenance, the chromatin state can be uncoupled from the nucleating sequences. Examples include X inactivation and paramutation (Brown and Willard, 1994; Chandler, 2007). **(B)** In this model, the heritable mark is found only at the nucleating sequence, but other marks spread into adjacent sequences. Again, this type of maintenance would give rise to parental state bias. However, the state cannot be maintained in the absence of the nucleating sequence. Examples include maintenance of silencing in *S. cerevisiae* (S. cer) and Polycomb repression in *Drosophila* (Busturia et al., 1997; Cheng and Gartenberg, 2000; Sengupta et al., 2004). **(C)** In the final model, there is no heritable mark, instead the state is continually reestablished. Because of this reestablishment, no parental state bias would be observed. Examples include gene activation in *S. cerevisiae* (Katan-Khaykovich and Struhl, 2002)


Model
Parental state bias?
Nucleating-sequence independent maintenance?
Examples
A
Heritable mark spreads into adjacent sequences
Yes
Yes
Nucleating Sequence
Adjacent Sequences
Adjacent Sequences
X inactivation
Paramutation
B
Heritable mark restricted to nucleating sequence
Yes
No
Nucleating Sequence
Adjacent Sequences
Adjacent Sequences
Silencing (S. cer)
Polycomb repression
C
No heritable mark - persistent reestablishment
No
No
Nucleating Sequence
Adjacent Sequences
Adjacent Sequences
Gene activation (S. cer)
Heritable, epigenetic, mark
Transient mark
Silenced parent
Expressed parent
Silenced progeny
Expressed progeny

chromatin states, including *S. pombe*, do not have DNA methylation (Antequera et al., 1984; Hall et al., 2002a; Pillus and Rine, 1989). Alternatively, this mark could include RNAs that direct establishment of a specific state at that site (through a mechanism discussed later). Another model would suggest that maintenance is caused by protein factors that do not spread, but remain associated with the nucleating through replication (Figure 2B). Finally, it is possible that these states are simply not maintained, but instead reestablished after every round of DNA replication. In this case the factors required for establishment would be indistinguishable from those required to propagate the state in the daughter cells (Figure 2C), as is the case with the perpetuation of active modifications in *S. cerevisiae* (Katan-Khaykovich and Struhl, 2002). In the latter two models, the DNA sequences that play a role in establishment would be required to maintain the state either for reestablishment or to maintain contact with the epigenetic marks throughout cell division.

The extent to which these domains are maintained, and the role of sequence therein, gets to the core of the use of the descriptor "epigenetic." The definition of epigenetic requires that the state be heritable independent of changes in genomic sequence (Berger et al., 2009; Bird, 2007; Gottschling, 2004; Ptashne, 2007). Furthermore, the most rigorous usage of the definition requires that the state be self-propagating, allowing the state to be maintained even in the absence of the initiating signal (Berger et al., 2009; Gottschling, 2004; Ptashne, 2007). As histone modifications are frequently referred to as epigenetic modifications, it is thus critical that we understand the extent to which they are heritable and self-propagating. This will be the focus of Chapter 3 of this thesis.

1.3 Silencing in yeast

In the following sections I describe what is known about role of genomic sequence in the specification of heterochromatin in yeast, Polycomb silencing in *Drosophila*, dosage compensation in metazoans, and paramutation in maize. As with heterochromatin in fission yeast, these chromatin states rely on genomic sequence for various aspects of their establishment, maintenance and spreading. The extent of the role of sequence in each phase varies greatly among these organisms and thus can help establish expectations for and frame the interpretation of the findings presented in this thesis.

Silencing in *S. cerevisiae*

Work in *S. cerevisiae* has contributed to our most complete understanding of silent chromatin and the role of DNA sequence in the establishment, maintenance and spreading of the silenced state. A major similarity between budding yeast silent chromatin and heterochromatin in other organisms is the ability of the state to spread in cis and repress transcription (Hecht et al., 1996; Renauld et al., 1993). Thus, while *S. cerevisiae* lacks the histone methylation of H3K9 commonly associated with heterochromatin in *S. pombe* and other eukaryotes there are similarities that make the studies here relevant to our findings in fission yeast.

The two *S. cerevisiae* silent mating type cassettes (HMR and HML) contain three silencers -- HMR-E, HML-E, and HML-I -- that are sufficient for the establishment of the silenced state (Brand et al., 1985; Mahoney and Broach, 1989), in a manner that depends on the direct interaction between protein factors and their binding sites contained within the silencer.

Together, these DNA-bound proteins recruit the SIR (silent information regulator) proteins that spread and silence the locus (reviewed in Rusche et al., 2003). Thus, *in S. cerevisiae* establishment of silencing requires DNA sequences that recruit -- through protein intermediates -- the silencing machinery.

To address whether the silenced state is maintained, the distribution of silencing was monitored in the progeny of silenced and expressed cells (Pillus and Rine, 1989). The progeny of silenced cells were more likely to be silenced than the progeny of an expressed cells (Pillus and Rine, 1989). These data serve as evidence that states can be maintained when the silencer is present. To address whether the silencer is required for maintenance, two different strategies were developed to uncouple the silencer from the adjacent, silenced, regions. Using both approaches, the silenced state was lost after cell division in the absence of the silencer (Cheng and Gartenberg, 2000; Cheng et al., 1998; Holmes and Broach, 1996). These studies demonstrate that in *S. cerevisiae* the maintenance of silencing through cell division requires the presence of the silencer. Thus, the histone hypoacetylation and SIR proteins that spread across the mating type loci are insufficient to maintain silencing independently.

The requirement of genomic sequence for maintenance of silencing is consistent with two of the three maintenance models presented earlier (Figure 2). The silenced state may be reestablished after each cell division (Figure 2C). However, this interpretation is unfavorable as there is clear evidence that the expression state of the parent affects the expression state in the progeny (Pillus and Rine, 1989). If the chromatin state were established *de novo* each generation, no such parental state bias should be observed. Alternatively, the epigenetic signals required for maintenance may be spatially restricted to the silencer (Figure 2B) such that loss of

the silencer signals results in loss of the silenced, heritable, epigenetic mark. This possibility of this type of inheritance will be discussed further in the Polycomb silencing section.

While it is evident that maintenance of silencing through cell division depends on the persistence of silencers, silencing can be maintained in G_1 arrested cells in the absence of the silencers (Cheng and Gartenberg, 2000; Holmes and Broach, 1996). This maintenance is attributed to the presence of a DNA sequence within the locus that behaves as a protosilencer (Cheng and Gartenberg, 2000). These data suggest that genomic sequences, here a protosilencer that is insufficient to recruit the silencing machinery, can influence the maintenance of the silenced state.

In addition to initiating and maintaining silent chromatin, specific sequences also exist that stop the spread of heterochromatin. A tRNAthr gene at HMR behaves as a chromatin barrier through a mechanism that requires the recruitment of Pol III (Donze and Kamakaka, 2001). In the absence of the tRNAthr gene, silenced chromatin spreads into adjacent sequences and can effect gene expression (Donze et al., 1999; Donze and Kamakaka, 2001).

Silencing in *S. pombe*

Silencing in *S. pombe* shares many similarities with silencing in higher eukaryotes, such that it is commonly referred to as heterochromatin, despite lacking cytologically visible regions of condensed chromatin, the classical and literal definition of the term (reviewed in Grewal and Elgin, 2002). As the focus of this thesis is on *S. pombe*, this section will describe in detail the molecular mechanisms by which heterochromatin is established, propagated and maintained. Finally, I will describe the role of genomic sequence in each of these phases.

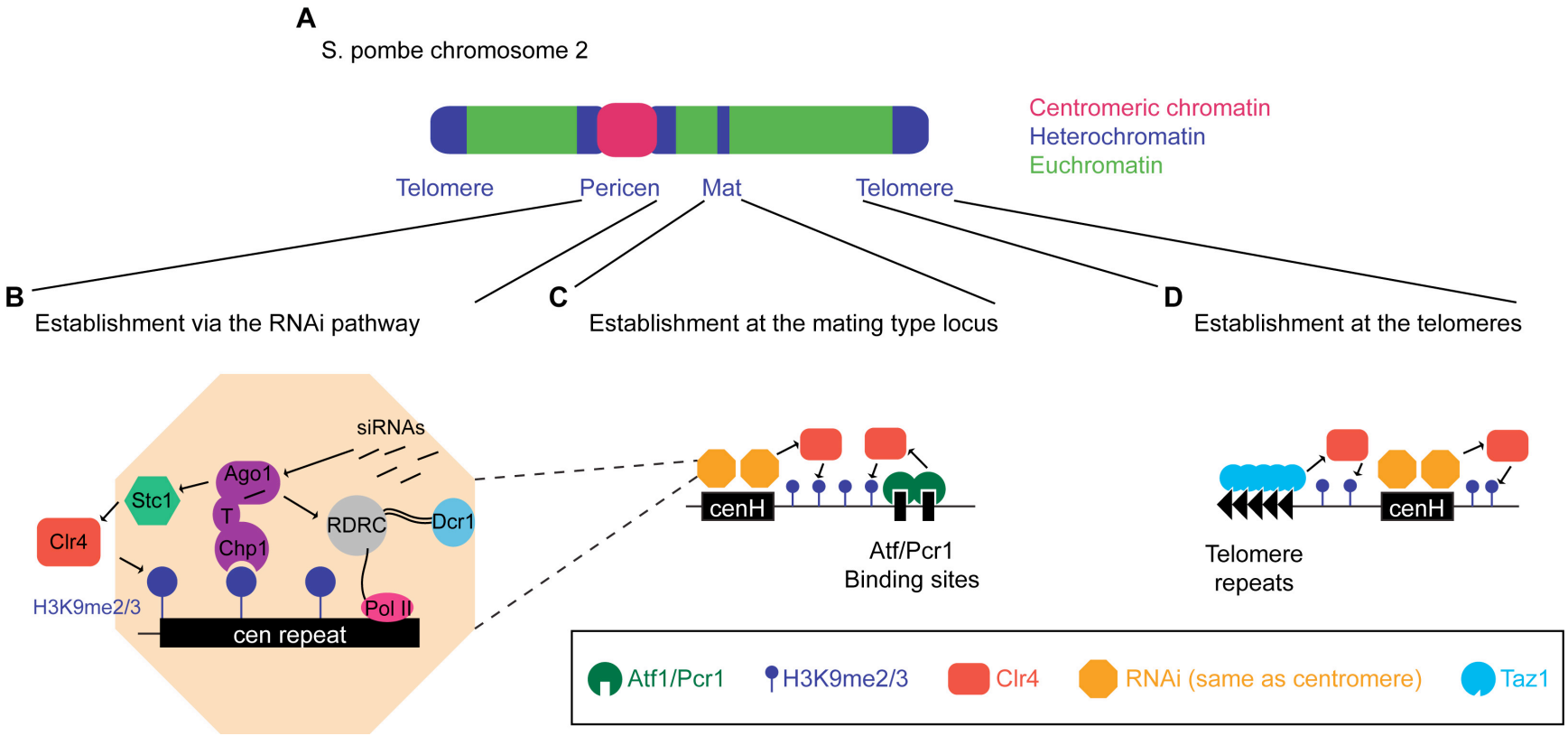
Heterochromatin in fission yeast is marked by nucleosomes that are methylated at histone H3 lysine 9 (H3K9me) (Nakayama et al., 2001a). Clr4, a histone methyltransferase, is required for the deposition of methyl groups on to H3K9 and thus is required for silencing in S. pombe (Nakayama et al., 2001a). How then is Clr4 and subsequently H3K9me2 recruited to regions of heterochromatin? In fission yeast, heterochromatin is found at the pericentromeric regions (here referred to as the "pericentromeres"), the telomeres, the rDNA array and the silent mating type loci (Figure 3A) (Cam et al., 2005). Recruitment of H3K9me at each of these loci utilizes the RNAi pathway described in the following section (Cam et al., 2005; Hall et al., 2002a; Kanoh et al., 2005; Volpe et al., 2002). However, the telomeres and silent mating type loci also utilize multiple silencing mechanisms that will be discussed later (Jia et al., 2004a; Kanoh et al., 2005).

RNAi-mediated heterochromatin formation

At the pericentromere, silencing and appropriate levels of H3K9me2 require members of the RNAi pathway (Figure 3B) (Volpe et al., 2002). Silencing via the RNAi pathway requires the transcription of centromere sequences, or sequences homologous to centromere sequences (Hall et al., 2002a; Kanoh et al., 2005; Volpe et al., 2002). These transcripts are processed by dicer (Dcr1) into small interfering RNAs (siRNAs) and bound by the RITS complex (RNA-induced initiation of transcriptional gene silencing) (Verdel et al., 2004). The RITS complex includes the chromodomain protein Chp1, Ago1 (which binds siRNAs), and Tas3, which connects the complex (Verdel et al., 2004). RITS recruits Clr4 through interactions mediated by the protein Stc1 (Bayne et al., 2010). This interaction is critical for the RITS-dependent recruitment of Clr4 and H3K9me

Figure 3: Establishment of heterochromatin domains in *S. pombe*

(A) Schematic of *S. pombe* chromosome three where domains of heterochromatin (telomere, pericentromere, and mating type locus) are shown in blue. Centromeric chromatin (cenH3-containing) is shown in pink and euchromatin is shown in green. **(B)** Establishment via the RNA pathway is shown here at the centromere. However, RNAi is also used to establish heterochromatin in a fashion that is redundant with Atf/Pcr1 at the mating type locus **(C)** and Taz1 at the telomere **(D)**.


A
S. pombe chromosome 2
Centromeric chromatin
Heterochromatin
Euchromatin
Telomere
Pericen
Mat
Telomere
B
Establishment via the RNAi pathway
C
Establishment at the mating type locus
D
Establishment at the telomeres
siRNAs
Ago1
Stc1
T
Chp1
RDRC
Dcr1
Clr4
H3K9me2/3
Pol II
cen repeat
cenH
Atf/Pcr1
Binding sites
cenH
Telomere
repeats
Atf1/Pcr1
H3K9me2/3
Clr4
RNAi (same as centromere)
Taz1

to the pericentromere. Tethering the RITS complex to a transcript is sufficient to establish silencing and heterochromatin (Bühler et al., 2006), suggesting that siRNA-directed targeting of RITS to sites of centromeric transcription is a major step in the formation of heterochromatin. Furthermore, expression of hairpin RNAs that can be processed into siRNAs is sufficient to establish heterochromatin at the homologous region of the genome (Iida et al., 2008; Simmer et al., 2010).

RITS interacts with both centromeric transcripts and RDRC (RNA-directed RNA polymerase complex), which contains the RNA-directed RNA polymerase, Rdp1 (Motamedi et al., 2004). This interaction is thought to couple recruitment of RITS to amplification of siRNAs by recruiting Rdp1 to single stranded transcripts, forming the dsRNA substrate required for processing by Dcr1.

What then provides the specificity, such that RITS binds to centromeric sequences as opposed to other transcripts being produced throughout the genome? The production of centromeric siRNAs depends on the presence of H3K9me. In *clr4*$^{-}$ strains siRNAs are lost and RITS fails to localize to the centromere (Cam et al., 2005; Noma et al., 2004). Chp1 serves to couple RITS recruitment to H3K9me. The Chp1 chromodomain recruits the RITS complex, including Ago1, to H3K9me found at the centromere (DeBeauchamp et al., 2008; Noma et al., 2004; Sadaie et al., 2004). These data suggest that siRNAs and H3K9me are mutually dependent. This system provides a self-reinforcing loop whereby H3K9me promotes the amplification of siRNAs, which in turn recruit more H3K9me.

RNAi-independent establishment of heterochromatin

Unlike silencing at the centromeres, which requires the RNAi pathway, silencing at the telomeres and at the mating type locus can occur in an RNAi-independent fashion (Jia et al., 2004a; Kanoh et al., 2005). In both cases this is due to redundant mechanisms of nucleation.

At the mating type locus, binding sites for the Pcf1/Atf1 transcription factor serve to establish and maintain heterochromatin in a pathway parallel to RNAi. Pcf1/Atf1 bind DNA and recruit Clr4 as well as the HP1 homologue, Swi6 (Figure 3C) (Jia et al., 2004b). Similarly, at the telomeres, silencing reflects the redundant efforts of the RNAi pathway and the telomere binding protein Taz1. Taz1 recruits Clr4, which in turn recruits Swi6 (Figure 3D) (Kanoh et al., 2005).

The RNAi pathway can also be bypassed by synthetically tethering Clr4 to a genomic locus (Kagansky et al., 2009). Expression of a chimeric protein including the catalytic domain of Clr4 and the Gal4 binding domain results in the recruitment of H3K9me to Gal binding sites. Thus, in this system, silencing, as well as recruitment of H3K9me, occurs in an RNAi-independent fashion.

Together, these studies show that the RNAi pathway is not the only mechanism of heterochromatin formation in *S. pombe*. Instead, the RNAi pathway can be bypassed by the direct, organic or synthetic recruitment of Clr4 proteins.

Heterochromatin and silencing

RNAi-dependent and -independent mechanisms are responsible for the recruitment of H3K9me to the appropriate genomic loci. However, the H3K9me mark itself is insufficient for silencing, as mutations that affect silencing but not H3K9me can be identified (Shimada et al.,

2009). This, consistent with the histone-code hypothesis, would suggest that H3K9me serves as a platform to recruit secondary proteins to enact silencing. Swi6, the *S. pombe* homologue of heterochromatin protein 1 (HP1), is recruited to heterochromatin by the specific binding of its chromodomain to H3K9me (Nakayama et al., 2001b). Swi6 recruits a number of factors, including the SHREC complex (Sugiyama et al., 2007; Yamada et al., 2005; Zofall and Grewal, 2006). SHREC (Snf2/HDAC-containing repressor complex) contributes to reduced levels of Pol II within heterochromatin (Motamedi et al., 2008; Sugiyama et al., 2007), suggesting that ability of heterochromatin to repress gene transcription may be at least partially due to exclusion of Pol II. The ability to limit Pol II access within heterochromatin requires both the histone deacetylase and chromatin remodeling activities of the SHREC complex (Sugiyama et al., 2007). Histone deacetylation is associated with a more compact form of chromatin (Tse et al., 1998). Furthermore, in the absence of SHREC nucleosome positioning within heterochromatin is dramatically altered (Sugiyama et al., 2007). Together, these data suggest that heterochromatin may cause silencing by recruiting an effector complex, SHREC, which compacts and organizes nucleosomes in a manner that occludes the associated DNA from access by Pol II (Motamedi et al., 2008; Sugiyama et al., 2007). The interplay between transcription-mediated establishment of heterochromatin and heterochromatic silencing of transcription will be discussed in following sections.

Spreading of heterochromatin in fission yeast

Consistent with observations of heterochromatin silencing in other organisms, transgenes inserted within or adjacent to heterochromatin in *S. pombe* are repressed in a

manner that requires the heterochromatin machinery (Ayoub et al., 1999; Kanoh et al., 2005; Partridge et al., 2000). In fission yeast, there are two major mechanisms that contribute to heterochromatin spreading. The first involves sequential recognition of H3K9me via chromodomain proteins and subsequent recruitment of Clr4 to propagate methylation of H3K9 (Figure 4A and 4B) (Bannister et al., 2001; Hall et al., 2002a; Nakayama et al., 2001a; Zhang et al., 2008). The latter involves RNAi-dependent spreading (Figure 4C) (Bühler, 2008; Irvine et al., 2006; Simmer et al., 2010). Both mechanisms allow the propagation of heterochromatin into non-nucleating sequences.

At the mating type locus, heterochromatin can spread from both types of nucleating sequences, Atf1/Pcr1 binding sites as well as the cenH RNAi nucleation site, to form a 20 kb heterochromatin domain (Hall et al., 2002a; Noma et al., 2004). The ability of heterochromatin to spread requires the chromodomain of Clr4, which specifically recognizes H3K9me (Zhang et al., 2008). One attractive model for this requirement is that Clr4 is recruited to sites of nucleation and then propagates by methylating adjacent nucleosomes, forming new binding sites for Clr4 and thus expanding the heterochromatin domain (Figure 4A).

Swi6 also plays a role in the spreading of heterochromatin, as increased Swi6 dosage results in increased spreading (Hall et al., 2002a). Swi6, like Clr4, contains a chromodomain that recognizes H3K9me. Swi6 is also capable of dimerization through its chromoshadow domain (Cowieson et al., 2000). As proposed in other organisms, sequential binding of Swi6 followed by dimerization and Swi6-dependent recruitment of additional Clr4 could also serve to propagate heterochromatin in a stepwise fashion (Figure 4B) (Bannister et al., 2001; Nakayama et al., 2001a). Together, the actions of Swi6 and Clr4 could serve to propagate heterochromatin over distances greater than 10 kb within the fission yeast genome.

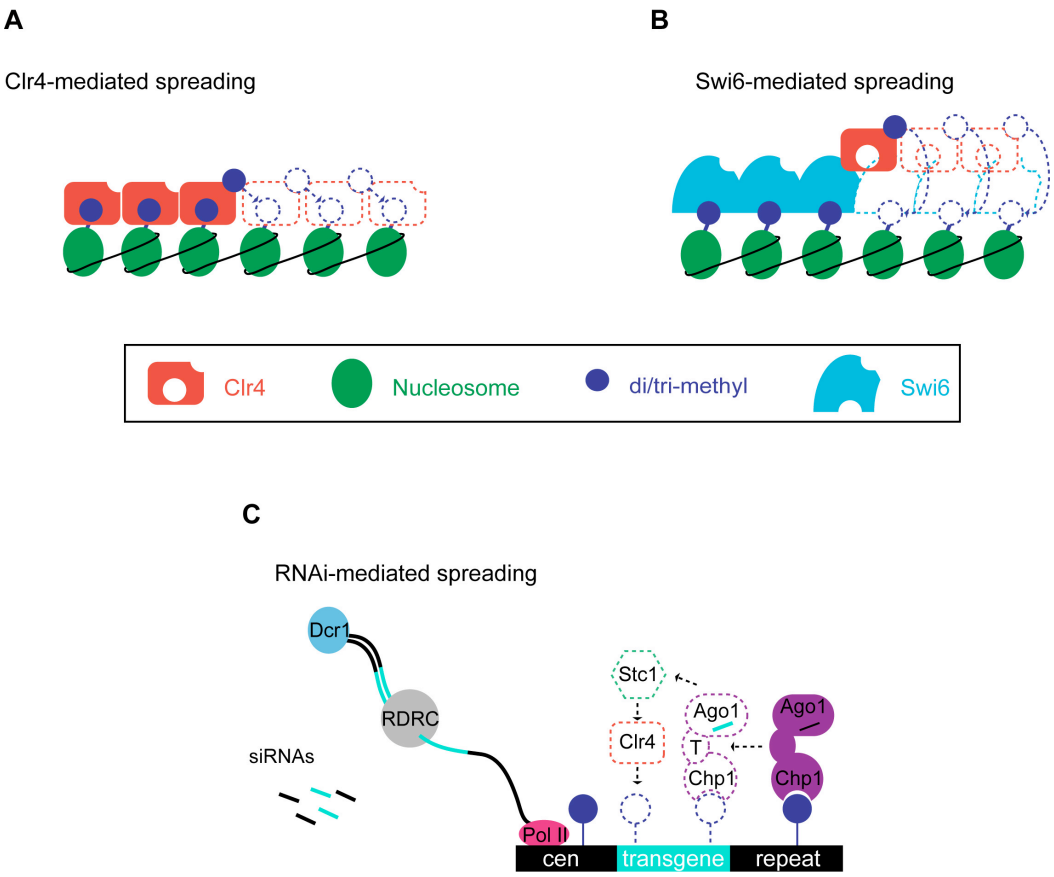


Figure 4: Mechanisms of heterochromatin spreading in fission yeast

There are three major mechanisms thought to contribute to the propagation of heterochromatin in fission yeast. **(A)** Clr4 has both the ability to recognize and write the H3K9me mark. Thus, by sequential rounds of binding and adding the methyl-mark heterochromatin can spread along the chromosome. **(B**) Alternatively, heterochromatin propagation could require the Swi6 chromodomain and its ability to recognize H3K9me and recruit Clr4. **(C)** The RNAi can also spread via both read-through transcription and interactions between Tas3 (T) in different RITS complexes.

In contrast to the mating type locus, spreading of heterochromatin into transgenes within the centromeric region occurs independent of Swi6. Here, spreading via RNAi plays an important role (Figure 4C) (Irvine et al., 2006). *ura4*$^{+}$ transgenes inserted within the centromere are silenced to varying extents. The strength of silencing increased in strains in which *ura4*$^{+}$ is inserted within a centromeric transcript, as read-through transcription results in the formation of ura4-homolgous siRNAs (Bühler, 2008; Irvine et al., 2006). Additional studies demonstrated that expression of siRNAs homologous to portion of a gene results in the production of siRNAs from the adjacent, non-homologous, regions of the transcript (Simmer et al., 2010). This supports the conclusion that production of siRNAs and the RNAi complex can spread into adjacent sequences. Furthermore, in transgenes inserted within the centromere, RITS spreads in a fashion that requires Tas3 self-association (Figure 4C) (Li et al., 2009). In contrast to the chromodomain model, RNAi-dependent spreading is likely to have more localized effects and contribute to silencing of genes embedded within heterochromatin.

Maintenance of heterochromatin in fission yeast

As in *S. cerevisiae*, heterochromatin in fission yeast is heritable through both mitosis and meiosis. Switching between silenced and expressed states at the mating type locus occurs at a low frequency, between 1-2% per generation (Grewal and Klar, 1996; Hall et al., 2002a). The stability of heterochromatin is influenced by the dosage of trans-factors (Nakayama et al., 2000). Together, these data suggest that heterochromatin is maintained through cell division by an epigenetic mechanism (Figure 2A and 2B). Interestingly, these states are heritable despite the restructuring of heterochromatin that occurs during DNA replication described below.

The observation that heterochromatin formation requires transcription runs counter to the prevailing belief that heterochromatin is a transcriptionally repressed state. However, these two states are reconciled by the observation that the presence of repressive chromatin and transcription are found at distinct stages of the cell cycle (Chen et al., 2008; Kloc et al., 2008). Throughout the majority of the cell cycle, the centromeres of *S. pombe* are enriched in H3K9me and depleted for Pol II. However, during S phase, the levels of H3K9me drop and Pol II levels increase. The switch between the repressive state and the transcriptionally active state corresponds with the phosphorylation of H3S10, which antagonizes the binding of H3K9me chromodomain proteins, such as Swi6 (Chen et al., 2008; Kloc et al., 2008). Thus, heterochromatin in fission yeast is dynamic, but nonetheless exhibits characteristics of being maintained through cell division.

What allows for this heritability is unknown. One possibility is that, as in *S. cerevisiae*, the heterochromatin nucleating sequences confer maintenance (Figure 2B). Alternatively, inheritance of siRNAs could be sufficient to initiate heterochromatin formation in subsequent generations (Figure 2A). Supporting this model is the observation that siRNAs generated from hairpin constructs are sufficient to establish silencing (Iida et al., 2008; Simmer et al., 2010). However, the persistence of silencing in these experiments depends on continued ectopic expression of the hairpin (Iida et al., 2008). These results would argue against the hypothesis that siRNAs alone can initiate a stably inherited heterochromatin domain. Finally, it is possible that the heterochromatic histone modifications themselves contribute to maintenance. Supporting this hypothesis, the CAF-1 histone chaperone complex, which interacts with both heterochromatin factors and the replication fork, is required for stable maintenance of

heterochromatin (Dohke et al., 2008). Inheritance of heterochromatin could be due to any of the above components alone or in combination with other factors.

Identification of trans-acting factors with specific roles in either maintenance or establishment provides further evidence that heterochromatin is maintained through a process that is distinct from establishment. For example, mutations in the RITS subunit Tas3 (*tas3*^{wg}) that abolish its ability to interact with Chp1 disrupt establishment but not maintenance of centromeric heterochromatin (Partridge et al., 2007). Interestingly, while *tas3*^{wg} mutants cannot reestablish heterochromatin in cells that were transiently depleted for Clr4, heterochromatin can be reestablished in cells that were transiently depleted for Dcr1. These genetic results suggest that the RNAi pathway acts downstream of H3K9me in the heterochromatin assembly pathway (Partridge et al., 2007).

The role of genomic sequence in the establishment, maintenance, and spreading of heterochromatin in fission yeast

There is a clear role for genomic sequence in the establishment of heterochromatin in fission yeast. RNAi-dependent establishment requires the transcription of repeats from promoters within the centromeric, or centromere-like, repeats (Irvine et al., 2006; Verdel et al., 2004; Volpe et al., 2002). These repeats, which share a high level of sequence homology, are found in multiple copies at the centromeric region and in a single copy at the telomere and mating type locus (Grewal and Klar, 1996; Kanoh et al., 2005). Centromeric and centromere-like repeats are sufficient to establish heterochromatin at ectopic sites (Ayoub et al., 2000; Hall et al., 2002a; Kanoh et al., 2005; Partridge et al., 2002; Volpe et al., 2003). These demonstrate the importance of the centromere and cen-like repeats in establishment of heterochromatin.

Whether there are specific sequence features of these repeats, and their associated transcripts, that allow for the nucleation of heterochromatin is unknown. However, one possible model is that the copy number of these repeats allows for the production of sufficient amounts of siRNAs to establish silencing at homologous copies in trans.

Additionally, the RNAi-independent mechanisms of assembling heterochromatin also require genomic sequence. These cases share conceptual similarities with silencing in *S. cerevisiae*, as silencing requires the binding of heterochromatin-recruitment proteins to specific DNA binding sites (Jia et al., 2004a; Kanoh et al., 2005), bypassing the need for the RNAi pathway. It is interesting that the specific proteins used to connect silencers to heterochromatin differ between the mating type locus and the telomeres (Jia et al., 2004a; Kanoh et al., 2005). Together, these studies show that establishment of heterochromatin in fission yeast requires DNA sequences that act either through transcription or through DNA-protein interactions to nucleate heterochromatin.

While it is clear that the heterochromatic state can be maintained (Hall et al., 2002a) and that maintenance requires different trans-acting factors than establishment (Partridge et al., 2007), the extent to which genomic sequence contributes to maintenance is unknown and will be a major focus of this thesis (Chapter 3).

In fission yeast, as in other organisms, heterochromatin can spread and propagate gene silencing, suggesting that there may be mechanisms through which the extent of this spread is regulated. Indeed, there is evidence for the presence of heterochromatin barriers in the fission yeast genome. The mating type locus is a 20kb domain of heterochromatin flanked on either side by inverted repeat elements, IR-R and IR-L (Nakayama et al., 2001a; Noma et al., 2001). In the absence of either DNA element heterochromatin spreads into adjacent, euchromatic,

territory altering gene expression of neighboring genes (Noma et al., 2001). These repeats contain 5 B-box sites, which are necessary for barrier activity (Noma et al., 2006). B-boxes are sequences found in the promoters of RNA Polymerase III (Pol III) transcribed genes and are recruit the Pol III transcription factor, TFIIIC (Huang et al., 2000). Interestingly, TFIIIC but not Pol III is recruited to these B-box sites, suggesting that the barrier functions independently of Pol III transcription (Noma et al., 2006). Instead, the barrier activity of these sites may be related to the ability of TFIIIC to tether genomic loci to the nuclear periphery (Noma et al., 2006).

The fission yeast centromeric regions consist of domains of pericentromeric heterochromatin apposed with domains of centromeric chromatin, a defining state of chromatin that is distinct from both euchromatin and heterochromatin and that is found exclusively at functional centromeres (Figure 3A) (Partridge et al., 2000). The transition between these two states is coincident with genes encoding tRNAs, suggesting that the transcribed tRNA genes may serve some role in maintaining domain boundaries. Indeed, removal of one of the tRNAs, tRNAala, results in spreading of heterochromatin into the domain of centromeric chromatin (Scott et al., 2006). The barrier activity is dependent on the tRNA promoter sequences and suggests that recruitment of a full RNA Pol III complex is required, including the Pol III transcription factor TFIIIC (Scott et al., 2006; Scott et al., 2007). However, unlike the IR elements at the mating type locus, recruitment of TFIIIC is not sufficient for barrier activity. Non-centromeric tRNAs can substitute for the tRNAala suggesting that this barrier activity is shared among tRNAs in general (Scott et al., 2007). Interestingly, the tRNAala exhibits partial barrier activity when studied at domains of ectopic heterochromatin, suggesting that there are both context-dependent and context-independent aspects of barrier activity (Scott et al., 2006).

In addition to the centromeric-pericentromeric frontier, pericentromeric heterochromatin is also adjacent to the euchromatin on the chromosome arms (Figure 3A). Again, between these two domains are specific DNA sequences required for appropriate specification of the boundary. The barriers found on chromosome 1 (referred to as IRC1-R and IRC1-L) contain an inverted repeat motif that is required for barrier activity (Noma et al., 2006). The inverted repeat does not recruit TFIIIC and thus functions by a third, and unknown, mechanism. However, the repeat element is coincident with a peak of "active" chromatin modifications, H3K4me and histone hyperacetylation. Recruitment of these marks may thus serve to limit the spread of heterochromatin (Noma et al., 2006). Consistent with this, the IRC1 boundaries are sensitive to the presence of the H3K9me demethylase, Lsd1, suggesting a role for active modifications in barrier activity (Lan et al., 2007).

Together, these studies show a role for genomic sequence in the organization of chromatin domains, specifically, in the ability to prevent heterochromatin from encroaching into adjacent domains. Interestingly, even within *S. pombe*, the mechanisms of barrier activity are diverse and suggest that barrier activity may have been co-opted from other uses, i.e. transcriptional activation or regulation of nuclear localization.

1.4 Heterochromatin and Polycomb repression in Drosophila

Position effect variegation

Position effect variegation (PEV), the spreading of heterochromatin and variable repression of gene expression in what is normally euchromatin, was first observed and characterized in strains of *Drosophila melanogaster* with rearranged X chromosomes (Muller

1930; Schultz PNAS 1936). Genetic screens identifying trans-factors that suppress or enhance variegation have created a robust data set of factors involved in the heterochromatin assembly and spreading process (reviewed in Girton and Johansen, 2008). Furthermore, approximately one third of the *Drosophila* genome is heterochromatic, suggesting that regulating the spread of heterochromatin is critical to maintaining appropriate gene expression in this organism (Smith et al., 2007).

The heterochromatic regions of *Drosophila* are composed largely of satellite DNAs, transposable elements, and other repetitive DNAs (Lohe and Brutlag, 1986; Smith et al., 2007). The exact manner in which these repetitive sequences establish heterochromatin is unknown. However, like fission yeast, heterochromatin formation depends on the RNAi pathway (Pal-Bhadra et al., 2004). There is evidence that the repetitive nature of the heterochromatic sequences is important to the establishment of heterochromatin as transgenes inserted in tandem in three or more copies can nucleate heterochromatin (Dorer and Henikoff, 1994) in an RNAi-dependent fashion (Pal-Bhadra et al., 2004). Additionally, the transposable element 1360, also called hoppel, is important for position effect variegation on the largely heterochromatic Drosophila Chromosome 4 (Sun et al., 2004) and is sufficient to nucleate heterochromatin at some ectopic sites (Haynes et al., 2006). These data suggest that there is a role for repetitive DNA sequences in the establishment of heterochromatin in Drosophila.

Both the ability of the 1360 element or tandemly repeated DNA to nucleate heterochromatin and the strength of PEV correlates with the proximity of the element to the pericentromere and its inclusion within a heterochromatic chromocenter (Dorer and Henikoff, 1997; Haynes et al., 2006; Talbert et al., 1994; Wakimoto and Hearn, 1990). Furthermore, the repetitive DNA located in proximity (within 20 kb) of the centromere is frequently associated

with HP1, an H3K9me-specific chromodomain protein in *Drosophila*, with an important role in heterochromatin, whereas repetitive DNA located on the chromosome arms (greater than 100 kb from the centromere) is not (de Wit et al., 2005). These data suggest that these repetitive sequences may not suffice as nucleating sequences but instead serve as protosilencers that disseminate silencing established at the centromere. This model of spreading, commonly referred to as hopping (Talbert and Henikoff, 2006), would suggest that specific genomic features attract heterochromatin and lead to a pattern of spreading that is discontinuous. Unlike the oozing model, where heterochromatin spreads through the modification of adjacent nucleosomes, the hopping model suggests that heterochromatin factors spread from the silencers by diffusion and collect specific sites in the genome (Talbert and Henikoff, 2006). Thus, spreading would reflect locus-specific features that may include nuclear localization, transcriptional activity, and repeat content. Two other observations support the hopping model in *Drosophila*. Spreading of HP1 on rearranged X chromosomes, which can occur over distances of 200 kb, shows substantial local variation (Vogel et al., 2009). Also, the extent of silencing of genes does not always correlate with the proximity of the gene to the source of heterochromatin (Belyaeva and Zhimulev, 1991; Csink et al., 2002; Talbert and Henikoff, 2000), suggesting discontinuous spreading.

Studies in *Drosophila* have also contributed significantly to our understanding of how boundaries between heterochromatin and euchromatin are enforced. Insulator elements, first characterized in Drosophila (Udvardy et al., 1985) serve to maintain appropriate patterns of gene expression. This is achieved in two ways; insulators block enhancers from acting on promoters and they prevent heterochromatin from encroaching into domains of heterochromatin (Labrador and Corces, 2002). The latter characteristic is referred to as barrier

activity and has been discussed in previous sections. Transgenes flanked by chromatin insulators are expressed in a manner that is independent of chromosomal location (Kellum and Schedl, 1991). Together, these data suggest that genomic sequence serves to both enhance and restrict the spread of heterochromatin in *Drosophila*.

Polycomb repression

In multicellular organisms, development requires the epigenetic regulation of gene expression, allowing genomes to achieve vastly different phenotypic outcomes. A major player in the epigenetic regulation of gene expression and therefore development is the Polycomb group (PcG) of proteins (Boyer et al., 2006; Bracken et al., 2006; Lee et al., 2006). Thus, there is much to learn from Polycomb about how chromatin states are established and maintained and the role of genomic sequence both of these stages. While PcG proteins are of functional importance to most higher eukaryotes (reviewed in Schwartz and Pirrotta, 2008), the most extensive work has been completed in flies, which will be the major system described here.

Early in development, transcriptional states are established by the segmentation gene products, which serve to activate or repress transcription (reviewed in Ringrose and Paro, 2007). After the segmentation gene products decay, these states are maintained throughout development, giving rise to different cell fates (Orlando et al., 1998). Thus, there are distinct establishment and maintenance phases, and PcG proteins play a role only in the latter (Simon et al., 1992; Struhl and Akam, 1985). Gene expression is maintained in the repressed state through recruitment of the PcG proteins (Moehrle and Paro, 1994).

While PcG binding varies depending on the cell type and as such is epigenetically regulated, there is still a role of genomic sequence in recruiting the PcG proteins. Polycomb response elements (PRE), the sequences bound by the PcG proteins, can function to maintain gene repression outside of their normal context (Chan et al., 1994; Muller and Bienz, 1991; Sengupta et al., 2004; Simon et al., 1993), suggesting a role of genomic sequence, along with lack of expression early in development, in PcG binding. Determining the sequences bound by PcG proteins is challenging because the profile of PcG binding varies with cell type and developmental stage. Despite the complexity, *in silico* predictive approaches have been successful in identifying potential PcG binding sites, many of which have been verified *in vivo* (Ringrose et al., 2003). Pairs of binding sites for the PcG proteins pleiohomeotic (Pho), GAGA factor (GAF), and Zeste (Z) were strong determinants of PcG binding (Ringrose et al., 2003). Recruitment of Pho to the PRE initiates recruitment of the Esc-E(z) complex, followed be recruitment of Polycomb (Pc) (Wang et al., 2004). The Esc-E(z) complex contains a SET domain required for methylation of H327me. In the absence of this SET domain, silencing of PcG targets is lost (Cao et al., 2002; Czermin et al., 2002; Müller et al., 2002). In addition to localization at the PRE, H3K27me spreads into adjacent sequences (Schwartz et al., 2006).

Despite the role of PcG in epigenetic memory, the silenced state cannot be uncoupled from the PRE (Busturia et al., 1997; Sengupta et al., 2004). This, much like silencing in *S. cerevisiae*, represents a case of sequence-dependent epigenetic inheritance. Whereby the chromatin modifications (H3K27me) and other marks that spread outside the DNA element are insufficient to maintain the state. Again, the same two models that suggest the silenced state is either established anew each generation or that the signals for maintenance are only found at the nucleating sequence, are applicable (Figure 2A and 2B). However, in Polycomb-repression

there is evidence favoring silencer-localized memory. Studies examining the dynamics of PcG-DNA interactions through the cell cycle demonstrated that, *in vitro*, PcG proteins remain bound through the DNA even after replication (Francis et al., 2009). Thus, epigenetic memory in Drosophila could be formed by the stable DNA-PcG interactions, serving as a heritable silenced mark (Figure 2A). This interaction would then be sufficient to reestablish the other factors required for silencing, including H3K27me.

1.5 Dosage compensation

Dosage compensation is the process whereby gene expression is regulated to equalize gene expression between the heterogametic and homogametic sexes in various organisms (reviewed in Chow and Heard, 2009; Gelbart and Kuroda, 2009; Meyer, 2005). Dosage compensation requires mechanisms of recruitment and/or spreading that allow chromosome-wide localization of the dosage compensation machinery and thus serves as an attractive system in which to study the interaction between chromatin state and genomic sequence. While the actual mechanism of equalizing gene expression is different in *C. elegans*, *Drosophila*, and mammals, they share many common features including a role for genomic sequence in the establishment, maintenance, and propagation of the epigenetic, dosage compensated, state. A comparison between the size of domains of dosage compensation and those described earlier in this thesis are included in the following table (Table 2).

Table 2: Comparison of domain sizes

Table including the size of chromatin domains, nucleating sequences, and whether the domains are continuous.

Organism	Domain (size)	Nucleating sequence(s)	Continuous
S. cerevisiae	Mating type cassettes (HMR 3.5kb, HML 5kb)	HMR-E, HML-I, HML-E (Brand et al., 1985; Mahoney and Broach, 1989),	Yes
S. pombe	Centromere, Chr 2 (~20kb) (Cam et al., 2005)	Cen repeats (Partridge et al., 2002)	Yes
	Mating type locus (20kb) (Hall et al., 2002a)	CenH, Atf1/Pcr1 (Jia et al., 2004a)	Yes
	Telomere, Chr 2 (45-55kb) (Kanoh et al., 2005)	CenH, Telomere repeats (Kanoh et al., 2005)	Yes
Drosophila	Position effect variegation (175 kb) (Vogel et al., 2009)	Repetitive DNAs, Hoppel elements (Sun et al., 2004)	local variation
	Polycomb silencing, Bithorax locus (75 kb) (Bender et al., 1983)	PREs (Muller and Bienz, 1991; Simon et al., 1993)	No
	Dosage compensation (X chromosome 22MB)	CES (Alekseyenko et al., 2008)	No
C. elegans	Dosage compensation (X chromosome 17.7 MB)	*rex* sites (McDonel et al., 2006)	No
Mammals	Dosage compensation (X chromosome ~150MB)	XIST (Hall et al., 2002b; Herzing et al., 1997; Lee and Jaenisch, 1997)	No

Dosage compensation in *C. elegans*

Dosage compensation in *C. elegans* is achieved by reducing gene expression on both X chromosomes in the XX hermaphrodite by approximately one half, to equal the level of expression on the single X in male worms. *The C. elegans* dosage compensation complex (DCC) is localized at discrete locations on both X chromosomes (Ercan et al., 2007; Jans et al., 2009) where it works to reduce gene expression.

The establishment of DCC binding requires specific DNA sequences called *rex* (recruitment on X) sites that are sufficient to bind the DCC when inserted as tandem, extrachromosomal arrays (Csankovszki et al., 2004; Jans et al., 2009; McDonel et al., 2006) and are bound by components of the DCC in their endogenous X chromosomal context (Ercan et al., 2007; Jans et al., 2009). The mechanism by which *rex* sites recruit the DCC is as yet unknown, but DCC localization to *rex* sites involves a DNA motif, called MEX (motif enriched on x), that is found at most *rex* sites, usually in multiple copies (Jans et al., 2009). Together, these data suggest a paramount role for DNA sequence in recruiting the DCC to the X chromosome. However, the strength of DCC motifs alone is insufficient to predict all sites of DCC binding. The additional factors that affect DCC binding may be either genetic (additional sequence preferences, outside of the MEX motif), or epigenetic in nature (for example, MEX motif accessibility could alter the probability or affinity of DCC binding).

In addition to rex sites, there is evidence that the DCC is located in regions of the X chromosome that cannot independently recruit the DCC complex (Csankovszki et al., 2009;

Ercan et al., 2007; Jans et al., 2009). This is consistent with a model in which the DCC is recruited to the X chromosome via *rex* sites and then spreads into adjacent sequences. The majority of these so-called *dox* sites (dependent on X, non-rex sites of DCC binding) lack robust MEX motifs and are preferentially located within the promoters of genes. Furthermore, the strength of DCC binding at dox sites correlates with the level of transcription (Ercan et al., 2007; Jans et al., 2009). Thus, transcription appears to play a key role in spreading of the DCC. Additional evidence that the DCC propagates without requirement for specific X-enriched DNA sequences came from the study of X;autosome translocations in which the DCC is enriched on the autosomal sequences (Ercan et al., 2009). However, there may still be a more subtle role of genomic sequence in the propagation of the DCC, as is observed in X inactivation in mammals and will be discussed in later sections.

The extent to which DCC is maintained, and the role of genomic sequence therein, is largely unknown. The observation that that the level of DCC binding at *dox* sites changes with the level of transcription suggest that, at these genes, DCC binding is dynamic (Ercan et al., 2009).

Dosage compensation in *Drosophila*

In contrast to *C. elegans*, expression is equalized between the sexes in Drosophila by upregulating gene expression off the X chromosome in males (Mukherjee and Beermann, 1965). Dosage compensation is initiated by two roX (RNA on the X) RNAs that are transcribed from the X chromosome and are included in the MSL (male-specific lethal) complex that specifically binds

the X chromosome to enact dosage compensation (Franke and Baker, 1999; Meller et al., 2000; Meller and Rattner, 2002).

Insight into the process of targeting the MSL complex to the X came from studies looking at the localization of the MSL complex in the absence of spreading (Sural et al., 2008). In these mutants, MSL binding was restricted to sites of recruitment, referred to as "chromatin entry sites" (CES) (Alekseyenko et al., 2008). Analysis of the sequence underlying these CES identified enrichment in a particular sequence motif called MRE (MSL recognition element) that is modestly enriched on the *Drosophila* X (Alekseyenko et al., 2008). Insertion of this motif on an autosome resulted in the recruitment of the MSL complex to that site, suggesting that it is sufficient for MSL binding (Alekseyenko et al., 2008). These data suggest that, like in *C. elegans*, dosage compensation in *Drosophila* relies on specific DNA sequences that recruit the dosage compensation machinery. However, the strength of the sequence motif does not perfectly predict sites of MSL binding, and there is a correlation between MSL binding and nucleosome depletion (Alekseyenko et al., 2008), suggesting that chromatin environment or additional sequence features may also play a role in establishment of dosage compensation.

Other parallels to dosage compensation in *C. elegans* become apparent when studying the effects of introduction of X sequences onto autosomes. In both systems the dosage compensation machinery is capable of spreading into autosomal sequences (Alekseyenko et al., 2008). This demonstrates that dosage compensation does not require X chromosome sequences for propagation, however X sequences may enhance the efficiency of spreading. Furthermore, in *Drosophila*, like *C. elegans* there is a preference for localization near genic sequences (Alekseyenko et al., 2008), suggesting that transcription plays a role in dispersing dosage compensation. This is consistent with the hopping model, discussed earlier in the context of

PEV. In this case, spreading of dosage compensation is discontinuous and hops to sites of active transcription

Dosage compensation in mouse and human

Mammals utilize yet a third mechanism of equalizing X dosage between males and females, the random inactivation of one of the two X chromosomes in females. Dosage compensation via X inactivation is highly relevant to heterochromatin silencing in fission yeast and other organisms, as both involve the near complete repression of transcriptional activity.

X inactivation requires the transcription of a non-coding RNA, called XIST (mouse: Xist) that coats the inactive chromosome in cis (Brown et al., 1992; Brown et al., 1991; Penny et al., 1996). Ectopic localization of XIST/Xist to autosomes results in recruitment of XIST/Xist RNAs, reduced gene expression, late replication, and chromatin modifications consistent with the inactive X chromosome, demonstrating that XIST/Xist expression is sufficient to induce X inactivation (Hall et al., 2002b; Herzing et al., 1997; Lee and Jaenisch, 1997). Here, the conceptual similarities between X inactivation and heterochromatin in fission yeast are clear; in both cases silencing is initiated by specific DNA sequences that contain non-coding RNAs and involves modified histones.

As the XIST sequence is sufficient to establish X inactivation (Hall et al., 2002b; Herzing et al., 1997; Lee and Jaenisch, 1997), is there a role for DNA sequence in propagating and maintaining the inactivation signal along the X chromosome? In addition to regions of the genome that are subject to X inactivation, other genes escape from X inactivation, suggesting that even within the X chromosome there is heterogeneity in dosage compensation status

(Carrel and Willard, 2005). Some of the genes that escape X inactivation are variable in a population, and whether there is a sequence basis to this variation is unknown.

There is significant evidence that X inactivation is more efficient in the context of the X chromosome than in X;autosome translocations or autosomal insertions on the X chromosome (Cattanach, 1974; Duthie et al., 1999; Hall et al., 2002b; Popova et al., 2006; Russell, 1963; White et al., 1998). Consistent with previous models (Gartler and Riggs, 1983), these studies suggest that there may be sequences on the X chromosome that stabilize gene inactivation. A more specific version of this model proposes that LINE elements, which are enriched on the X chromosome relative to autosomes (Korenberg and Rykowski, 1988), may serve to enhance the distribution of X inactivation (Lyon, 1998). In an X;4 translocation, X inactivation (as assayed by three markers of the inactive X chromsome; AcH4, H3K27me3, and Xist localization) fails to spread past a 20Mb "block" of low LINE density (Popova et al., 2006). In addition, L1 and L2 LINE elements are enriched in regions associated with genes that are subject to X inactivation (Carrel et al., 2006; Wang et al., 2006b). Testing the role of other DNA sequences in addition to LINE elements, a computational approach identified groups of sequence features that are capable of predicting the X inactivation status of genes with ~80% accuracy (Wang et al., 2006a).

It is interesting to note that the nature of this stabilization could be achieved by enhancing spreading of X inactivation or stabilizing the maintenance of X inactivation, as suggested by the spread and retreat hypothesis, which suggests that X inactivation spreads over the entire chromosome and is then maintained only at discrete sites (Hall et al., 2002b; Popova et al., 2006). The extent to which these two mechanisms are utilized is unknown.

In addition to enhancing X inactivation, certain sequences may also protect genes from X inactivation. This framework is conceptually similar to heterochromatin barriers; however, the

chicken β-globin HS4 barrier, which protects against position effect variegation on the active X, fails to protect genes on the inactive X chromosome from X inactivation (Ciavatta et al. PNAS 2006). Thus, barrier activity and prevention of the spread of X inactivation appear to be distinct capacities.

Sequences that would prevent X inactivation from acting on genes could act in a gene specific or domain-wide scale, as genes that escape from inactivation are frequently found in clusters (Carrel and Willard, 2005; Miller and Willard, 1998). Evidence that Jarid1C, a gene that escapes X inactivation in mouse, maintains expression even when inserted within a domain of subject genes is consistent with the first model (Li and Carrel, 2008). Furthermore, genes that escape X inactivation and are on the border between escape and subject domains bind CTCF, a protein with roles in organizing higher order chromatin structure (Libby et al., 2008). These sites may help to partition regions of subject and escape genes.

Finally, there is evidence that the XIST nucleating sequence is dispensable for maintenance, as X inactivation can persist upon uncoupling of XIST (Brown and Willard, 1994). This suggests that, unlike silencing by the PcG proteins or in *S. cerevisiae*, the chromatin state can be maintained independent of the nucleating sequence (Figure 2A).

1.6 Paramutation in maize

Paramutation is a phenomenon originally observed in maize, whereby interaction between epialleles, alleles attributed not to differences in sequence but to epigenetic differences, causes a heritable, but not sequence-based, reduction in expression even after the two alleles segregate from each other (Brink, 1956). For example, at the maize b1 locus there

are two epialleles – B', which gives rise to weak pigmentation and is the allele (called the paramutagenic allele) that partially silences other alleles in trans, and B1, which results in dark pigmentation and is the allele (called the paramutable or paramutant allele) that can be partially silenced by the presence of a parmutagenic epiallele (reviewed in Chandler et al., 2000). Interestingly, the two epialleles are genetically identical, suggesting that paramutation involves heritable, but non-sequence encoded, information. Furthermore, interaction of the two alleles in heterozygotes leads to the conversion of B1 to B'* (the asterisk denotes an epiallele that has recently undergone paramutation, that is, conversion from B1 to B') (Chandler et al., 2000; Hollick et al., 1995). After segregation of B'* and B*, B'* maintains reduced expression and is paramutagenic. Thus, paramutation involves heritable changes in expression that can be inherited even in the absence of the factors that established the state (Figure 2A).

It is unknown how the initial states giving rise B1 to B' are established. However, it is known that to participate in paramutation requires the presence of seven tandemly repeated copies of an 853 base pair repeat that is not found elsewhere in the genome upstream of the transcription start site (Stam et al., 2002). Genetically distinct alleles with only one copy of the repeat cannot become paramutagenic (Stam et al., 2002). This suggests that while DNA sequence is insufficient for paramutation, it is in fact required.

Initial insights into the role of these repeats in paramutation demonstrated that the repeats are transcribed and that paramutation requires an RNA-dependent RNA polymerase which is involved in the generation of siRNAs (Alleman et al., 2006). These data suggest that production of siRNAs homologous to the repeats may play a role in silencing of the paramutant allele. However, transcription is insufficient for paramutation, as both B1 and B' alleles are transcribed at similar levels (Alleman et al., 2006). Thus, other features work in concert with

transcription to establish and maintain the paramutagenic state. One feature that does correlate with the paramutagenic and paramutable states is a closed chromatin conformation, as indicated by reduced nuclease accessibility and DNA methylation (Stam et al., 2002). One model is that either the conformation of chromatin or DNA methylation is the heritable signal and that RdRP is required for perpetuation of either of these marks.

1.7 Thesis overview

Together these studies demonstrate that there are many mechanisms of epigenetic inheritance, ranging from nucleating-sequence independent as described here for paramutation and X inactivation as well as nucleating sequence-dependent as observed in *S. cerevisiae* and Polycomb repression. Furthermore, genomic sequence (protosilencers, barriers, nucleating sequences etc.) exerts a range of effects on the spreading of chromatin states.

The goal of this thesis is to dissect the role of DNA sequence in the spatial (spreading) and temporal (maintenance) propagation of heterochromatin in *S. pombe*. The goal of Chapter 2 is to determine the extent to which heterochromatin spreading is affected by genomic content. This work will serve to classify the types of genomic sequence that alter heterochromatin spreading. The work presented in Chapter 3 will address, in a highly tractable model organism with heterochromatin similar in nature to that of higher eukaryotes, the extent to which maintenance of the heterochromatic state depends on DNA sequence. This work will illuminate the mechanisms of epigenetic inheritance and will move the field towards and understanding of the requirements for maintenance of heterochromatin. Finally, in Chapter 4, I will highlight the

important conclusions from these studies, discuss relevant models that may explain the findings therein, and propose future experiments to expand on the discoveries made here.

2. The impact of local genome sequence on defining heterochromatin domains

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2.1 Author summary

Epigenetic packaging of DNA sequence into chromatin is a major force in shaping the function of complex genomes. Different types of chromatin have distinct effects on gene expression, and thus chromatin state imparts distinct features on the associated genomic DNA. Our study focuses on the transition between two opposing chromatin states: euchromatin, which generally correlates with gene expression, and heterochromatin, which is typically refractive to gene expression. While heterochromatin is capable of spreading into euchromatic domains, the parameters that influence such spreading are unknown. We established heterochromatin at ectopic sites in the genome and evaluated whether specific DNA sequences affected the extent of heterochromatin spreading and the transition between heterochromatin and euchromatin. We found that the nature of the genomic DNA neighboring the heterochromatic sequence dramatically affected the extent of heterochromatin spreading. In particular, the presence of genes antagonized the spread of heterochromatin, whereas neutral sequence elements were incorporated into the domain. This study demonstrates that genome sequence and chromatin identity are inextricably linked; features of both interact to determine the structural and functional fate of underlying DNA sequences.

2.2 Introduction

Correct patterns of gene expression are established by orchestrated interactions among *cis*-regulatory elements, *trans*-acting factors and the surrounding chromatin environment. How these interactions are coordinated and to what extent genomic sequence serves as a blueprint,

directing these interactions towards normal growth and development, remain major questions in genome biology.

Chromatin has classically been divided into two functionally distinct types: heterochromatin and euchromatin. Genes inserted within, or proximal to, major heterochromatin domains can exhibit either variegated or complete silencing (Allshire et al., 1994; Baur et al., 2001; Festenstein et al., 1996; Koering et al., 2002; Pedram et al., 2006; Pravtcheva et al., 1994; Schultz, 1936). This repression, referred to as position effect variegation (PEV), results from the propagation of heterochromatin marks along the chromosome, placing the euchromatic gene into a chromatin context that is incompatible with normal gene expression (Locke et al., 1988; Muller, 1930; Schultz, 1936). While PEV and the factors that contribute to it have been most thoroughly elucidated in yeast and flies, position-dependent gene silencing has been observed in a range of organisms including both mice and humans (Allshire et al., 1994; Festenstein et al., 1996; Milot et al., 1996; Schultz, 1936). Indeed, there are examples of human disease that can be attributed to gene silencing associated with aberrant formation of heterochromatin (Coffee et al., 2002; Eiges et al., 2007; Herman et al., 2006; Otten and Tapscott, 1995). Together, these studies highlight the important relationship between chromatin context and gene expression and suggest that eukaryotes have developed mechanisms to counter the spread of repressive heterochromatin (Donze et al., 1999; Muller, 1930; Noma et al., 2001; Schultz, 1936). However, the nature of these mechanisms and the extent to which they utilize specific DNA sequences remains incompletely understood.

Several studies have pointed towards the importance of genome sequence in shaping epigenetic states. For example, insulators are specific DNA sequences that protect genes from the regulatory effects of neighboring domains, thus enforcing domain boundaries (Roseman et

al., 1993). As presently defined, insulator activity has two components: the ability to prevent cross-talk between an enhancer and promoter (enhancer blockers) and the ability to stop the spread of repressive heterochromatin (heterochromatin barriers) (Kellum and Schedl, 1991; Roseman et al., 1993; Sun and Elgin, 1999; West et al., 2002). First identified and characterized in flies (Kellum and Schedl, 1991; Udvardy et al., 1985), insulators have since been identified in vertebrates (Chung et al., 1993; Filippova et al., 2005; Lunyak et al., 2007).

Elucidating the role of genome sequence in shaping chromatin domains requires an experimental system in which heterochromatin nucleation can be initiated in a controlled manner. To this end, we have examined heterochromatin spreading from a *de novo* nucleation site in the fission yeast, *Schizosaccharomyces pombe*. The unique advantage of this system, in addition to its genetic tractability, is the presence of well-defined DNA sequences, referred to here as heterochromatin-nucleating sequences, that are sufficient to induce heterochromatin formation *de novo* (Hall et al., 2002a; Partridge et al., 2002; Scott et al., 2006). Moreover, introduction of a *de novo* heterochromatin domain at a euchromatic locus permits a simplified view of this process, in contrast to native domains of heterochromatin that result from the complex interplay of multiple sites of nucleation and heterochromatin barriers (Cam et al., 2005; Noma et al., 2006; Scott et al., 2006). Analysis of the resulting *de novo* heterochromatin domains clearly implicates primary DNA sequence in defining both the magnitude and extent of the heterochromatin domain. The conceptual framework that emerges from this study provides a basis for exploring the nature of complex genomes and the impact of genome sequence on the establishment and maintenance of chromatin domains, in organisms ranging from yeast to mammals.

2.3 Results

The L5 element nucleates a *de novo* heterochromatin domain encompassing adjacent genomic sequences

Previous studies in *S. pombe* have demonstrated that a fragment of pericentromeric DNA, called L5, is capable of nucleating heterochromatin, marked by di-methylation at H3K9 (H3K9me2) and the presence of the HP1 homologue, Swi6p, at an ectopic site through an RNAi-dependent mechanism (Partridge et al., 2002; Volpe et al., 2003). Integration of the L5 element leads to the repression of an adjacent reporter gene in a manner that appears largely similar to that observed at the endogenous centromere (Partridge et al., 2002; Scott et al., 2006; Volpe et al., 2003). What is unknown, however, is the extent to which L5-nucleated heterochromatin is capable of extending past the reporter construct into endogenous genomic sequences.

To address this question, we created a construct containing the 1.6kb L5 element upstream of an *ade6*$^{+}$ reporter gene. This construct was then integrated at the euchromatic *ura4*$^{+}$ locus in order to create *ura4*::L5-*ade6*$^{+}$ strains. In addition to the L5-containing construct, a control construct bearing only the *ade6*$^{+}$ gene was also integrated at the *ura4*$^{+}$ locus (*ura4*::*ade6*$^{+}$). The effect of L5-integration on the chromatin environment of sequences within the *ura4* locus was characterized by quantifying H3K9me2 and Swi6p levels throughout the region using chromatin immunoprecipitation (ChIP). In the presence of the L5 element, H3K9me2 was enriched ~2- to >10-fold over both the reporter gene and the surrounding genomic neighborhood (Figure 5A and Figure 6A), extending 4kb proximal and 10kb distal to L5. The pattern of Swi6p enrichment is remarkably similar to the level of H3K9me2, consistent with previous reports demonstrating that H3K9me2 and Swi6p have tightly overlapping distributions

Figure 5: L5 initiates formation of de novo heterochromatin domains at two distinct loci.

(A) Levels of H3K9me2 (black) and Swi6p (grey) were assayed via ChIP and are shown relative to levels at *act1*$^{+}$, such that the dotted line indicates no enrichment. The enrichment data are plotted versus the *ura4* genomic region; grey arrows represent genes, each given a letter identifier, and the direction of the arrowhead indicates the direction of transcription. Non-coding RNAs are shown as asterisks. The disrupted *ura4* gene is shown as a broken arrow surrounding L5 and *ade6*$^{+}$. Tick marks are spaced every 1kb. The *de novo* heterochromatin domain is defined as regions that are greater than 2-fold enriched in H3K9me2. Error bars represent the standard error of the mean. **(B)** *ade6*$^{+}$ expression is reduced in strains containing L5. Shown is the phenotypic assessment of *ade6*+ expression using a serial dilution assay. Each row represents an individual strain plated on adenine limiting media. *ade6*$^{+}$ and *ade6*$^{-}$ strains demonstrate the phenotypic effects of *ade6* expression and are compared to a representative *ura4::ade6*$^{+}$ control strain and three independent *ura4*::L5-*ade6*$^{+}$ strains. **(C)** H3K9me2 and Swi6p enrichment are plotted in dark and light grey, respectively, at the *spbc2f12.03* locus. Genes in this genomic location are labeled numerically and are represented by open arrows. The disrupted *spcbc2f12.03* gene is represented by a broken arrow.

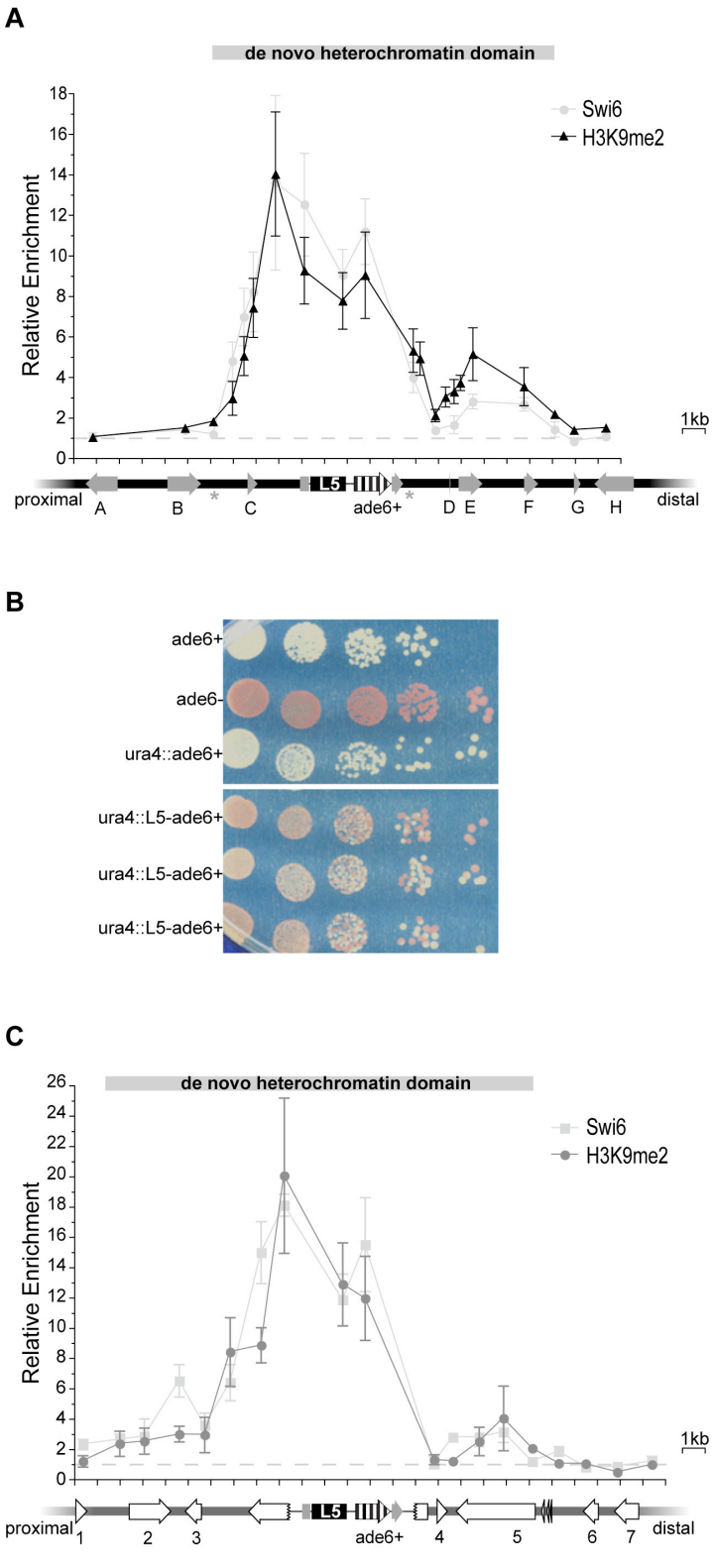
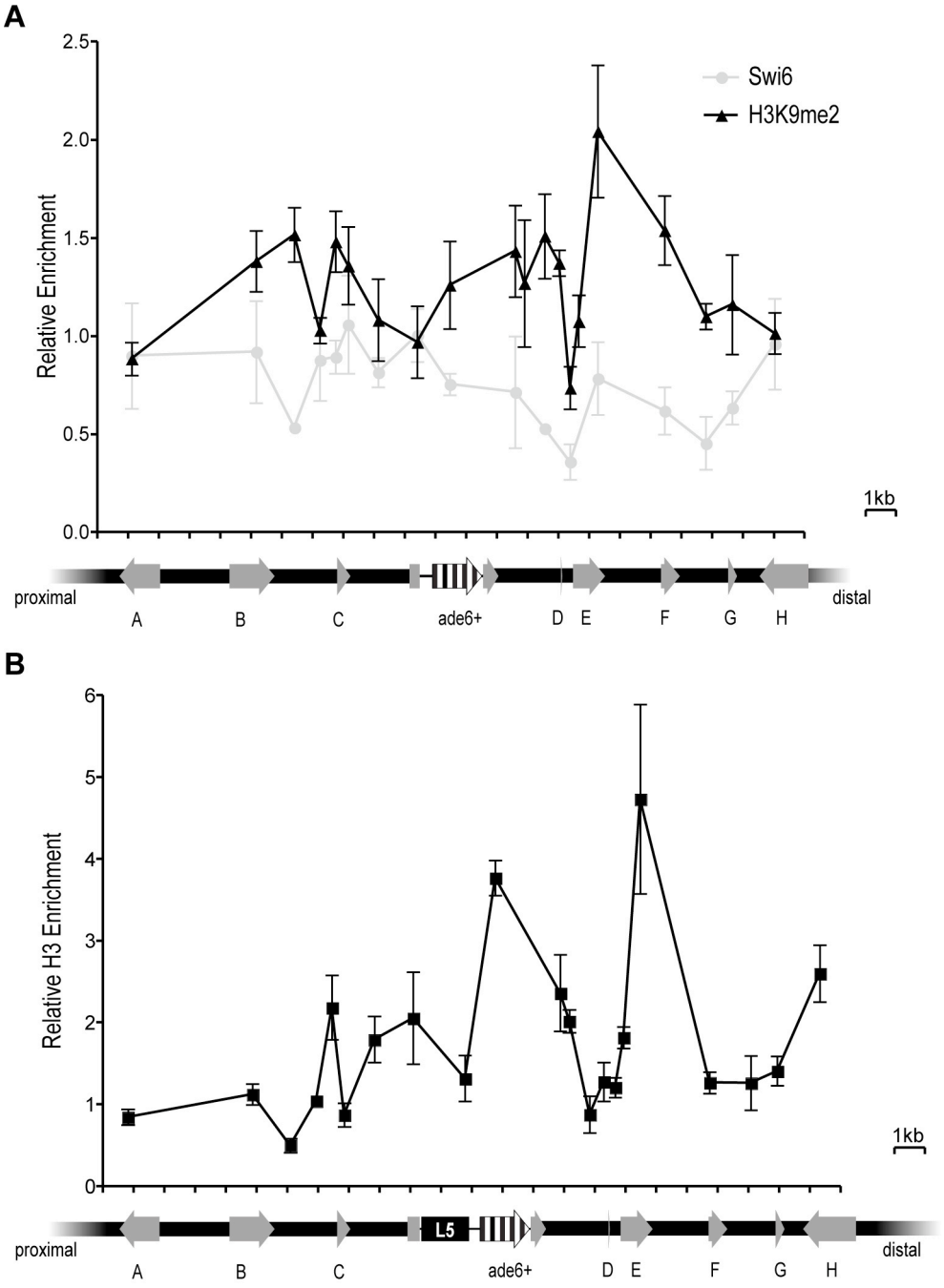

A
de novo heterochromatin domain
Swi6
H3K9me2
Relative Enrichment
1kb
proximal
A
B
C
L5
ade6+
D
E
F
G
H
distal
B
ade6+
ade6-
ura4::ade6+
ura4::L5-ade6+
ura4::L5-ade6+
ura4::L5-ade6+
C
de novo heterochromatin domain
Swi6
H3K9me2
Relative Enrichment
1kb
proximal
1
2
3
L5
ade6+
4
5
6
7
distal

Figure 6: The *ura4* genomic region is not enriched in H3K9me2 in the absence of L5

(A) The *ura4* locus is depicted, and assayed for the presence of H3K9me2 (black) and Swi6p (grey), in the absence of the L5 element. **(B)** Nucleosome occupancy was characterized using an antibody to the c-terminus of histone H3. The data are expressed relative to the nucleosome occupancy at the *act1*$^{+}$ locus.


A
Swi6
H3K9me2
Relative Enrichment
2.5
2.0
1.5
1.0
0.5
0.0
1kb
proximal
distal
A
B
C
ade6+
D
E
F
G
H
B
Relative H3 Enrichment
6
5
4
3
2
1
0
1kb
L5
proximal
distal
A
B
C
ade6+
D
E
F
G
H

within heterochromatin domains (Figure 5A and Figure 6A) (Cam et al., 2005). These data demonstrate that heterochromatin assembly is not limited to the L5-element and the reporter gene; instead, heterochromatin spreads bi-directionally into adjacent, formerly euchromatic, sequences, resulting in a *de novo* heterochromatin domain that spans approximately 15kb. Throughout, we will describe the properties of a heterochromatin domain by its extent, the distance over which heterochromatin is enriched, and its magnitude, the level of heterochromatin enrichment at a given location.

The presence of heterochromatin causes reduced expression within the *de novo* domain

Because the chromatin state of genes near *ura4* changes upon insertion of L5, we sought to determine whether gene expression at the ectopic locus was also altered. Quantitative RT- PCR (qRT-PCR) was used to quantify the levels of mRNA in the presence of L5 relative to control strains lacking L5. As expected from earlier studies (Partridge et al., 2002), we observed an L5-dependent decrease in *ade6*$^{+}$ expression; however, the reduction in expression was moderate (34±5%), indicating that silencing is incomplete in these strains (Table 1). In addition to *ade6*$^{+}$, two genes within the *de novo* heterochromatin domain, located 2.7kb proximal and 4.9kb distal from the L5 element, also exhibited a decrease in expression in the presence of L5, 43±10% and 52±8%, respectively. Gene expression outside of the *de novo* heterochromatin domain was also analyzed (Figure 5A). As predicted, three genes (A, B, H) had no significant difference in transcript abundance in the presence of L5 (Table 3). The remaining gene, G, as well as gene F that lies within the *de novo* domain, exhibit a discordant relationship between the enrichment of heterochromatin marks and the level of gene expression. Together, these results suggest that gene-specific features may have a greater influence on the level of

Table 3: Relative gene expression in the *ura4* and *spbc2f12.03* loci

Gene[1]	Gene name	Distance from L5 (bp)[2]	Relative mRNA (+L5/-L5)	N[3]	p[4]
A	alg11[5]	8393	1.02 ± 0.11	8	0.853
B	CC330.07	6245	1.21 ± 0.10	11	0.101
C	spcc330.06	2723	0.57 ± 0.10	14	**0.002**
	ade6	340	0.67 ± 0.05	13	**0**
D	tDNAgly	4535			
E	mug135	4943	0.48 ± 0.08	8	**0.001**
F	CC330.03	7819	0.90 ± 0.20	17	0.628
G	CC330.19	9984	0.66 ± 0.03	8	**0.008**
H	rhp7	10902	1.08 ± 0.15	4	0.731
1	mlo3[6]	10609	0.83 ± 0.09	5	0.172
2	byr2	8193	0.67 ± 0.15	6	0.284
3	mrpl7	4875	0.81 ± 0.15	6	0.289
	ade6	340	0.48 ± 0.10	5	**0.001**
4	rpl1701	4028	0.58 ± 0.08	5	**0.007**
5	BC2f12.05	9177	0.62 ± 0.18	6	0.087
6	rpl802	11334	1.03 ± 0.08	5	0.752
7	ceg1[7]	13164	1.08 ± 0.11	7	0.335

[1]From Figure 5A and 5C
[2]Distance of the translation start site from the nearest edge of L5
[3]Number of independent RNA isolations included in analysis
[4]p-value resulting from comparison between *ura4*::*ade6*$^+$ and *ura4*::L5-*ade6*$^+$ strains
[5]Essential for viability (Umeda et al., 2000)
[6]Mutation in mlo3 results in a growth defect (Thakurta et al., 2005)
[7]Essential for viability (Pei et al., 2001)

gene repression, as compared to the centromere, where gene repression is more complete (Allshire et al., 1995).

To further explore the extent of *ade6*$^{+}$silencing, we utilized a phenotypic assay for *ade6*$^{+}$expression. This assay allows the extent of silencing to be resolved on a sub-colony level, as opposed to the population level queried by qRT-PCR. Under conditions of limiting adenine, yeast that are mutant, or silenced (Allshire et al., 1994), for *ade6*$^{+}$ accumulate a metabolic intermediate that results in red pigmentation. In contrast, cells in which *ade6*$^{+}$is expressed at wild type levels remain white. Results from the phenotypic assay indicate that there is significant heterogeneity among colonies in the *ura4*::L5-*ade6*$^{+}$ strains (Figure 5B). Similar to classic PEV, the colony phenotypes ranged from white to red (Muller, 1930). However, distinct from PEV in *Drosophila*, we also observed intermediate phenotypes of pink and red with white sectors, consistent with PEV as observed in yeast (Allshire et al., 1994; Ayoub et al., 1999; Ayoub et al., 2000).

de novo heterochromatin domains are sensitive to genomic location

We next wanted to determine whether the magnitude and extent of a *de novo* heterochromatin domain depends upon its location in the *S. pombe* genome or whether domain properties are intrinsic to the L5 element itself. To explore this, we identified a second integrant of the *ura4*::L5-*ade6*$^{+}$construct on chromosome 2 (*spbc2f12.03*::*ura4*::L5-*ade6*$^{+}$). Comparison of H3K9me2 and Swi6p enrichment at L5 and *ade6*$^{+}$ between the two sites of integration reveals similar patterns of enrichment, suggesting that the nucleation of heterochromatin and local spreading are not sensitive to the changes in genomic location from *ura4* to *spbc2f12.03* (Figure 5C).

We next compared the magnitude and extent of the *de novo* heterochromatin domains formed at these two genomic locations. Distal to L5, the patterns of heterochromatin enrichment are markedly similar between the *ura4* and *spbc2f12.03* loci. In contrast, heterochromatin is observed 9kb proximal to L5 at the *spbc2f12.03* locus as compared to only 4.9kb at the *ura4* locus (compare Figures 5A and 5C). While this expansion at the *spbc2f12.03* locus is only modestly enriched in H3K9me2, it is also marked by the presence of Swi6p (Figure 5C), suggesting there is a 4.1kb expansion of the heterochromatin domain relative to the *ura4* locus. Thus, the proximal boundary of the *de novo* heterochromatin domain at the *ura4* locus is influenced by genomic location as opposed to reflecting an intrinsic limitation of the L5-element.

To determine whether the *de novo* heterochromatin domain at *spbc2f12.03* alters gene expression, qRT-PCR was used to analyze mRNA levels at the *spbc2f12.03* ectopic locus. Repression is observed at *ade6*$^{+}$ and the nearby *rpl1701*$^{+}$ gene, but not genes 2, 3 and 5 (Figure 5C, gene 4; Table 3). Thus, analogous to gene F at the *ura4* locus, the recruitment of heterochromatic marks to an ectopic locus is not always associated with significant gene silencing.

Cis-acting sequences shape the *de novo* heterochromatin domain

The experiments described above demonstrate that the extent of an L5-dependent *de novo* heterochromatin domain can vary between different locations in the genome. To explore whether these differences are attributable to *cis*-acting factors, we engineered constructs in which different DNA sequences were placed adjacent to L5. These constructs were then inserted

at the *ura4* locus to examine the role of sequence in defining the heterochromatin domain, without altering its location in the genome.

First, 5kb of *S. pombe* DNA taken from a region between two divergently transcribed genes (*spcc320.02*$^{+}$ and *spcc320.03*$^{+}$) was positioned between L5 and *ade6*$^{+}$. This region was selected because it is one of the larger regions in the *S. pombe* genome in which known protein-coding genes are absent and because it normally lacks heterochromatic modifications (Cam et al., 2005). Having established that this region maintains the absence of H3K9me2 when moved to the *ura4* locus in strains lacking L5 (Figure 7), H3K9me2 levels were queried over this DNA in the presence of the L5 element. Heterochromatin was robustly enriched over the 5kb insert DNA (Figure 8A). Strikingly, the magnitude of H3K9me2 enrichment is comparable to the level observed at *S. pombe* centromeres (Figure 8A), suggesting that H3K9me2 can reach and sustain high levels of occupancy over the entire region. This is in contrast to the pattern of heterochromatin spreading over the gene-rich *ura4* and *spbc2f12.03* neighborhoods (Figure 5A and 5C). The difference between the magnitudes of heterochromatin enrichment between these DNA sequences supports the role of *cis*-acting DNA sequences, potentially the genes themselves, in shaping the characteristics of heterochromatin domains.

Consistent with this hypothesis, there is a significant reduction in H3K9me2 enrichment coincident with the start of the *ade6*$^{+}$ gene, and the level of enrichment at this location is similar to the level observed when *ade6*$^{+}$ is adjacent to L5 (compare Figure 5A and Figure 8A). One interpretation of these data is that only low levels of heterochromatin can exist in transcriptionally active regions. Thus, when heterochromatin spreads from the spacer DNA into the *ade6*$^{+}$ gene, the *ade6*$^{+}$ gene behaves as a heterochromatin attenuator. Alternatively, this

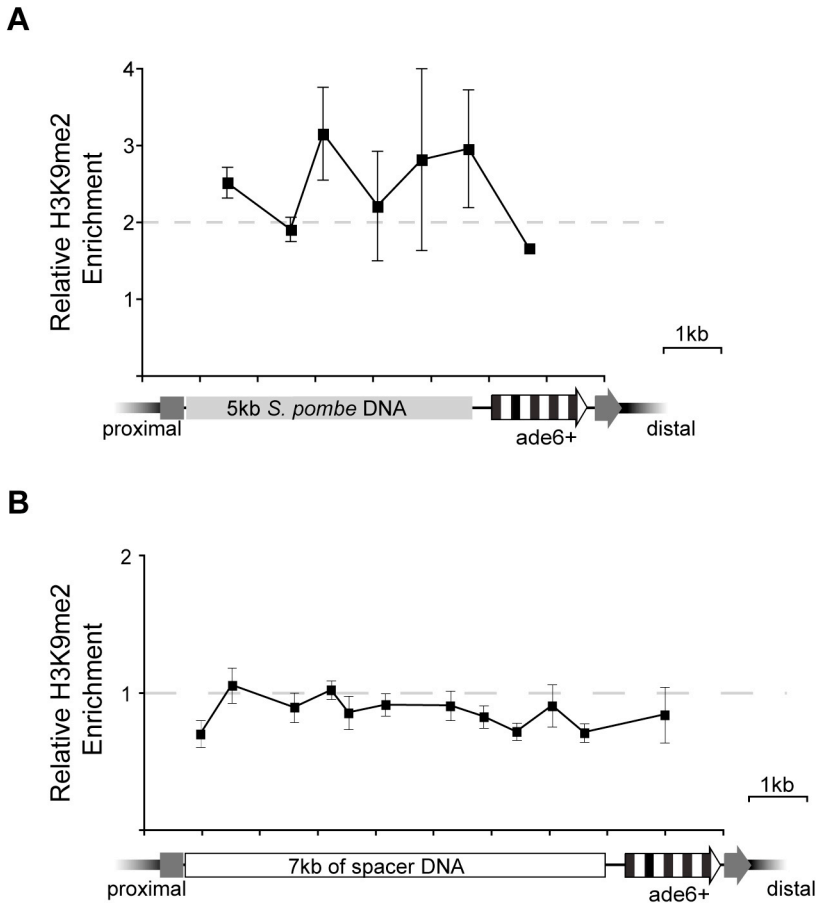


Figure 7: *S. pombe* intergenic and lambda spacer fragments do not recruit H3K9me2 in the absence of L5

H3K9me2 enrichment over *S. pombe* intergenic (A) and lambda (B) sequences in the absence of L5. The intergenic spacer DNA is in duplicate copies in the genome (at the ura4 locus as well as its endogenous locus) thus no enrichment is represented by a value of 2.

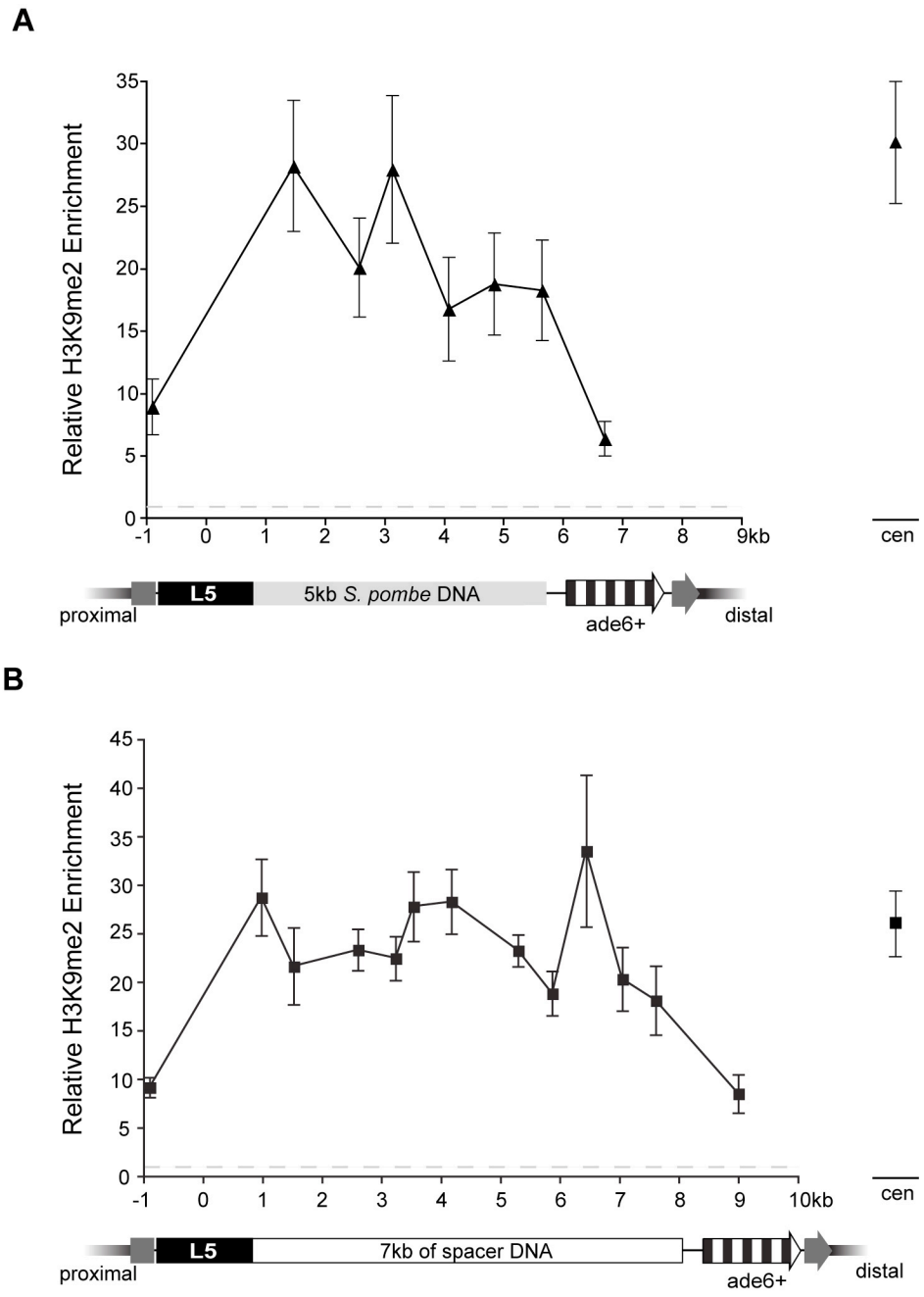


Figure 8: H3K9me2 is highly enriched over intergenic and spacer DNA fragments

H3K9me2 enrichment is plotted versus *S. pombe* intergenic DNA as shown in light grey **(A)** and DNA from the phage lambda as shown in white **(B)**. The dark grey arrow represents the disrupted *ura4* gene. The data points on the far right represent the level of H3K9me2 enrichment observed at the pericentromeric repeats in the respective strains, as internal controls.

may indicate that the extent of spreading is constrained such that heterochromatin cannot spread, with high levels of enrichment, farther than 5.6kb from L5.

To address this latter possibility, a longer spacer sequence was selected and inserted between L5 and *ade6*$^{+}$. Sequences from lambda phage were chosen, as they have been used in previous epigenetic studies as spacer DNA (Chung et al., 1997). No significant enrichment was observed over the length of the 7kb insert in the absence of L5, suggesting that these sequences do not nucleate heterochromatin on their own (Figure 7B). In contrast, when the L5 element is present, robust enrichment in H3K9me2 was observed over the length of the lambda fragment, at levels similar to that of the centromeres and the 5kb *S. pombe* spacer fragment (Figure 8B). Moreover, when we extended our analysis to include the levels of H3K9me2 enrichment at endogenous sequences in *ura4*::L5-7kb-*ade6*$^{+}$ strains we found that it was remarkably similar to the levels observed in strains lacking spacer DNA (Figure 9). Thus, the addition of spacer DNA (up to 7 kb) does not constrain the extent of a *de novo* heterochromatin domain. Instead, our data are consistent with a model in which both the extent and magnitude of a heterochromatin domain are dictated by features of the underlying DNA sequence.

The boundaries of *de novo* heterochromatin domains are marked by the presence of highly transcribed genes

Because endogenous sequences can influence the extent of a heterochromatin domain, we next sought to determine the factors that mediate the interaction of DNA sequence and chromatin. Based on the observation that some barriers require formation of a transcription

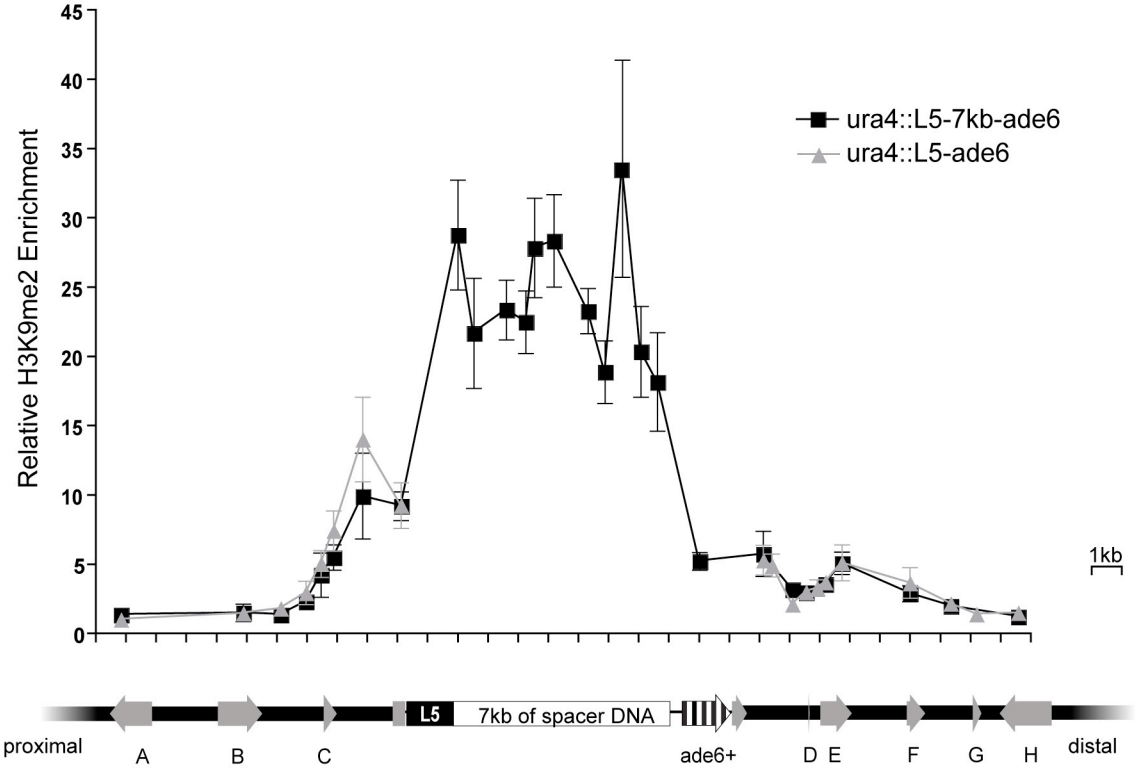


Figure 9: Heterochromatin spreading in the *ura4* locus is unaffected by the presence of lambda spacer DNA

Analysis of H3K9me2 in the ura4 locus in *ura4*::L5-7kb-*ade6*$^{+}$ strains (black) and *ura4*::L5-*ade6*$^{+}$ strains. To facilitate comparison between the two strains H3K9me2 enrichment for the *ura4*::L5-*ade6*$^{+}$ is depicted with a gap over the lambda insert.

complex (Donze and Kamakaka, 2001; Lunyak et al., 2007; Noma et al., 2006; Scott et al., 2006; Scott et al., 2007), we investigated the relationship between domain size and transcriptional activity.

The level of transcriptional activity within both the *ura4* and the *spbc2f12.03* regions could be assessed using previously reported data sets (Lackner et al., 2007; Noma et al., 2006). Transcriptional activity was inferred from both the steady state levels of mRNA and the level of RNA Polymerase II (Pol II) and RNA Polymerase III (Pol III) enrichment at the promoter. Between the two regions, there were five loci that were transcriptionally exceptional: one gene that was transcribed by Pol III and four genes with unusually high levels of Pol II transcriptional activity (Table 4) (Lackner et al., 2007; Noma et al., 2006).

The *ura4* genomic neighborhood includes a Pol III-transcribed $tDNA^{Gly}$ (gene D in Figure 5A), which is coincident with an H3K9me2 gap. This gap could be attributed to general nucleosome depletion or, alternatively, to nucleosomes shielded from H3K9me2 modification by the Pol III transcription complex. Supporting the former hypothesis, tDNA genes are generally depleted of nucleosomes when compared to the genome average (Parnell et al., 2008). To distinguish between these two possibilities, an antibody to the C-terminus of histone H3 was used to characterize nucleosome occupancy surrounding to the $tDNA^{Gly}$ gene. Indeed, the level of H3 enrichment at the $tDNA^{Gly}$ was reduced relative to sequences in the surrounding neighborhood (Figure 6B), indicating that the observed H3K9me2 gap is due to decreased nucleosome occupancy surrounding the $tDNA^{Gly}$.

The *ura4* genomic neighborhood includes the gene *spcc330.06*$^{+}$(gene C in Figure 5A), which is highly expressed and enriched in Pol II (at the 94th percentile genome-wide) at its promoter (Table 4) (Lackner et al., 2007). This gene is located within a striking transition in

Table 4: Transcription levels at the wild type *ura4*$^{+}$ and *spbc2f12.03*$^{+}$ loci

Gene	Steady state mRNA levels[1]	RNA Pol II Enrichment[1]
A	1769	1.14
B	761	0.91
C	10588	3.41
E	618	0.76
F	377	
G	296	0.64
H	942	0.88
1	8746	3.28
2	347	0.64
3	1885	0.88
	1845	1.47
4	9130	3.81
5	2220	0.88
6	9810	4.75
7	364	0.80
ade6+	5472	0.91

[1]From (Lackner et al., 2007)

H3K9me2 enrichment from 14-fold to <2-fold enrichment over a distance of only 2.7kb (Figure 5A). In contrast to the nucleosome gap discussed above, this transition marks a boundary of heterochromatin enrichment and cannot be explained by reduced nucleosome occupancy (Figure 6B). We hypothesize that this gene may behave as a heterochromatin barrier and, more broadly, that highly expressed genes in general may be effective heterochromatin barriers.

Within the *spbc2f12.03* genomic neighborhood, three genes are highly transcribed (genes 1,4 and 6 in Figure 5C). One of these genes (gene 6) is distal to the boundary of the *de novo* heterochromatin domain, and as such is uninformative. However, genes 1 and 4 (Figure 5B) are located at boundaries of the *de novo* heterochromatin domain, consistent with the hypothesis that highly expressed genes weaken and/or stop the spread of *de novo* heterochromatin.

Introduction of a gene within spacer DNA attenuates heterochromatin spreading independent of level of transcription

To directly test whether the presence of transcribed genes can influence the extent of a *de novo* heterochromatin domain, we constructed a chimeric reporter gene composed of the strong, repressible, *nmt1*$^{+}$ promoter driving expression of the *his3*$^{+}$ open reading frame (Pnmt1-*his3*$^{+}$) (Maundrell, 1990). This construct was then inserted within the 7kb spacer fragment, and heterochromatin spreading was monitored over the spacer sequences and the inserted gene. As expected, H3K9me2 was highly enriched over the spacer DNA proximal to Pnmt1-*his3*$^{+}$, consistent with the levels observed in uninterrupted spacer strains (Figure 10). However, the magnitude of H3K9me2 enrichment decreases over the Pnmt1-*his3*$^{+}$sequences and remains reduced over the distal portion of the spacer DNA (Figure 10). These data demonstrate that the

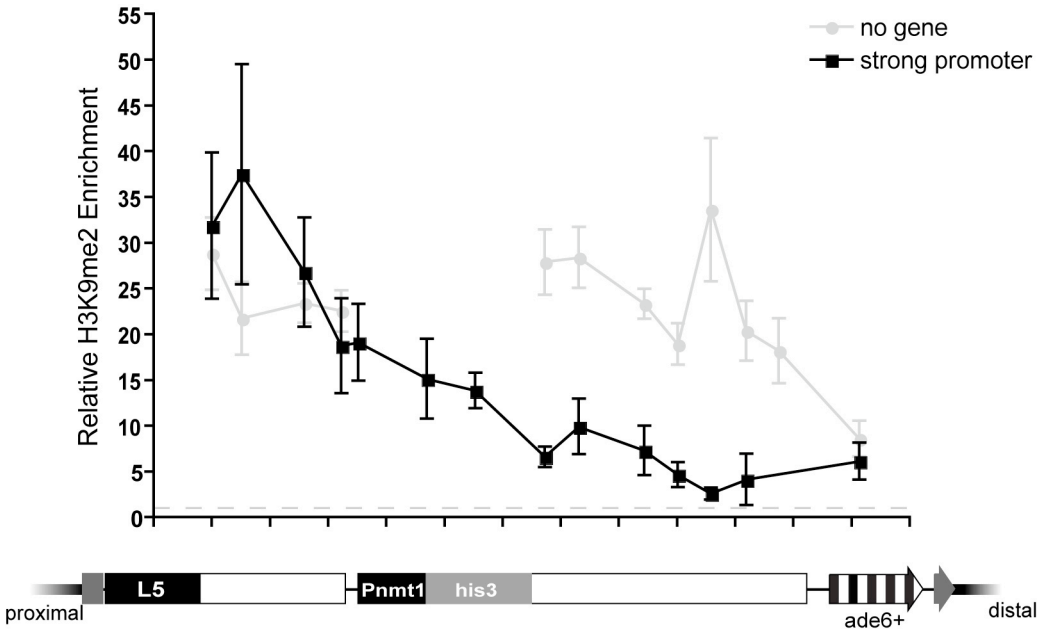


Figure 10: The presence of Pnmt1-*his3* attenuates heterochromatin spreading

H3K9me2 enrichment in is shown for strains in which Pnmt1-*his3*$^{+}$ has been inserted within lambda (black) or containing uninterrupted spacer DNA (grey).

insertion of genic sequences within the spacer DNA attenuates the spread of heterochromatin and further support the hypothesis that the presence of genes within the *ura4* and *spbc2f12.03* neighborhoods limits heterochromatin spreading. It is interesting, however, that the Pnmt1-*his3*$^{+}$ construct, despite being more highly transcribed than *spcc330.06*$^{+}$ (Figure 11A), does not exhibit complete barrier activity (Figure 10), suggesting that factors other than high levels of transcriptional activity are required for complete barrier activity.

Because the presence of genes antagonizes heterochromatin spreading, we sought to determine whether a high level of transcriptional activity is required for attenuator activity. To test this we took advantage of an engineered allele of the *nmt1*$^{+}$ promoter that results in reduced transcription efficiency (Basi et al., 1993), and cultured these strains in medium containing thiamine, which results in further repression of the *nmt1*$^{+}$ promoter (Maundrell, 1990). Despite a ~570 fold decrease in expression the weakened Pnmt1-*his3*$^{+}$ gene still exhibited significant attenuation ability, indistinguishable from the strongest allele (Figure 11B). Thus, other features of the *nmt1*$^{+}$ promoter may serve to attenuate the spread of heterochromatin. Indeed, the region of the promoter that is required for thiamine repression binds a protein complex independent of thiamine conditions (Zurlinden and Schweingruber, 1997). This protein complex, or other complexes that localize to the promoter independent of thiamine and transcription efficiency, may serve to attenuate the spread of heterochromatin.

Increased L5-copy number does not alter the heterochromatin domain

Having demonstrated the impact of genome sequence on the extent of spreading from a heterochromatin-nucleating sequence, we wanted to determine whether changes to the

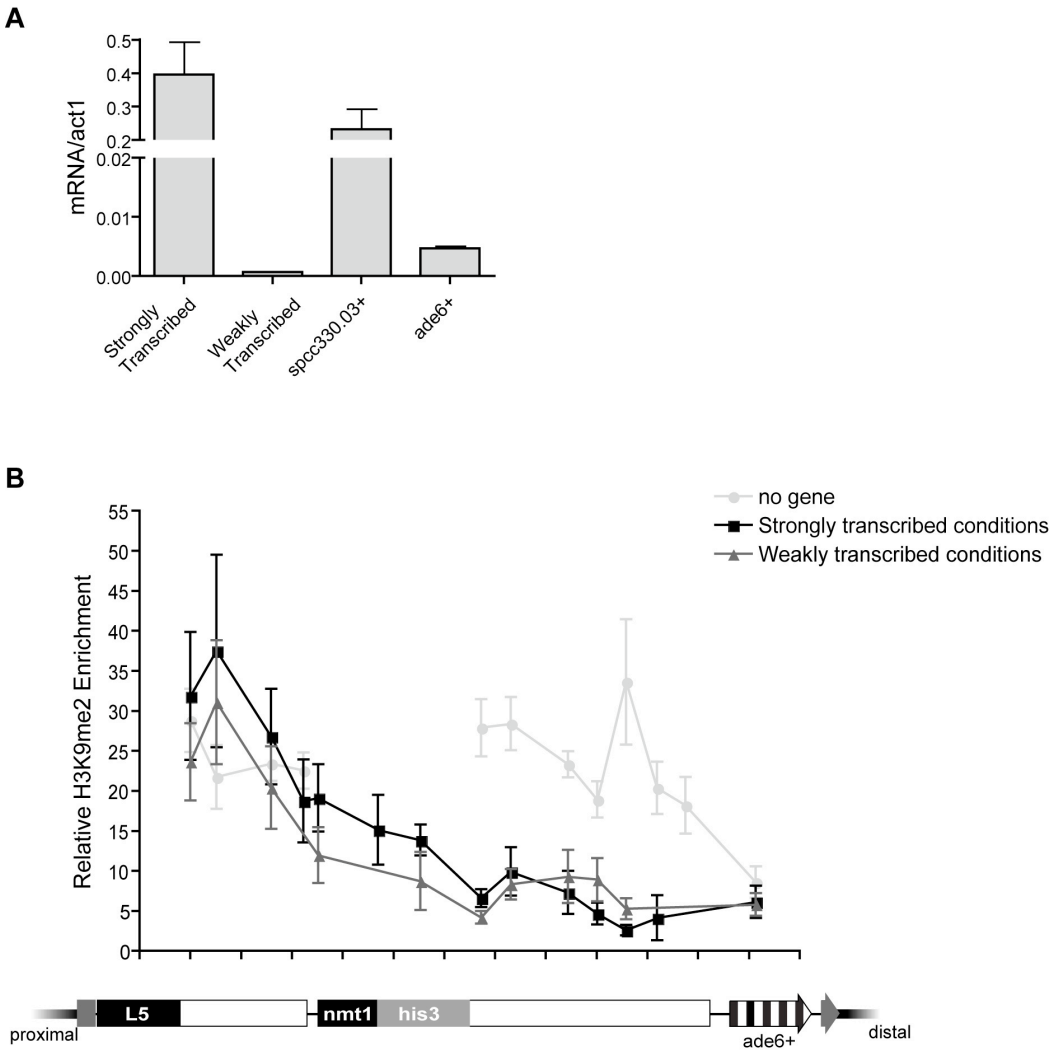


Figure 11: Insertion of a gene within lambda attenuates heterochromatin independent of the level of transcription

(A) Levels of steady state mRNA relative to *act1*$^{+}$. *his3*$^{+}$ mRNA was isolated from highly transcribed (strong *nmt1* allele, no thiamine) and weakly transcribed conditions (weak *nmt1* allele, thiamine) in *swi6- ura4*::L5-7kb::(Pnmt1-*his3*$^{+}$)-*ade6*$^{+}$ strains. For comparison the level of *ade6*$^{+}$ and *spcc330.03*$^{+}$ mRNA are shown for *ura4*::*ade6*$^{+}$ strains. **(B)** Relative H3K9me2 enrichment for strongly and weakly transcribed conditions.

sequence content, in terms of L5 copy number, would alter the properties of a *de novo* heterochromatin domain. Thus, two copies of L5 were inserted in tandem at the *ura4* locus. The magnitude and extent of heterochromatin enrichment in these strains was markedly similar to strains bearing one copy of L5 (Figure 12A), suggesting that the copy number of L5 does not notably enhance heterochromatin enrichment or spreading within a *de novo* domain.

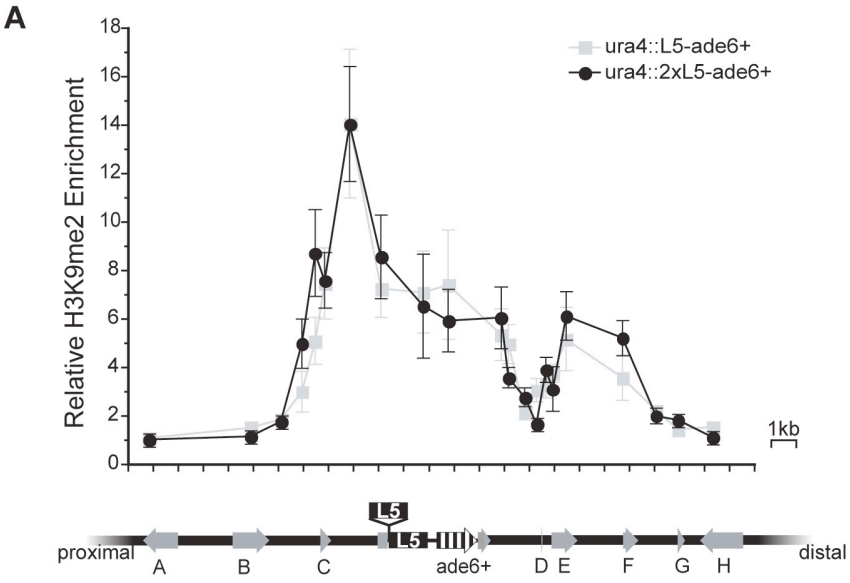
The boundaries of the *de novo* heterochromatin domain are insensitive to increased dosage of *swi6*$^{+}$

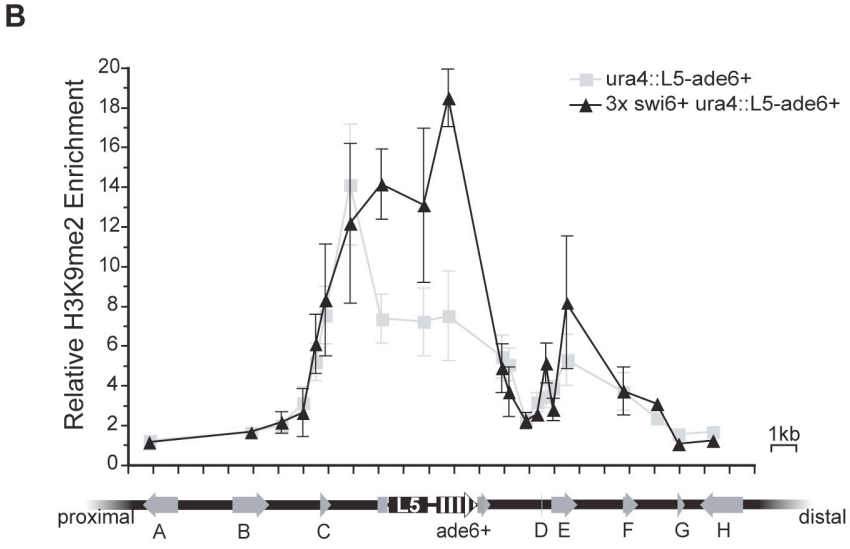
We also wanted to address the possible role of *trans*-acting factors in regulating the extent of the heterochromatin domain, either by competition with other heterochromatic regions for limiting heterochromatin components (Eissenberg et al., 1992) or by competition between heterochromatic and euchromatic factors for the same nucleosome substrate (Ebert et al., 2004). We hypothesized that increasing the dosage of heterochromatin proteins (or reducing the amount of competing factors) should result in the expansion of a heterochromatin domain (Ebert et al., 2004; Eissenberg et al., 1992; Kimura et al., 2002; Locke et al., 1988; Zhang et al., 2006b).

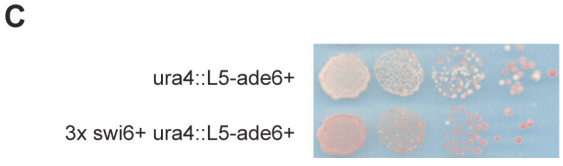
Swi6p is a dosage-dependent modifier of heterochromatin levels at the *S. pombe* mating-type locus as well as a limiting factor in heterochromatin formation (Hall et al., 2002a; Nakayama et al., 2000). Thus, we analyzed the magnitude and extent of the *ura4 de novo* heterochromatin domain in strains bearing three copies of *swi6*$^{+}$ (Hall et al., 2002a). We confirmed that the level of *swi6*$^{+}$ mRNA is increased by 2.7-fold in these strains (data not shown). While the local magnitude of H3K9me2 proximal to L5 was increased in these strains (Figure 12B), the increased dosage of *swi6*$^{+}$ did not result in the expansion of the

Figure 12: The *de novo* heterochromatin domain is shaped by the dosage of trans-acting factors

(A) H3K9me2 enrichment in the presence of an additional copy of L5 is shown in black circles. The extra copy of L5 is shown above the *ura4* genomic region, with insertion site indicated, compared to strains bearing only one copy of L5 (light grey squares). **(B)** H3K9me2 enrichment in strains bearing extra copies of the *swi6*$^{+}$ gene, shown in black triangles. **(C)** Serial dilution analysis of *ade6*$^{+}$ expression in wild type and 3x *swi6*$^{+}$ strains


A
Relative H3K9me2 Enrichment
ura4::L5-ade6+
ura4::2xL5-ade6+
1kb
L5
L5
proximal
A
B
C
ade6+
D
E
F
G
H
distal


B
Relative H3K9me2 Enrichment
ura4::L5-ade6+
3x swi6+ ura4::L5-ade6+
1kb
L5
proximal
A
B
C
ade6+
D
E
F
G
H
distal


C
ura4::L5-ade6+
3x swi6+ ura4::L5-ade6+

heterochromatin domain. Consistent with the increased level of H3K9me2 enrichment, *ade6*$^{+}$ expression was further reduced in these strains, resulting in an increased proportion of red colonies (Figure 12C and Figure 13). In contrast, increased *swi6*$^{+}$ dosage did not significantly affect expression of other genes within the *ura4*$^{+}$ neighborhood (Figure 13). This suggests that, while the level of Swi6p influences both the local concentration of H3K9me2 and the level of gene expression, the extent of the *de novo* heterochromatin domain is not sensitive to increased dosage of *swi6*$^{+}$.

Functional distinction between local heterochromatin formation and spreading over spacer DNA

In the absence of known transcribed elements, H3K9me2 spreads unattenuated over distances at least up to 7kb (Figure 8B), resulting in a consistent level of H3K9me2-enrichment at *ade6*$^{+}$ independent of the presence of spacer DNA (Figure 14C). What remains to be addressed is whether the level and the stability of gene silencing differ between strains containing spacer DNA versus *ura4*::L5-*ade6*$^{+}$.

To explore this question, we compared the levels of *ade6*$^{+}$expression by qRT-PCR and found that when *ade6*$^{+}$was located 7kb away from the L5 element, distal to the lambda spacer, silencing was no longer observed despite the presence of H3K9me2 (Figures 14A and 14C). This finding was confirmed using the phenotypic *ade6*$^{+}$ assay, which revealed a much lower level of silencing in *ura4*::L5-7kb-*ade6*$^{+}$ strains (Figure 14B). These data suggest that, even when the levels of H3K9me2-enrichment are similar (Figure 14C), heterochromatin formed proximal to L5 and heterochromatin formed over spacer DNA can have different effects on gene expression.

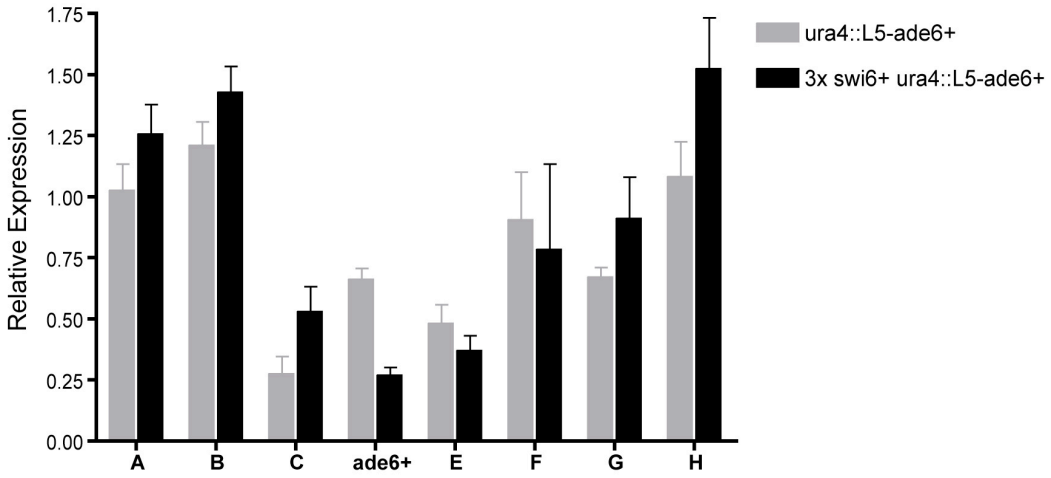


Figure 13: Increased *swi6*$^{+}$ copy number results in decreased *ade6*$^{+}$ expression but does not alter expression of other genes within the de novo heterochromatin domain

Steady state mRNA levels are depicted relative to *ura4*::*ade6*$^{+}$ strains for wild type strains (grey) and strains bearing 3 copies of *swi6*$^{+}$ (black).

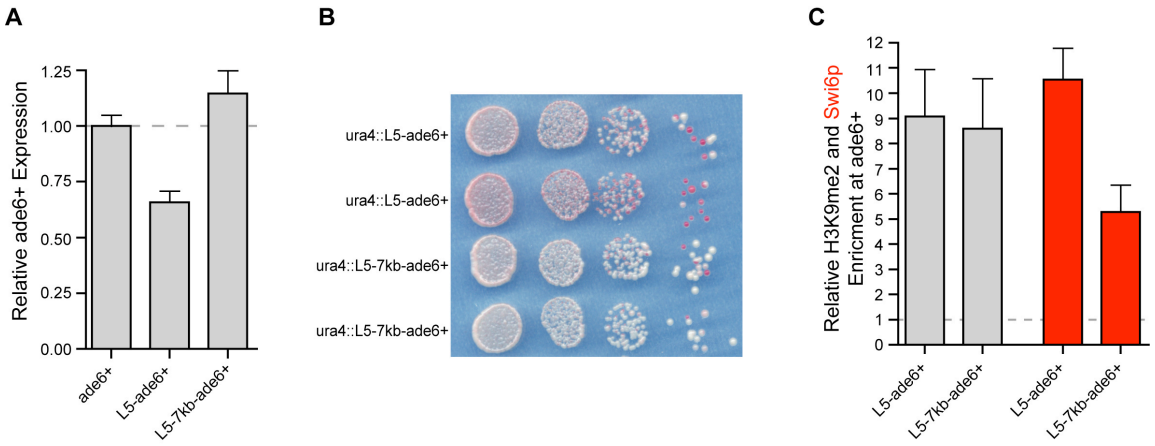


Figure 14: Local versus spreading over spacer DNA exerts different effects on *ade6*$^+$ expression

(A) Levels of *ade6*$^+$ expression in control (*ura4*::*ade6*$^+$) strains compared to both *ura4*::L5-*ade6*$^+$ and *ura4*::L5-7kb-*ade6*$^+$strains **(B)** Serial dilution assay comparing the extent of *ade6*$^+$ expression in both *ura4*::L5-*ade6*$^+$ and *ura4*::L5-7kb-*ade6*$^+$strains. **(C)** The level of H3K9me2 enrichment at *ade6*$^+$ in *ura4*::L5-*ade6*$^+$ and *ura4*::L5-7kb-*ade6*$^+$ strains is shown as gray bars. The level of Swi6p enrichment is plotted in red.

We suspected that the differences in silencing could be attributed to the levels of Swi6p at the *ade6*$^{+}$ gene in *ura4*::L5-*ade6*$^{+}$ as compared to *ura4*::L5-7kb-*ade6*$^{+}$ strains. To address this hypothesis, we assessed the level of Swi6p enrichment over the lambda spacer DNA and distal *ade6*$^{+}$ gene. We observed a significant decrease in Swi6p enrichment relative to the level of H3K9me2 across spacer and *ade6*$^{+}$ DNA when compared to *ura4*::L5-*ade6*$^{+}$ and *spbc2f12.03*::*ura4*::L5-*ade6*$^{+}$ strains, as well as to other heterochromatic loci (Figure 14C and Figure 15). This reduction is consistent with the decreased levels of silencing and could be a function of long distance spreading or a sequence-dependent affect of spacer DNA.

In addition to the total level of gene silencing, another manner in which the reduced levels of Swi6p in spacer strains could affect gene expression is by altering the stability of gene repression. When transgenes are placed within the centromere, or at locations throughout the mating type locus, their phenotypic stability (silenced or expressed) can vary with location and Swi6p dosage (Allshire et al., 1995; Ayoub et al., 1999; Hall et al., 2002a; Nakayama et al., 2000). To address whether the silenced and expressed states are stable to equivalent degrees in cases of local (high levels of Swi6p) versus spreading over spacer DNA (reduced levels of Swi6p) we chose colonies that were either silenced or expressed, as determined by their *ade6*$^{+}$ expression phenotype (i.e. entirely red or entirely white, respectively). The stability of the silenced state was determined by the proportion of progeny that exhibited silencing after a period of overnight growth. We examined the phenotypic stability of *ade6*$^{+}$ and *ade6*$^{-}$ controls, and as expected, the progeny maintained the appropriate phenotype (Figure 16A). However, when expressed colonies were chosen from *ura4*::L5-*ade6*$^{+}$ strains, only ~62% maintained the

completely expressed phenotype, while the remaining colonies switched to a partially or completely

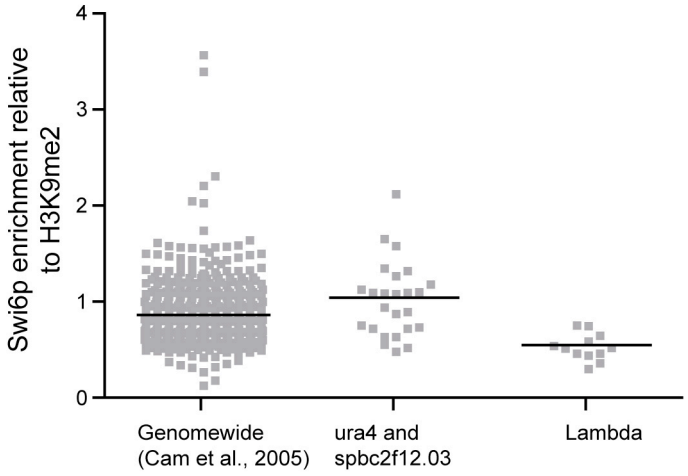
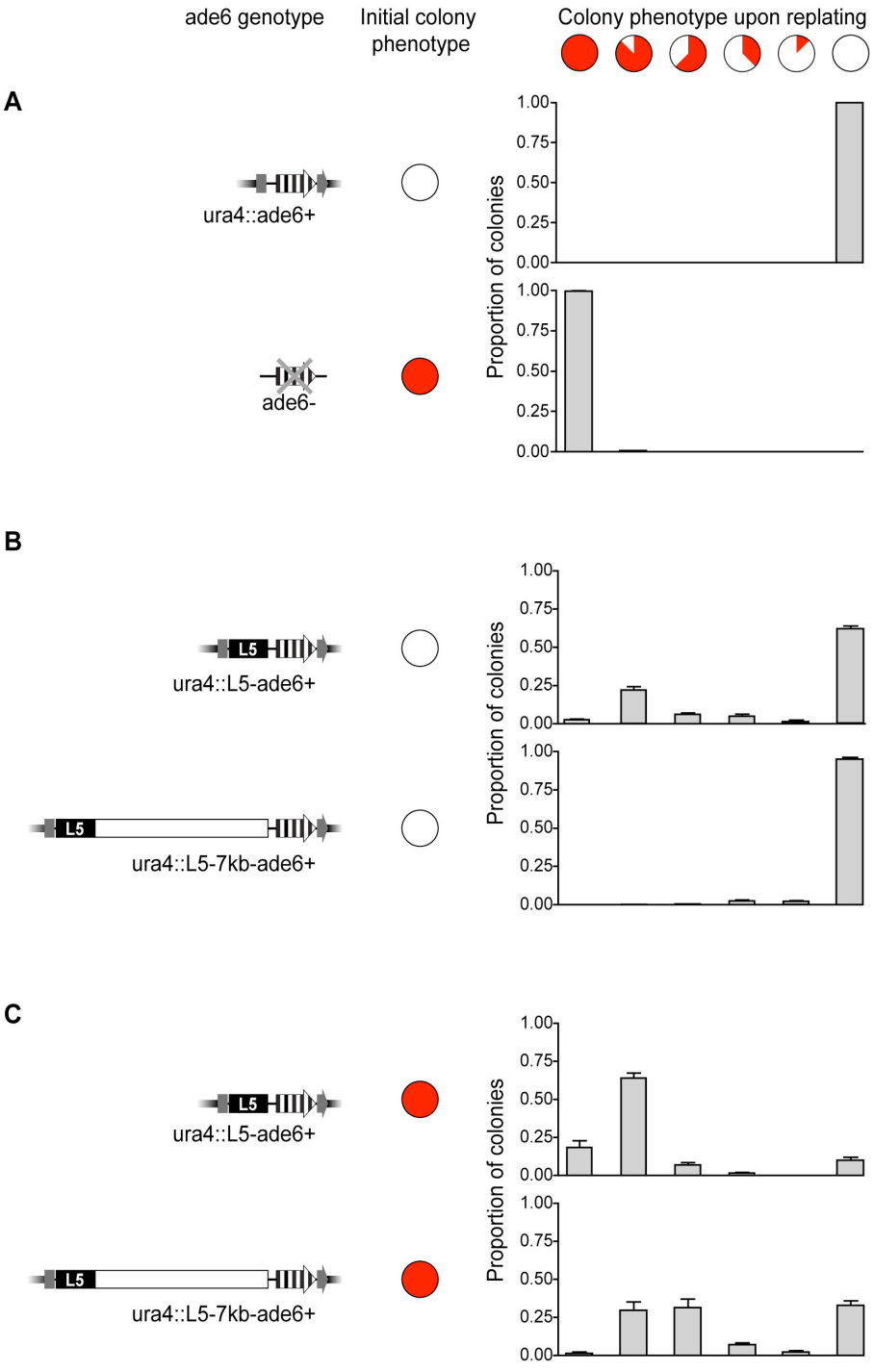


Figure 15: The ratio of Swi6p/H3K9me2 is reduced over spacer DNA

The ratio of Swi6p/H3K9me2 is reduced over spacer DNA. Scatter plot of the levels of Swi6p/H3K9me2 for heterochromatic regions genomewide (Cam et al., 2005), or within *de novo* heterochromatin domains.

Figure 16: The extent and stability of ade6+ silencing is altered in spacer strains

(A-C) Colonies were selected on the basis of the initial colony phenotype (either all entirely red or entirely white) for each of the given genotypes. After a 24-hour period of growth, strains were re-plated and the phenotypes of the resultant colonies were scored and classified based on the proportion of the colony that exhibited silencing, as indicated schematically above the graphs. Intermediate silencing phenotypes included sectoring and homogenous, intermediate levels of pigmentation. The graphs in this figure represent the proportion of colonies in each class based on genotype and initial colony color.


ade6 genotype
Initial colony phenotype
Colony phenotype upon replating
A
ura4::ade6+
ade6-
Proportion of colonies
1.00
0.75
0.50
0.25
0.00
B
L5
ura4::L5-ade6+
ura4::L5-7kb-ade6+
C
L5
ura4::L5-ade6+
ura4::L5-7kb-ade6+

silenced phenotype. This is in stark contrast to the *ura4*::L5-7kb-*ade6*$^{+}$ strains, in which 95% of the progeny maintained the expressed state (Figure 16B), suggesting that the establishment of silencing (i.e., switching from an *ade6*$^{+}$ expressed state to a silenced state) occurs less often when *ade6*$^{+}$ is distal to 7kb of spacer DNA and less enriched in Swi6p, despite comparable levels of the epigenetic mark H3K9me2.

When silenced colonies were selected from *ura4*::L5-*ade6*$^{+}$ strains, ~18% of the progeny exhibited phenotypes indicative of complete silencing and 90% exhibited at least partial silencing. In contrast, silenced colonies from *ura4*::L5-7kb-*ade6*$^{+}$ strains were less likely to give rise to progeny that exhibited complete or partial silencing (~1% and 69%, respectively), suggesting that maintenance of silencing is also less frequent when *ade6*$^{+}$ is separated from L5 by spacer DNA and reduced in Swi6p localization (Figure 16C). These data provide evidence that the level of Swi6 impacts the establishment and maintenance of silencing, despite consistent levels of H3K9me2.

2.4 Discussion

Ectopic gene silencing and/or heterochromatin formation has previously been studied in mammalian systems (Ayyanathan et al., 2003; Brink et al., 2006; Snowden et al., 2002; Verschure et al., 2005). Ectopic X inactivation, for example, has been shown to affect gene expression on a large scale (White et al., 1998). Typically, however, the complex nature of the mammalian genome restricts the focus of these studies to local heterochromatin formation and single gene repression. In this study, the compact nature of the *S. pombe* genome and our ability to robustly query for the presence of heterochromatin allowed us to rigorously test the

response of multiple DNA sequences to encroaching heterochromatin. Our data demonstrate a clear effect of genomic sequence in shaping both the extent and magnitude of a heterochromatin domain and demonstrate that, while the eukaryotic genome is permissive to the negative transcriptional effects of heterochromatin, euchromatic sequences can counteract encroaching heterochromatin.

de novo heterochromatin domains are shaped by DNA sequences that vary in their ability to promote or antagonize heterochromatin spreading

The relationship between the size of a heterochromatin domain and the presence of specific heterochromatin barriers has been previously established in a number of eukaryotic organisms (Donze and Kamakaka, 2002). Our study extends this conclusion, demonstrating that DNA sequences exert a range of effects on heterochromatin domains. For example, the *ade6*$^{+}$ gene dampens heterochromatin enrichment independent of both genomic location and distance from L5, but is insufficient to completely stop heterochromatin spreading (Figures 5A and 5C, Figures 8A and 8B). In contrast, intergenic and spacer DNA sequences promote the assembly of robust H3K9me2. We propose that there is a spectrum of effects, ranging from antagonistic to cooperative, that genomic sequence can exert on heterochromatin (Figure 7). This model incorporates the complexity and context dependence of genomic sequence and its relationship to heterochromatin and is applicable to sequences in yeast, as seen here, or in more complex genomes, as will be discussed below.

For this model, we have subdivided the discrete extremes of DNA sequences noted previously (that is, heterochromatin-nucleating sequences and heterochromatin barriers) into subclasses that include attenuators, neutral elements and protosilencers/boosters. While this is

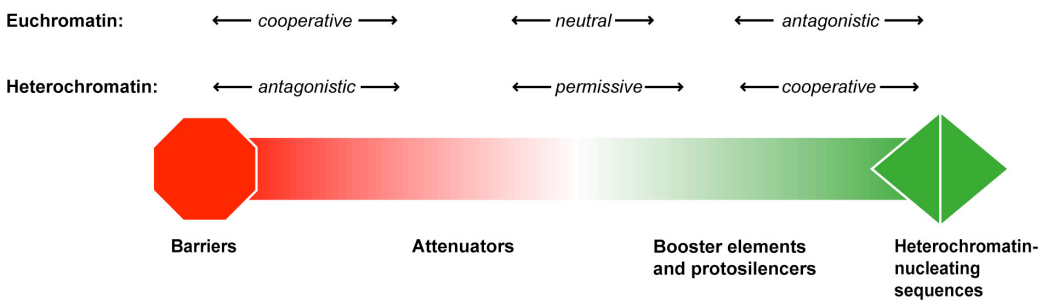


Figure 17: The continuum of DNA sequence, its effects on heterochromatin spreading, and the balance between opposing heterochromatic and euchromatic forces

The range of DNA sequences and the magnitude of their interaction with DNA is depicted along a gradient ranging from heterochromatin barriers (red) to heterochromatin nucleating sequences (green). The italic text above the figure describes the effect of a specific DNA sequence on both heterochromatin and euchromatin. Below the figure is a classification system that subdivides the continuum.

helpful for purposes of discussion, we do not wish to impose strict definitions, especially in light of data from this study, suggesting that particular sequences may be placed at multiple points along the continuum, depending on their context.

Heterochromatin antagonists: barriers and attenuators

Our data confirm that heterochromatin can spread from L5 in both directions over euchromatic DNA, resulting in a *de novo* heterochromatin domain encompassing multiple endogenous genes and altering gene expression (Figure 5A and 5C, Table 3). However, gene repression within the *de novo* domain is moderate, at most about 50%. The incomplete silencing observed within *de novo* heterochromatin domains, as well as the boundaries of these domains, may be a consequence of the factors present within euchromatic domains that antagonize the propagation of heterochromatin.

The boundaries of *de novo* heterochromatin domains are marked by three highly transcribed genes, implicating Pol II transcription in barrier activity (Figure 5A and 5C, Table 3) (Lackner et al., 2007). The *ade6*$^{+}$ gene is also transcribed and enriched in Pol II, albeit to a lesser extent than the three putative barriers. These four sequences may rely on transcription to counteract the spread of heterochromatin from the L5 heterochromatin-nucleating sequences. However, high levels of transcription are insufficient for complete barrier activity (Figure 10). Furthermore, we find that, in the case of Pnmt1-*his3*$^{+}$, the presence of genes can attenuate the spread of heterochromatin independent of the level of transcription (Figure 11B). We conclude that DNA sequences modify heterochromatin spreading through the sequence-dependent recruitment of other mediating factors, such as transcription complexes, and dictate whether a

sequence behaves as a true barrier or falls in the range of heterochromatin attenuators (Figure 17). These findings are consistent with previous results implicating transcription factors and promoters with barrier activity (Bi and Broach, 1999; Donze and Kamakaka, 2001; Ferrari et al., 2004; Fourel et al., 2001; Ohtsuki and Levine, 1998; Sekinger and Gross, 1999). Sequence could also influence heterochromatin directly, as is the case with some examples of nucleosome positioning (Segal et al., 2006) or could reflect selective pressure to maintain domain boundaries (Table 3).

In addition to protein-coding genes, the *ura4 de novo* heterochromatin domain includes a $tDNA^{Gly}$ gene (discussed below) and non-coding RNAs (Figure 5A). The *ura4* locus is not unique in its transcriptional makeup, as recent studies have provided insight into the vast amount of transcription occurring in the *S. pombe* genome outside of canonical protein coding genes (Dutrow et al., 2008; Wilhelm et al., 2008). Additionally, the *ura4* neighborhood also includes solo long terminal repeats (LTRs) (Cam et al., 2008). How these features interact with heterochromatin spreading, and whether they shape the formation of *de novo* heterochromatin domains warrants further genome-wide studies.

Transcription by Pol III complexes has an established relationship with barrier activity in yeast genomes (Donze and Kamakaka, 2001; Noma et al., 2006; Scott et al., 2006; Scott et al., 2007). A $tDNA^{Ala}$ within the *S. pombe* centromere 1 prevents the spread of heterochromatin into the abutting domain of centromeric chromatin. In contrast, the $tDNA^{Gly}$ gene is not coincident with the domain boundary of the ectopic heterochromatin domain formed at $ura4^{+}$; however it is deficient in H3K9me2 enrichment, due to the absence of a nucleosome(s) (Figure 5A and Figure 6B). Nucleosome depletion has been shown previously to restrict heterochromatin spreading (Bi et al., 2004); in this context, the nucleosome gap may weaken the spread of

heterochromatin, resulting in the gradual attenuation observed distal to the $tDNA^{Gly}$. We suggest that, like the *ade6*$^{+}$ gene, the $tDNA^{Gly}$ behaves as heterochromatin attenuator in our experimental system. It is interesting to note that, while other tDNAs substituted at the centromere recapitulate barrier activity, re-positioning of $tDNA^{Ala}$ at a euchromatic locus resulted in an attenuation of heterochromatin spreading, but not complete barrier activity (Scott et al., 2006). Together, these data establish a mechanistic link between heterochromatin barriers and attenuators, and implicate genomic context as an additional factor in determining where a sequence falls along the continuum of effects on heterochromatin (Figure 17).

DNA sequences prevent expansion of heterochromatin domains

Whether the effect of DNA sequence could be abrogated by increased dosage of heterochromatin proteins was also examined. Increased *swi6*$^{+}$ resulted in increased levels of H3K9me2 over sequences adjacent to L5, as well as enhanced repression of *ade6*$^{+}$, consistent with an increase in local heterochromatin (Figure 12B). However, this change in heterochromatin enrichment is not accompanied by an expansion of the domain. Further expansion of the domain is likely prevented by the barrier and attenuator activity of adjacent sequences, indicating that these sequences are robust to the increasing magnitude of heterochromatin in these strains. This is the also the case with models of PEV in mouse where enhancing heterochromatin formation is insufficient to cause PEV when a transgene is flanked by chromatin insulators (Festenstein et al., 1999). Alternatively, enhanced propagation of heterochromatin could be limited by selection against increased silencing of genes within the *de novo* heterochromatin domain.

Active and passive heterochromatin advocates: protosilencers, boosters, and neutral sequences

As heterochromatin antagonists are characterized by different strengths, we propose that DNA sequences also differ in their ability to initiate or promote heterochromatin spreading. The identification of protosilencers, sequences that can actively contribute to gene silencing but only in specific "silencing-conducive" environments (Fourel et al., 2002), supports this hypothesis. DNA sequences that are permissive to heterochromatin spreading can be conceptually subdivided into those that rely on active mechanisms, like those above, and those that passively allow heterochromatin but do not actively propagate the heterochromatic state (Figure 17). The spacer and *S. pombe* intergenic fragments may fall into this class of sequence elements. Both sequences allow formation of large heterochromatin domains with levels of H3K9me2 enrichment similar to that observed at the centromere (Figures 8A and 8B). Alternatively, these sequences may contain elements that enhance heterochromatin spreading, and thus would belong in the former class of sequences that actively promote heterochromatin spreading.

Interestingly, while high levels of H3K9me2 are sustained over the length of the lambda spacer DNA, the ratio of Swi6p/H3K9me2 is reduced, relative to both genome-wide data and data from the *ura4* and *spbc2f12.03 de novo* heterochromatin domains (Figure 14C and Figure 15) (Cam et al., 2005). The reduced levels of Swi6p correlate with reduced ability to establish and maintain silencing at *ade6*$^{+}$ when compared to *ura4*::L5-*ade6*$^{+}$ strains (Figure 16). These data suggest that lambda spacer DNA exerts a sequence-specific effect on the associated heterochromatin domain that results in reduced levels of gene repression.

Genome sequence affects chromatin state in higher eukaryotes as well as fission yeast

The spreading of heterochromatin from L5 shares at least conceptual similarities with the spreading of gene silencing and, presumably, facultative heterochromatin from an ectopic X inactivation center in mammalian systems (Lee et al., 1996; White et al., 1998). Furthermore, as we demonstrate in fission yeast, genome sequence is also implicated in the organization of chromatin on the mammalian X chromosome (Straub and Becker, 2008). The inactive X chromosome is organized in alternating domains of genes that are subject to inactivation (silenced) and domains of genes that escape from X inactivation (expressed) (Carrel and Willard, 2005; Miller and Willard, 1998), as well as by domains of different types of heterochromatin (Chadwick and Willard, 2004; Valley et al., 2006). A CTCF site on the mouse inactive X chromosome, located within such a transition region, exhibits insulator activity in transgene assays (Filippova et al., 2005), thus implicating DNA sequence in maintaining the boundaries of expression domains. Moreover, the presence of specific DNA features on the X chromosome can be used to accurately predict whether a gene will be subject to, or escape from, X inactivation (Carrel et al., 2006; Wang et al., 2006b). However, as with the intergenic and spacer fragments in this study, it is unknown whether the sequences correlated with gene silencing passively permit the silent state, or whether they actively promote the propagation of gene silencing. Finally, LINE-1 elements have been proposed to behave as protosilencers, or booster elements, relaying transcriptional inactivation from sites of nucleation (Lyon, 1998; Wang et al., 2006b). While such evidence points to the importance of DNA sequence in regulating domains of gene expression on the X chromosome, the presence of barriers and other sequences in mammals has yet to be addressed fully.

2.5 Materials and methods

Fission yeast strains

The genotypes for strains used in this study are as listed (Appendix A). Fission yeast media were prepared using standard procedures (Moreno et al., 1991). For repression of the *nmt1* promoter 15uM thiamine was added (Siam et al., 2004). A strain bearing the *ade6*$^{DN/N}$ allele (a loss of function mutation created by a 153bp deletion of the *ade6*$^{+}$ open reading frame (Ekwall et al., 1997)) was generated (Kfy539) and was transformed via electroporation (1.5kV, 200Ω, 25uF) on a BioRad Gene Pulser II. Transformed cells were selected on PMG media lacking adenine (Moreno et al., 1991). Colonies derived from strain Kfy539 were then patched onto media containing 2g/L of 5-fluoro-orotic acid (FOA) (MP Biomedicals) to select for disruption of *ura4*$^{+}$. The resulting strains were screened, using Southern analysis, for appropriate integration of *ade6*$^{+}$. Additionally, BW17 transformants were screened by Southern blot for the maintenance of the 7kb lambda DNA fragment. At least three independent transformants of each genotype were maintained (with the exception of the random integrant, Kfy812) and used for further analysis. All transformants were then crossed into a *swi6*$^{+}$ strain and the *ura4::*L5-*ade6*$^{+}$ allele was selected for on the basis of FOA resistance. To create *swi6+333* strains, *ura4::*L5-*ade6*$^{+}$ strains were crossed into SPG1232 (Shiv Grewal)(Hall et al., 2002a). To create *ura4*::L5-7kb::(Pnmt1-*his3*$^{+}$)-*ade6*$^{+}$ the Pnmt1-*his3*$^{+}$ construct was transformed into Kfy589, colonies were selected for on the basis of growth on media lacking histidine. After integration within lambda was confirmed by Southern analysis, these strains were crossed into a *swi6*$^{+}$ strain.

Plasmids

To create plasmid BW5, *ade6*$^{+}$ was amplified from *S. pombe* genomic DNA using primers BWP34F and BWP34Rb (Appendix B) to add StuI, SpeI, ClaI, and BglII sites to the 5' end of the product and Sac1, Sma1 and Stu1 sites to the 3' end. The PCR product was then digested with StuI and inserted into the StuI site of *ura4*$^{+}$ in pUC13/18. The *ade6*$^{+}$ open reading frame and upstream region were sequenced to ensure no mutations had been introduced during cloning.

Plasmid BW7 was constructed through digestion of YL317 with SpeI and ClaI and subsequent purification of the L5-containing fragment (Scott et al., 2006). L5 was then inserted into the SpeI/ClaI site of BW5. Plasmids BW32 and BW34 contain 4.9kb of *S. pombe* intergenic DNA taken from between SPCC320.02 and SPCC320.03 inserted into the BglII site of BW5 and BW7, respectively. The intergenic fragment was digested from the cosmid SPCC320 using XbaI, subcloned into pUC13/18, and then digested with BamHI before inserting into the appropriate plasmid. Plasmids BW30 and BW17 were created by digesting the lambda phage genome (NEB) with BamHI and purifying the 7.2kb fragment, which was then ligated into the BglII sites of BW5 and BW7, respectively. To create BW20 an additional copy of L5, as a BamHI – BglII fragment, was inserted into the BglII site of BW7.

Plasmids BWP40 and BWP41 were created by replacing the GFP ORF with *his3*$^{+}$ within the plasmids pFA6a-kanMX6-P3nmt1-GFP and pFA6a-kanMX6-P41nmt1-GFP, respectively (A gift from Jian-Qiu Wu) (Bähler et al., 1998). A subfragment of the lambda spacer DNA was liberated from BW17 by digestion with BglII and cloned into pUC1318. The Pnmt1-*his3*$^{+}$ containing fragment was then inserted within the PstI site in the lambda fragment.

Confirming and mapping random integrants

To identify random integrants that did not disrupt the *ura4* locus, we selected transformants on the basis of growth on PMG –adenine and death on FOA. These strains were then confirmed via Southern blot to have a single *ade6*$^{+}$ insertion and the site of integration was mapped using an inverse PCR protocol modified from (Ochman et al., 1988). Genomic DNA (2μL) was digested with MboI or Nde1 and incubated for 3.5 hours at 37°C. The digest was heat inactivated at 65°C for 20 minutes. 2μL of the digest was added to a standard ligation reaction (T4 ligase, NEB) and incubated overnight at room temperature. Inverse PCR was performed using primers E367/BWP89F for the Nde1 digest and BWP37F/BWP32F for the Mbo1 digest. The PCR products were purified and sequenced using the PCR primers listed above.

Serial dilution analysis and scoring of *ade6*$^{+}$ phenotypes

Strains were grown overnight with shaking in YES media at 32°C and diluted to a concentration of 1e6 cells/mL. Cultures were diluted serially (1:9) and plated on YES media lacking adenine.

To assess the stability of silencing, colonies that were scored as either completely white or completely red were identified using a Leica MZ7.5 microscope and grown for 24 hours in YES media before plating on YE plates lacking adenine.

For both protocols, plates were grown for three nights at 32°C and shifted to 4°C for 24 hours before photographing or counting.

Real time RT-PCR

Total nucleic acid was isolated from logarithmically growing cells in YES media at 32°C, and was then subjected to DNAse treatment and RT-PCR using oligodT as a primer. Expression was analyzed by quantitative PCR using SYBR Green on a Bio-rad myCycler, using primers specific to the wild type copy of *ade6*$^{+}$ (BWP85F/R). Levels of mRNA from *ade6*$^{+}$, and other genes queried, were expressed relative to *act1*$^{+}$(BWP74F/R). The standard curve was generated using genomic DNA isolated from strain Kfy1. In order to be included in this study a PCR experiment had to have a PCR efficiency between 90-110% and a correlation coefficient >0.99.

Chromatin immunoprecipitation

The H3K9me2 ChIP protocol was adapted from (Pidoux et al., 2004). Logarithmically growing cells from control and experimental strains were treated with 1% paraformaldehyde for 15 minutes. The cell wall was then destroyed through bead beating twice for two minutes in buffer containing protease inhibitors. The resulting material was then sheared to an average fragment size of 600bp using sonication. Chromatin preps were then subdivided into three tubes: an input sample that was used to check shearing, an IP sample to which protein beads and antibodies to H3K9me2 (from Takeshi Urano) were added, and a mock sample to which only protein beads were added. The mock and IP samples were incubated overnight, and the beads were isolated and subjected to a series of washes. Finally, DNA was purified from all three samples (IP, mock, and input) with phenol-chloroform extraction and ethanol precipitation using glycogen as a carrier.

H3 ChIPs were preformed as above using an antibody to H3 (abcam 1791).

Swi6p ChIPs were performed using the above protocol modified from (Huang and Moazed, 2003). 2.5e8 cells were shifted to room temperature for two hours prior to fixation. Cells were fixed with 3% paraformaldehyde for 30 minutes at room temperature. 1μL of antibody (from Shiv Grewal) was incubated with the IP sample overnight, prior to incubation with protein beads for two hours.

Quantitative PCR was used to assay levels of query/*act1*$^{+}$ in IP reactions relative to a no-antibody control.

2.6 Acknowledgements

We wish to thank Shiv Grewal for the swi6+333 strains and the anti-Swi6p antibody, Takeshi Urano for the anti-H3K9me2 antibody, Jian-Qiu Wu for the pFA6a series plasmids, Terilyn Gaither for technical assistance and Laura Rusche for helpful discussion and critical reading of the manuscript.

3 Uncoupling of genomic and epigenetic signals in the establishment, spreading and inheritance of heterochromatin domains in fission yeast

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3.1 Introduction

It is of great biological interest to understand how behavior to arises from a fixed set of genomic instructions (Blasco, 2007; Feinberg, 2007; Surani et al., 2007). While regulatory factors can control both the spatial and temporal regulation of the genome, an equally critical role for the underlying DNA is also evident, as the genomes of complex, multi-cellular organisms contain sequence-specific landmarks that can serve as targets of such regulation (Alekseyenko et al., 2008; McDonel et al., 2006; Simon et al., 1993). Thus, it is evident that the establishment of dynamic chromatin states, characterized in complex genomes by DNA methylation, by locus-specific and state-specific histone variants, and by post-translational modifications of histones, requires integration of both genomic and non-genomic signals (reviewed in Straub and Becker, 2008), the latter of which are commonly referred to as "epigenetic". However, while these genomic and non-genomic signals must work in concert to establish different chromatin states, the relative contribution of each to the transmission of the resulting chromatin domains through mitosis and meiosis is unclear. Further, given the rigorous definition of epigenetic as heritable changes that are not due to changes in sequence and that persist in the absence of the establishment signals, few examples of epigenetic states have been observed (Gottschling, 2004).

To address whether maintenance and inheritance of the heterochromatic state can be uncoupled from the DNA sequence(s) required to establish it, we have capitalized on an ectopic silencing assay to establish a *de novo* heterochromatin domain at a formerly euchromatic location in the genome of the fission yeast *Schizosaccharomyces pombe* (Partridge et al., 2002;

Wheeler et al., 2009). *De novo* domains are nucleated by the L5 repetitive element and are characterized by transcriptional repression of surrounding genes and by the presence of heterochromatin markers, including H3K9me2-modified nucleosomes and the HP1 homolog, Swi6 (Wheeler et al., 2009). Gene silencing is detectable histochemically in this assay; adjacent to the L5 element is an *ade6*$^{+}$ reporter gene, the repression of which can be visualized as the presence of red pigment within a colony. L5-mediated silencing is subject to variegation, and colonies with expressed (white), silenced (red), and intermediate (pink and sectored) phenotypes are observed (Wheeler et al., 2009).

3.2 Results and discussion

While the requirement for L5 sequences to nucleate formation of heterochromatin is well established (Partridge et al., 2002; Wheeler et al., 2009), the parameters that govern the stability, maintenance and transmission of the heterochromatic state remain unknown. Further, while the presence of variegating phenotypes implies a switching equilibrium between expressed and repressed states at the *ade6*$^{+}$ reporter locus, the extent to which this reflects inheritance of the epigenetic state and/or the re-establishment of heterochromatin at every cell division has not been addressed previously.

To address these questions and to characterize the stability of the expressed and silenced states within the ectopic heterochromatin domain, single colonies were selected, and the phenotypes of the resulting mitotic progeny were analyzed at regular intervals over a period of 600 cell divisions. In the early generations of growth, the parental expression state is maintained in the progeny; that is, a culture derived from a silenced colony remained nearly

entirely silenced, while a culture derived from an expressing colony retained a tendency to continue to express the *ade6*$^{+}$ reporter gene during the initial generations of growth (Figure 19B). These data indicate that maintenance of the parental state is favored over switching between states and, further, argues against the simplest model in which each cell has an equal probability of becoming silenced each generation. Thus, the parental state influences the state of the progeny, consistent with earlier observations at other loci (Grewal and Klar, 1996).

To explore the potential role of the L5 element in the maintenance of the silent state, we engineered strains in which LoxP recombination sites flank the L5 element (Partridge et al., 2002; Volpe et al., 2003; Wheeler et al., 2009). Transformation of these strains with a cre recombinase expression vector resulted in the efficient excision of L5 (Iwaki and Takegawa, 2004) (Figure 18B and 18C). Following the removal of L5, cultures derived from colonies expressing *ade6*$^{+}$ retained the expressing state in 100% of cells as expected, since they lack the genomic element known to be required for establishment of heterochromatin in this system (Figure 19C and 19D). Cells exhibiting the phenotypically silenced state efficiently maintained silencing through the early generations of the time-course, despite removal of the L5 heterochromatin-nucleating sequence (Figure 19C and 19D, generations 50 and 100). Maintenance of the transcriptionally silent state depends on the presence of a functional heterochromatin pathway (Figure 18D). Together, these data demonstrate that heterochromatin maintenance can be uncoupled from the heterochromatin nucleating sequence and, therefore, that an epigenetic component is sufficient to maintain the transcriptionally silenced state in the absence of L5.

This finding contrasts with studies of transcriptional repression in budding yeast and flies, where maintenance of a repressive chromatin state requires the continued presence of genomic nucleating sequences (Busturia et al., 1997; Pillus and Rine, 1989). Rather, despite the

Figure 18: *ade6*$^{+}$ and *ade6*$^{-}$ strains grow equivalently in rich media and cre-mediated excision is efficient

(A) *ade6*$^{+}$ (black diamonds) and *ade6*$^{-}$ (open circles) strains were grown in independent cultures or in a 47:53 mixture of *ade6*$^{+}$: *ade6*$^{-}$ (grey squares and grey triangles). The mixed cultures were maintained in two different types of media; rich media and adenine limiting media, which is used to resolve *ade6*$^{+}$ phenotypes. After approximately 10 generations each culture was plated on adenine limiting media and the proportion of colonies that were red (*ade6*$^{-}$) was determined. **(B)** PCR primers were designed to distinguish the L5^{+} allele from ΔL5. All three primers, shown as black arrows were added to a single PCR reaction. The L5^{+} allele results in a PCR product 446bp whereas ΔL5 results in a larger PCR product of 583bp. **(C)** Example PCR showing the expected fragment sizes for ΔL5 and L5^{+} controls. Additionally, 3 strains post-transformation with the cre plasmid, in all three the L5 element has been excised. **(D)** Wild-type and *swi6*$^{-}$ strains before and after transformation with the cre-plasmid.

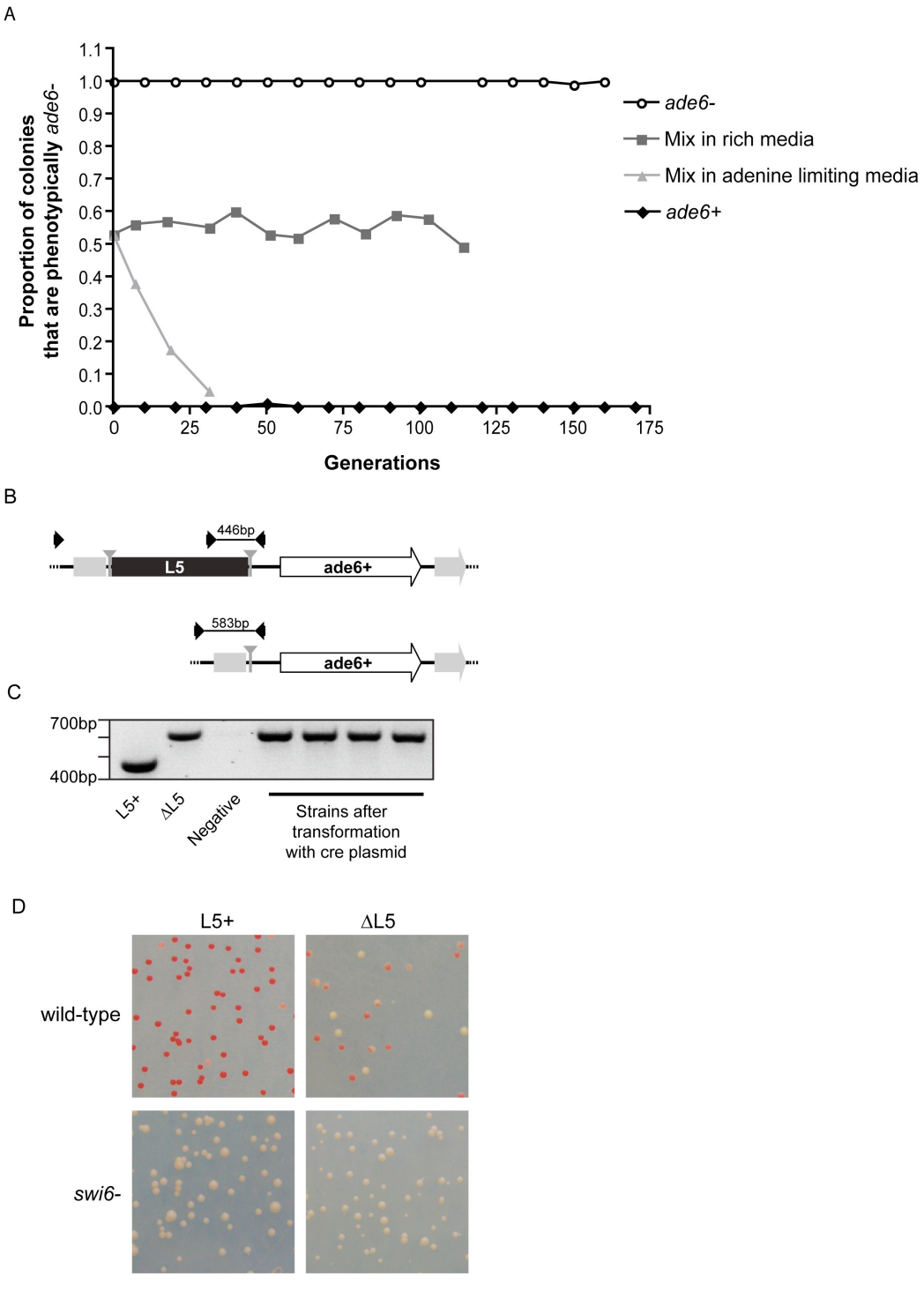
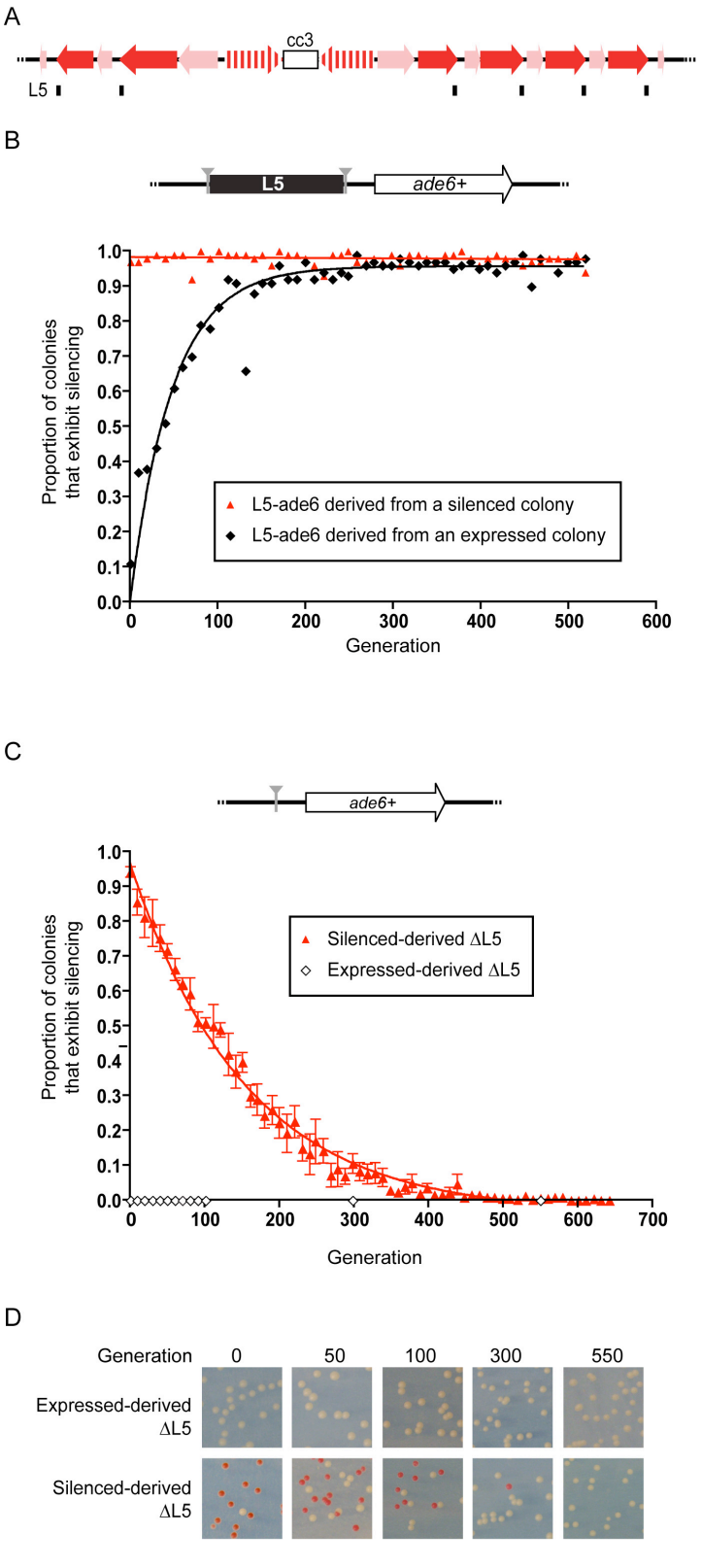

A
Proportion of colonies that are phenotypically ade6-
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
25
50
75
100
125
150
175
Generations
ade6-
Mix in rich media
Mix in adenine limiting media
ade6+
B
446bp
L5
ade6+
583bp
ade6+
C
700bp
400bp
L5+
ΔL5
Negative
Strains after transformation with cre plasmid
D
L5+
ΔL5
wild-type
swi6-

Figure 19: Heterochromatic silencing within a *de novo* heterochromatin domain exhibits parental state bias and can be inherited in the absence of the L5 nucleating sequence

(A) Schematic of *S. pombe* chromosome 3 where red arrows represent dg repeats, pink arrows represent dh repeats, striped arrows represent IMR repeats, and the central core is shown as an open rectangle. L5 and L5-homologous sequences are shown as black rectangles. **(B)** Cultures were derived from silenced and expressed L5-*ade6*$^{+}$ colonies and allowed to grow exponentially. The proportion of the culture that exhibited silencing as determined by counting the number of colonies that had any phenotypic evidence of silencing. The red-derived culture is shown as red triangles and the white-derived culture is shown as black diamonds with the corresponding exponential association curve $y=y_{max}(1-\exp(-0.02*x))$, $R^2=0.9245$. **(C)** Cultures were derived from ΔL5 colonies that were either *ade6*$^{+}$-expressed (shown as open diamonds) or *ade6*$^{+}$-silenced (shown as red triangles) and the proportion of progeny that exhibit silencing over time was monitored (as shown in **D**). The red line represents the exponential decay curve fit to the red-derived ΔL5 data, $y=0.99\exp(-0.0073*x)$, $R^2=0.96$.


A
cc3
L5
B
L5
ade6+
Proportion of colonies that exhibit silencing
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
100
200
300
400
500
600
Generation
L5-ade6 derived from a silenced colony
L5-ade6 derived from an expressed colony
C
ade6+
Proportion of colonies that exhibit silencing
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
100
200
300
400
500
600
700
Generation
Silenced-derived ΔL5
Expressed-derived ΔL5
D
Generation
0
50
100
300
550
Expressed-derived ΔL5
Silenced-derived ΔL5

absence of previously established maintenance factors such as DNA methylation or histone H3.3 in *S. pombe* (Ahmad and Henikoff, 2002; Feng et al., 2006; Ng and Gurdon, 2008; Wilkinson et al., 1995), our data are more similar to a sequence-independent pathway of epigenetic inheritance, characteristic of paramutation in maize (Chandler, 2007) or maintenance of mammalian X inactivation in the absence of the *XIST* gene (Brown and Willard, 1994).

At later generations (300-550; Figure 19C and 19D), the proportion of transcriptionally silent colonies is significantly reduced, suggesting that heterochromatin maintenance decays over time. The switch between epigenetic states (that is, loss of the silenced phenotype) occurs slowly (half-life: 99.8 generations), with an estimated loss rate of 0.7% per generation. Furthermore, enrichment of the heterochromatic histone modification, H3K9me2, at *ade6*$^{+}$ also gradually decreases with time (Figure 20A).

The maintenance of the expressed state is unperturbed following L5 excision, demonstrating that the sequence-specific L5 element is necessary for the reestablishment of the heterochromatin state (Figure 19C and 19D). Therefore, while the transcriptionally silent state can be maintained epigenetically, once silencing is lost it cannot be reestablished in the absence of the L5 heterochromatin-nucleating sequence. These strains also lack detectable levels of H3K9me2 (Figure 20A). Thus, the key feature that distinguishes an L5-containing heterochromatin domain from an epigenetically maintained heterochromatin domain is the ability to reestablish heterochromatin after it has been lost (Figure 19). Reestablishment provides the robustness needed to ensure stable maintenance of a heterochromatic domain in the face of the dynamic nature of heterochromatin (Grewal and Jia, 2007).

The above data demonstrate uncoupling of genomic and epigenetic signals needed for the establishment and maintenance of chromatin states in mitosis. However, the extent to

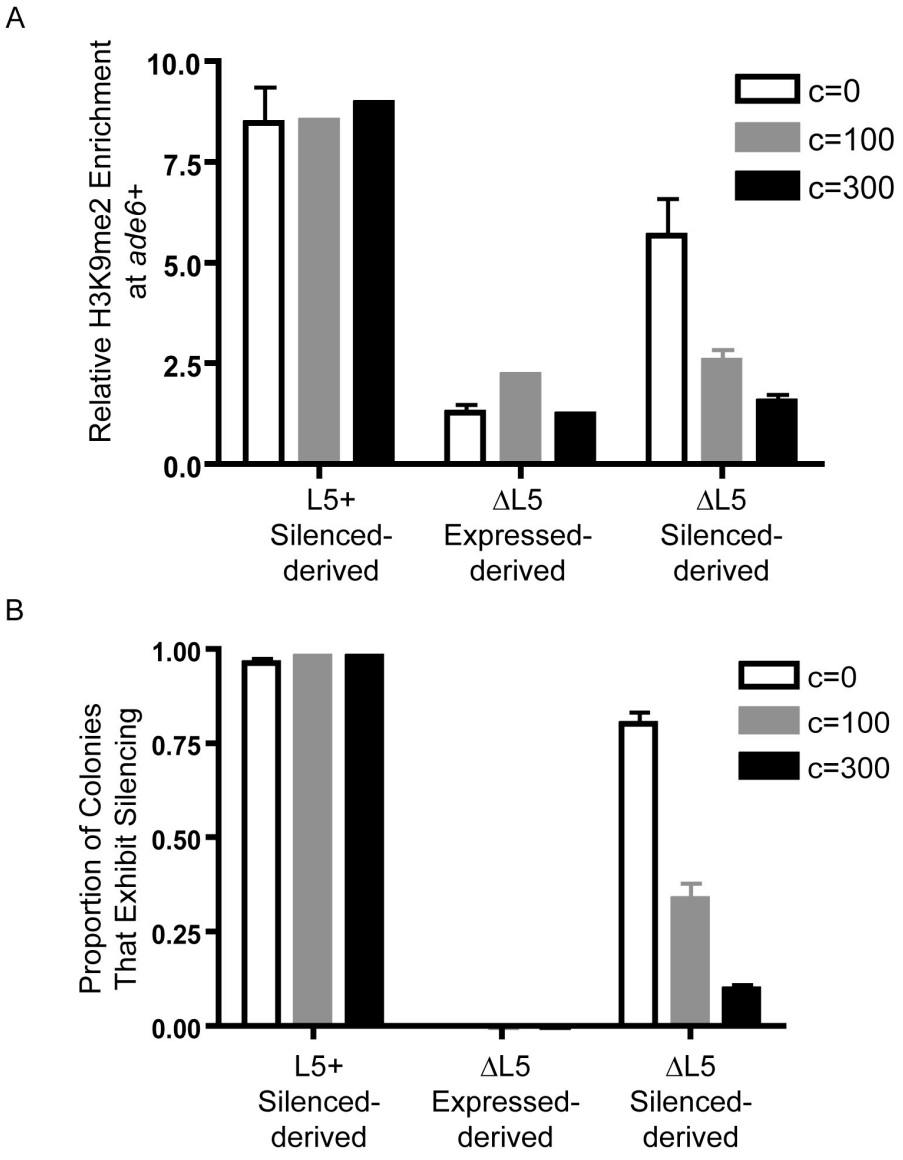


Figure 20: The loss of silencing in ΔL5 strains is concomitant with loss of H3K9me2

(A) Levels of H3K9me2 were assessed at *ade6*$^{+}$ over time. ChIP cultures were derived from time-course cultures corresponding to generations 0, 100, and 300 (white, grey and black). The genotypes are listed below with the phenotype of the colony used to inoculate the initial time-course culture. **(B)** ChIP cultures shown used in (A) were also plated immediately before ChIP to determine the proportion of colonies that exhibited silencing.

which and the manner by which heterochromatin can be inherited through meiosis and subsequent sporulation is unknown. While the progeny of strains containing the L5 element (L5+) exhibit silencing following meiosis and subsequent sporulation (Figure 21A), it has not been determined whether this silencing is due to inheritance of the epigenetic state or to inheritance of the genomic L5-element itself, followed by reestablishment. To determine whether the transcriptionally silent state can be inherited through meiosis in the absence of L5 (ΔL5), two phenotypically silenced ΔL5 strains were mated, followed by tetrad dissection and growth of the individual progeny. Remarkably, 93% of the meiotic progeny exhibited silencing after meiosis (Figure 21A). The inheritance of the transcriptionally silent state among meiotic products approximated a binomial distribution (Figure 21B), suggesting that the silencing state in a given progeny is independent of the status of the other three progeny. Thus loss of the epigenetic state appears stochastic, and inheritance of heterochromatin is epigenetically maintained at a high frequency through meiosis, even in the absence of the known heterochromatin nucleating sequences.

In addition to assembling heterochromatin locally at the *ade6*$^{+}$ gene, L5-mediated heterochromatin spreads into endogenous sequences, creating a *de novo* heterochromatin domain (Wheeler et al., 2009). In the presence of the L5 element, adjacent sequences are enriched in H3K9me2, which can spread approximately 4.0 kb proximal and 12 kb distal to the L5 element (Figure 22A). Even following excision of L5, chromatin remains enriched in H3K9me2 at adjacent sequences, demonstrating that both local transcriptional silencing and heterochromatin assembly at the *de novo* domain can be maintained in the absence of L5-dependent heterochromatin establishment. Interestingly, the extent to which H3K9me2 is

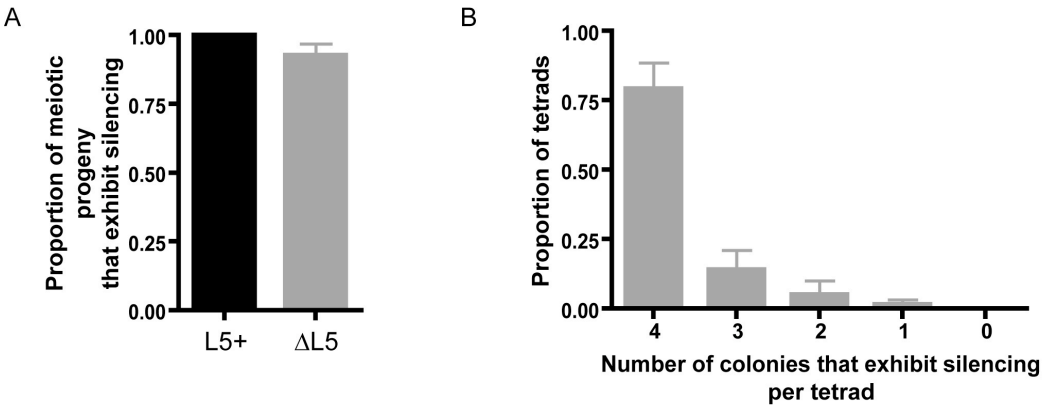
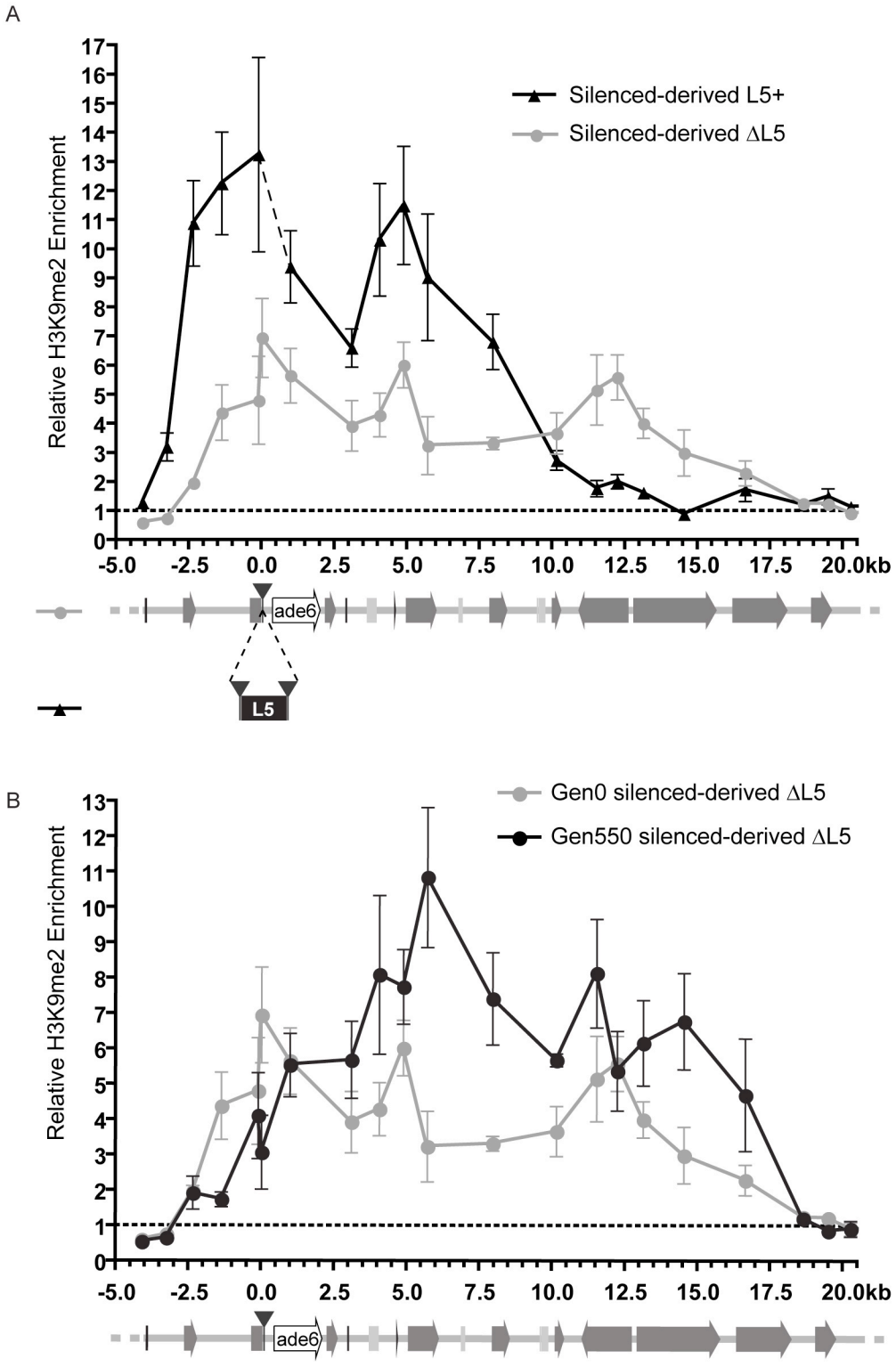


Figure 21: Silencing is inherited through meiosis in a sequence-independent fashion

(A) The number of meiotic progeny that exhibit silencing per meiosis was determined for all ΔL5 strains derived from red colonies. (**B**) The number of progeny per meiosis that exhibited silencing was scored.

Figure 22: Heterochromatin is maintained throughout the de novo heterochromatin domain

(**A**) The relative enrichment of H3K9me2 throughout the *de novo* heterochromatin domain in ΔL5 (grey circles) or $L5^{+}$ (black triangles) strains derived from silenced colonies is depicted relative to a map of the *ura4* genomic region. The $L5^{+}$ and ΔL5 populations were on average 96% and 80% silenced, respectively. To facilitate comparison between the two strains, the L5 element is depicted below the *ura4* region, and the data over this region in ΔL5 strains are represented by a dashed line. The LoxP sites are shown as dark grey triangles, genes are shown as grey arrows in the direction of transcription, LTRs are shown as light grey boxes, and non-coding RNAs and tRNAs are shown as black boxes and arrows, respectively. More detailed information about the *ura4* region is provided in Figure 24A. (**B**) The level of H3K9me2 enrichment in cultures derived from gen550 ΔL5 colonies (generation 550, Figure 19B and 19C) is shown as black circles. As above, the H3K9me2 profile for ΔL5 red colonies from generation 0 (Figure 19B and 19C) is shown as grey circles.


A
Relative H3K9me2 Enrichment
Silenced-derived L5+
Silenced-derived ΔL5
-5.0
-2.5
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0kb
ade6
L5
B
Relative H3K9me2 Enrichment
Gen0 silenced-derived ΔL5
Gen550 silenced-derived ΔL5
ade6

maintained varies throughout the *de novo* heterochromatin domain, perhaps reflecting the influence of genomic context on the stability of heterochromatin (Wheeler et al., 2009). Proximal to the site of L5 excision, H3K9me2 enrichment is greatly reduced in ΔL5 strains as compared with L5+ strains. In contrast, more distally, the level of H3K9me2 enrichment in ΔL5 strains actually *exceeds* the level of enrichment in $L5^+$ strains. This unexpected pattern of heterochromatin distribution is even more readily apparent over time, as assessed by the distribution of H3K9me2 in the progeny of ΔL5 colonies selected at generation 550 (Figure 22B), by which time silencing is infrequent (Figure 19C and 19D). In these strains, the relative enrichment of H3K9me2 is increased at least two-fold over levels seen in strains immediately following excision of the L5 element (Figure 22B). We considered that the increased level of H3K9me2 enrichment distally might increase the efficiency of heterochromatin; however, the rate of maintenance of *ade6*$^+$ silencing was slightly reduced in cultures derived from gen550 colonies (Figure 23). We speculate that repeated cell division in the absence of L5-dependent reestablishment of heterochromatin may permit time-dependent redistribution of the H3K9me2 marks, resulting in the observed pattern of enrichment throughout the *de novo* heterochromatin domain, even after 550 cell divisions. This model implies the possible existence of genomic interactions between L5 and sequences throughout the domain that are perturbed in the absence of L5.

To test whether colonies that had lost silencing at the *ade6*$^+$ gene maintained H3K9me2 elsewhere in the *de novo* domain, H3K9me2 enrichment levels were queried from six independent *ade6*$^+$-expressing colonies. No detectable H3K9me2 enrichment was present within the 25 kb region surrounding the *ade6*$^+$ gene (Figure 24). Thus, there is no evidence for

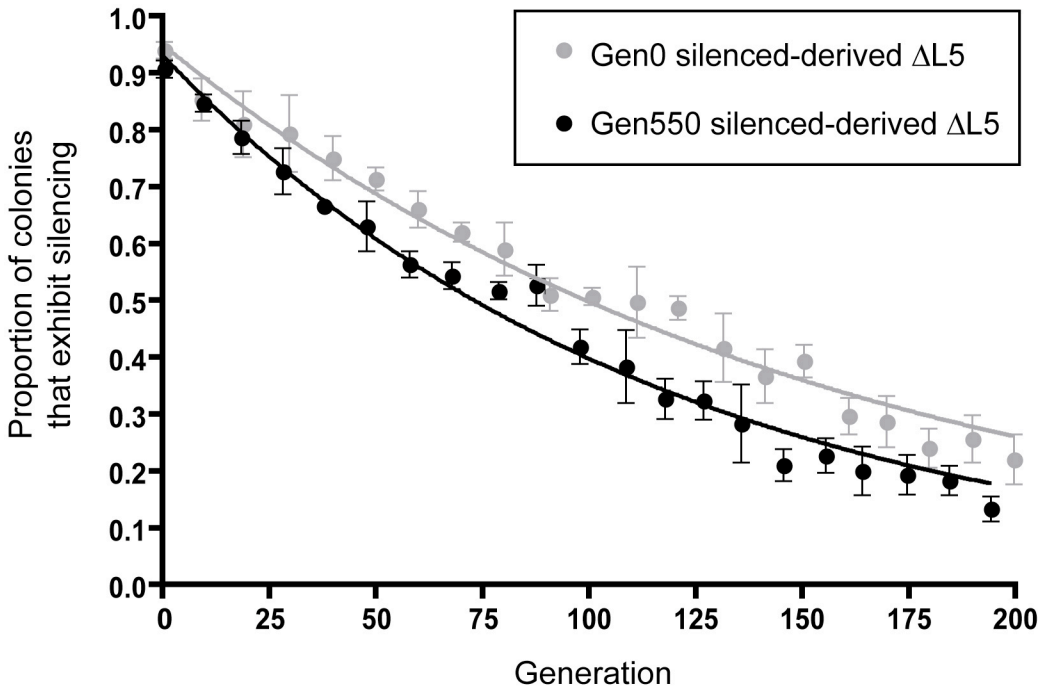


Figure 23: Maintenance is reduced in gen550 ΔL5cultures

Proportion of ΔL5 colonies that exhibit silencing over time. Silenced-derived ΔL5 cultures, as shown in Figure 22, are shown as grey circles. The associated exponential decay curve, recalculated from generations 1-200 is shown as the grey line $y=0.95*\exp(-0.0065*x))$, $R^2= 0.91$. Cultures derived from gen550 silenced colonies are shown as black circles with the corresponding decay curve $y=0.93*\exp(-0.0085*x))$, $R^2= 0.94$.

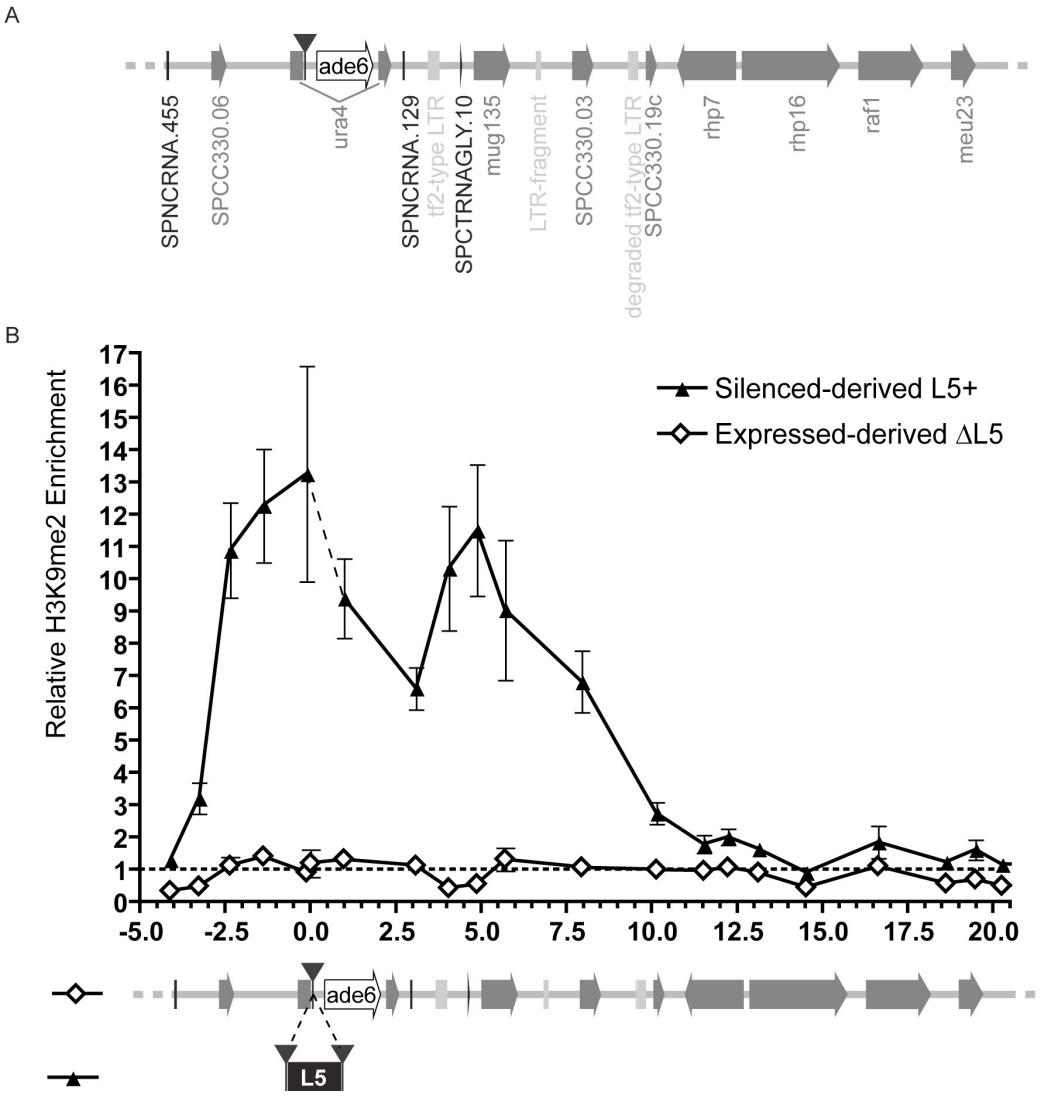


Figure 24: Expressed-derived strains do not maintain H3K9me2 elsewhere in the *de novo* heterochromatin domain

(A) Detailed schematic of the *ura4* neighborhood. **(B)** H3K9me2 enrichment in silenced-derived L5^{+} strains (black triangles) and expressed-derived ΔL5 strains (open diamonds).

maintenance of heterochromatin within the sequences surrounding *ade6*$^{+}$ locus in *ade6*$^{+}$-expressing colonies.

Combined with our earlier work (Wheeler et al., 2009), these data provide insight into the nature of genomic elements involved in the establishment and maintenance of heterochromatin and epigenetic silencing in fission yeast. To complement these findings, it is important to explore the role of specific *trans*-acting factors, as has been demonstrated previously with mutations that specifically affect heterochromatin establishment, but not maintenance (Partridge et al., 2007; Sadaie et al., 2004).

The CAF-1 complex, comprised of Pcf1 and Pcf2 subunits, has established roles both in the deposition of histone H3-H4 onto newly replicated DNA and in the replication and propagation of heterochromatic domains in various organisms (Quivy et al., 2008; Smith and Stillman, 1989; Song et al., 2007). Absence of a functional CAF-1 complex reduces the stability of chromatin states in *S. pombe* (Dohke et al., 2008). To explicitly test the role of the CAF-1 complex in mitotic maintenance of heterochromatin in the *de novo* heterochromatin domain studied here, we excised the L5 element in *pcf1*$^{-}$ and *pcf2*$^{-}$ mutant strains. After excision, we observed a reduced number of colonies that exhibit silencing (Figure 25A). Next, we monitored *ade6*$^{+}$ expression over time to analyze heterochromatin stability in these strains. Both *pcf1*$^{-}$ and *pcf2*$^{-}$ ΔL5 colonies lost silencing seven-fold faster than the wild type ΔL5 controls, demonstrating that the CAF-1 complex plays an important role in the maintenance of heterochromatin in the absence of sequence-specific reestablishment of heterochromatin (Figure 25, Figure 26A and 26B). Previous studies have documented an association between the CAF-1 complex and the histone H3K9 methylase SetDB1 in higher eukaryotes (Sarraf and Stancheva, 2004). Our data confirm

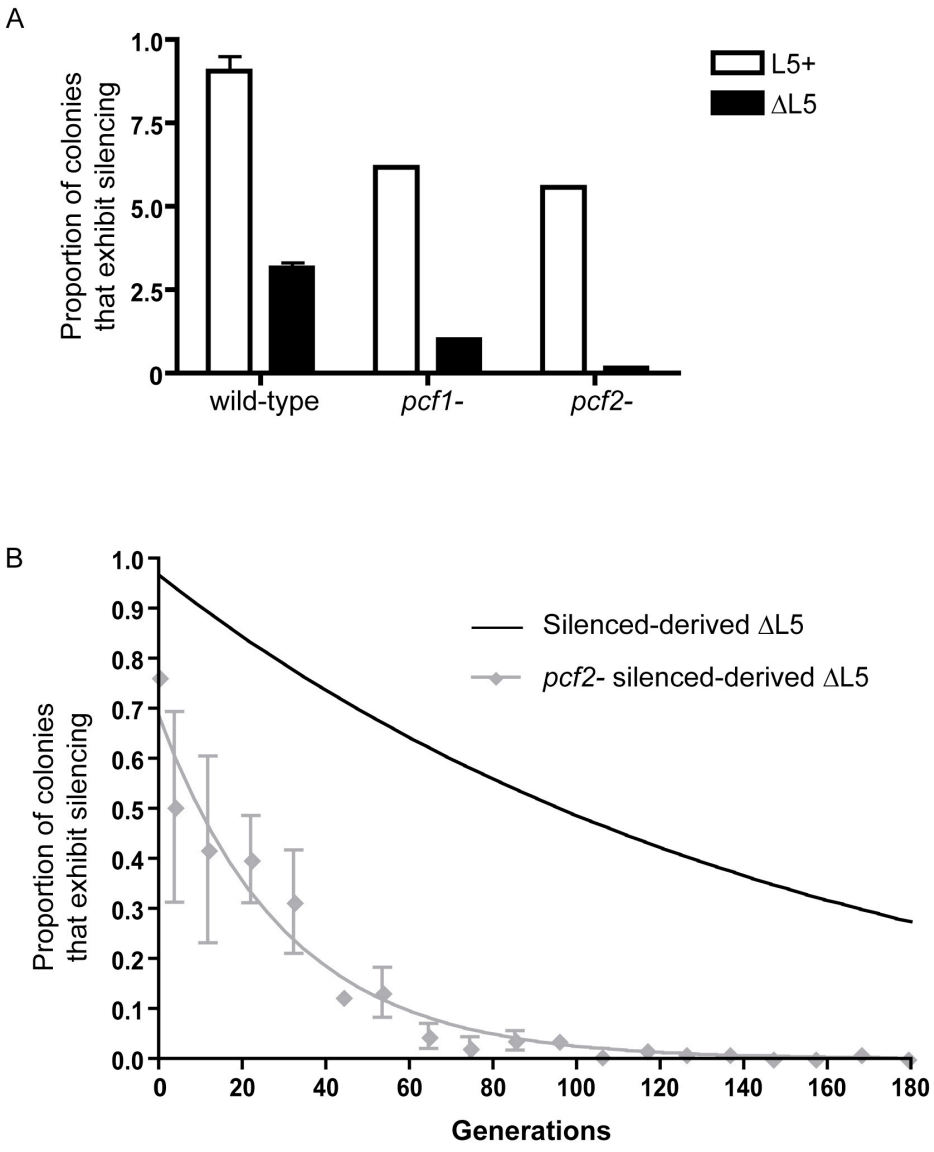


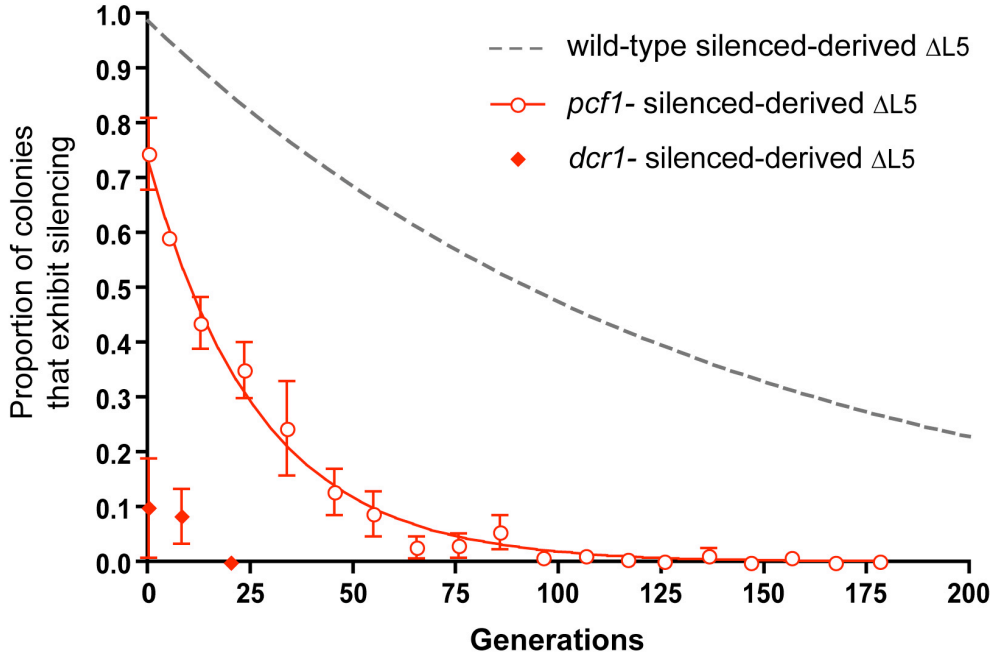
Figure 25: *pcf1*$^{-}$ and *pcf2*$^{-}$ strains exhibit lower amounts of silencing after transformation with the cre plasmid than wild type

(A) The distribution of silencing in mock transformed strains (L5^{+}, white bars) of all three genotypes compared to the distribution of silencing after transformation (ΔL5, black bars). **(B)** Proportion of colonies that exhibit silencing after excision as above except strains lacking *pcf2*$^{+}$ as shown as grey diamonds with the corresponding exponential curve, y=0.69exp(-0.033*x)), R^2= 0.87

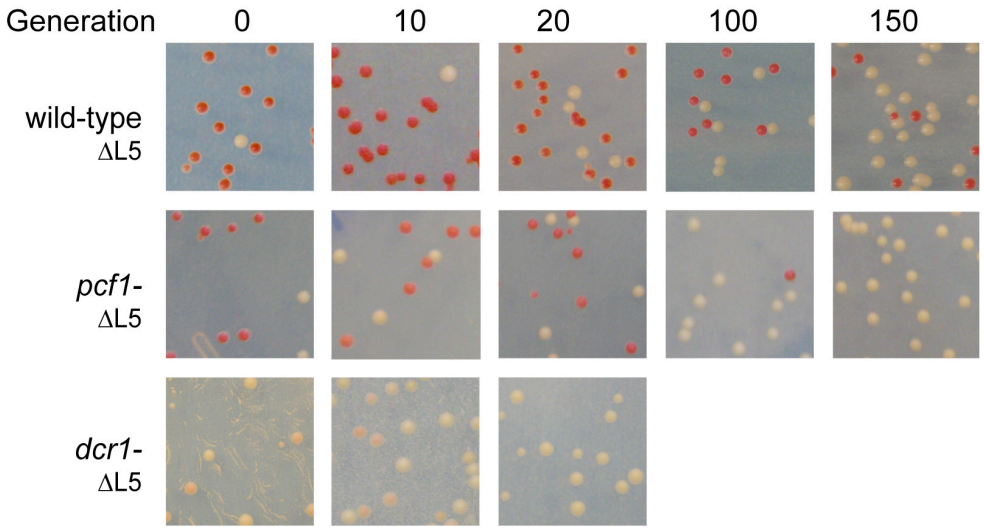
Figure 26: *pcf1*$^{+}$ and *dcr1*$^{+}$ are required for efficient maintenance of H3K9me2 in the absence of L5

(A) Proportion of colonies that exhibit silencing after excision in wild-type, shown as the grey dashed line, and strains lacking *pcf1*$^{+}$, as shown as red open circles with the corresponding exponential curve, y=0.73exp(-0.037*x)), R^2= 0.96. The loss of silencing that occurs in ΔL5 strains after loss of plasmid-born *dcr1*$^{+}$is indicated by red diamonds. (**B**) The corresponding phenotypic data for each genotype are shown.

A


wild-type silenced-derived ΔL5
pcf1- silenced-derived ΔL5
dcr1- silenced-derived ΔL5
Proportion of colonies that exhibit silencing
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
25
50
75
100
125
150
175
200
Generations

B


Generation
0
10
20
100
150
wild-type ΔL5
pcf1- ΔL5
dcr1- ΔL5

and extend this functional link between replication and chromatin assembly and further demonstrate a requirement for CAF-1 in epigenetic inheritance in ΔL5 strains.

The RNAi pathway plays an important role in both the establishment and maintenance of heterochromatin at fission yeast centromeres (Martienssen et al., 2005). Loss of functional Dcr1p following meiosis results in the alleviation of transcriptional silencing, both within the centromere and adjacent to endogenous L5 elements in the pericentromeric region (Volpe et al., 2003; Volpe et al., 2002). However, even in a *dcr1*$^{-}$ mutant strain, reduced levels of H3K9me2 can persist over endogenous centromeric sequences (Sadaie et al., 2004). To extend these studies, we established ectopic heterochromatin in *dcr1*$^{-}$ strains by expressing Dcr1p from a plasmid that rescues silencing defects in *dcr1*$^{-}$ strains (Macrae et al., 2006). L5 was subsequently excised, and the transcriptional state of *ade6*$^{+}$ was monitored in strains in the presence or absence of the plasmid-borne *dcr1*$^{+}$. Loss of *dcr1*$^{+}$ expression resulted in an immediate decrease in *ade6*$^{+}$ silencing; only faint pink colonies were observed, and apparent silencing was completely lost by generation 20 (Figure 26A and 26B).

These data confirm the involvement of *dcr1*$^{+}$ and the RNAi pathway in heterochromatin and extend our understanding by identifying a role for Dcr1p during the maintenance of the epigenetic state even in the absence of heterochromatin reestablishment. Previous studies have documented the presence of small interfering RNAs homologous to reporter genes inserted at the centromere (Buhler et al., 2007). Our data further suggest that non-centromeric sequences can also become substrates for RNAi when placed within a heterochromatic context. An important mechanistic distinction between our data and those described previously is that siRNAs have been thought to be generated by read-through transcription from centromeric promoters (Irvine et al., 2006). In the experimental system used here, however, transcripts

originating from centromeric promoters embedded within L5 are no longer possible in the absence of L5. An alternative model posits that euchromatic sequences within the *de novo* domain are "taught" to behave as heterochromatic sequences by the transient presence of L5 and generate transcripts that are subsequently processed by the RNAi pathway. This model is supported by the recent finding that small interfering RNA synthesis, and subsequent heterochromatin assembly, occurs following the expression hairpin RNA that is homologous to a euchromatic target (Simmer et al., 2010).

These data demonstrate that cellular "memory" of chromatin state is the sum of both genomic and non-genomic marks, including in different organisms sequence-specific nucleating sequences, DNA methylation, post-translational modifications of histones, histone variants, and, as demonstrated in this study, non-histone chromatin proteins. Additionally, we have demonstrated that heterochromatin in fission yeast can be uncoupled from and inherited independent of the signals required for its establishment, thus adhering to the most rigorous definition of "epigenetic." The heterochromatic state in fission yeast exhibits the same self-propagation achieved in other organisms via distinct pathways (Brown and Willard, 1994; Ptashne, 2009).

Notwithstanding the uncoupling of genetic and epigenetic signals demonstrated here, it is likely that genomic context – independent of any heterochromatin nucleating sequences – plays a role in the persistence of cellular memory by altering the strength of selection for or against the maintenance of heterochromatin or by altering the intrinsic stability of heterochromatin itself. This possibility invites approaches to identify different sequence elements that can sustain or antagonize inheritance of heterochromatin genome-wide and to

thus provide further insight into the nature of genomic code(s) that underlie different chromatin states.

3.3 Materials and methods

To create the L5^{flox}-*ade6*$^{+}$ construct, oligos containing the LoxP sequence were cloned in the same orientation into the SpeI and ClaI/BglII sites flanking the L5 element in plasmid BW7 (Wheeler et al., 2009). The resulting plasmid, BW38, was sequenced to ensure that no errors had been introduced during cloning.

The pREP41-cre expression plasmid was a gift from K. Takegawa (Iwaki and Takegawa, 2004). To adapt this plasmid for use in strains bearing the *leu2*$^{+}$ maker, a *ura4*$^{+}$ marker was inserted in the PstI site of pREP41-cre, allowing for selection on media lacking uracil.

Fission yeast strains

The genotypes for strains used in this study are listed in Appendix A. Strains Kfy1265-Kfy1267 were created by transforming plasmid BW38 into Kfy501 and selecting for growth on PMG –adenine plates (Moreno et al., 1991). Appropriate integration of the construct at the *ura4* locus was confirmed phenotypically, by growth on media containing 2g/L of 5-fluoro-orotic acid (FOA) (MP Biomedicals), as well as by Southern blot. All other strains in this study were generated through standard genetic crosses with strains carrying the *ura4*::L5^{flox}-*ade6*$^{+}$ allele. Strains mutant for the components of the *S. pombe* CAF-1 complex were a gift from Dr. Murakami (Dohke et al., 2008).

All liquid cultures were grown in YES media (Moreno et al., 1991), unless otherwise indicated. For resolving *ade6*$^{+}$ expression phenotypes, cells were plated on PMG 1/10th adenine plates and incubated at 32ºC for 4 days, shifted to 4ºC for one night, and then counted under a Leica MZ7.5 microscope.

Excision of L5 using cre recombinase

To induce excision of L5, *ura4*::L5^{flox}-*ade6*$^{+}$ strains were transformed with the pREP41-cre plasmid via electroporation (1.5kV, 200Ω, 25uF) on a BioRad Gene Pulser II. Unexcised control strains were derived from the same culture as the pREP41-cre transformants, however no plasmid was added to transformation reaction. To allow for excision to occur prior to plating strains were grown for 24-hours in liquid PMG media –leucine (pREP41-cre) or PMG complete (no DNA control). pREP41-cre transformants and were then plated twice on PMG –leucine and PMG complete, respectively. No DNA control strains were plated directly onto PMG 1/10th adenine plates. Excision of L5 was confirmed in pREP41-cre transformants using a PCR strategy (BWP33F, BWP33R, and BWP246F; Appendix B). Strains in which L5 was excised, ΔL5, were streaked onto PMG complete plates to allow for loss of the pREP41-cre plasmid. Finally, individual colonies from the complete plates were streaked onto PMG 1/10th adenine.

Time course of *ade6*$^{+}$ expression

YES cultures were inoculated with a single colony of appropriate genotype and *ade6*$^{+}$ phenotype. The time course data are compiled from one white and one red L5^{+}colony and three red and three white ΔL5 colonies. Cultures were allowed to double for approximately 10 generations, as determined by counting cell density with a hemacytometer. When appropriate

density was achieved a subset of the culture was plated PMG $1/10^{th}$ adenine plates. These plates were then used to calculate the proportion of colonies that exhibited silencing. In addition to plating, 2000 cells from the culture were used to inoculate a new culture that was allowed to double for approximately ten generations before plating. Using this scheme, cells were maintained in logarithmic growth throughout the time course.

Chromatin Immunoprecipitation

H3K9me2 ChIP was preformed using a protocol modified from (Huang and Moazed, 2003). $2.5*10^8$ cells were fixed for 15 minutes in 1% paraformaldehyde. Cells were lysed by two rounds of bead beating for 30 seconds in lysis buffer containing protease inhibitors. Chromatin was sheared to an average DNA fragment size of 600 bp, precleared, and divided into input and IP samples. The IP sample was incubated overnight with 5μL of anti H3K9me2 antibody (Active Motif, 39239). Protein-A beads were added to the IP samples and incubated for two hours at 4ºC before washing. DNA was isolated from IP and input samples, and the level of enrichment was quantified using real-time PCR as the ratio of query/*act1*$^+$ for IP relative to input samples. Standard curves were generated from genomic DNA isolated from the wild-type strain. Data were analyzed with iCycler iQ Optical System Software.

dcr1$^+$ analysis

dcr1$^-$ L5$^+$ strains (Kfy1455 and Kfy1456) were generated by standard genetic crosses. To add back *dcr1*$^+$ these strains were transformed with the plasmid pREP2-*dcr1*$^+$ a gift from F. Li (Macrae et al., 2006). Upon reestablishment of silencing cultures derived from red L5$^+$ pREP2-*dcr1*$^+$ *dcr1*$^-$ strains were transformed with the cre plasmid and excision of L5 was confirmed as

described. Strains were maintained on media lacking uracil to select for the plasmid. To determine the phenotypic effect of loss of *dcr1*$^{+}$ strains were moved to media containing 5-FOA to select for loss of the plasmid. Time course cultures were grown in liquid PMG –uracil or PMG complete +FOA to select for strains that maintained or lost pREP2- *dcr1*$^{+}$, respectively.

3.4 Acknowledgements

We thank Janet Partridge, Yota Murakami, Robin Allshire, Fei Li and Kaoru Takegawa for strains and reagents critical to the execution of these experiments. We thank members of our labs, Laura Rusche and Beth Sullivan for helpful discussions, and Terilyn Gaither for technical support. This work was supported by funds from the Duke Institute for Genome Sciences & Policy.

4. Conclusions and future work

4.1 Summary

In this thesis, I have taken advantage of specific genomic sequences that can establish heterochromatin at euchromatic sites in the genome in order to study the role of DNA sequence in the spreading and maintenance of heterochromatin in fission yeast. Here, I will summarize the major findings presented in Chapters 2 and 3.

The work in Chapter 2 demonstrated that the ability of heterochromatin to spread along the chromatin fiber is largely influenced by the genomic content of the sequences over which it spreads. These data demonstrate that features of a chromatin domain reflect the effects of DNA sequences that establish the domain and sequences that enhance or restrict its spread and provide a link between chromatin state and the underlying genome content. These studies extend those that have identified heterochromatin barriers in the genomes of fission yeast and other eukaryotes and suggest that DNA sequences, in addition to serving as barriers, can have a range of effects on the spreading of heterochromatin (summarized in Figure 17) (Chung et al., 1993; Donze et al., 1999; Donze and Kamakaka, 2001; Noma et al., 2001; Noma et al., 2006; Scott et al., 2006; Scott et al., 2007). This work provides evidence for a class of genomic sequences that can serve as heterochromatin attenuators. While attenuators are insufficient to completely stop the spread of heterochromatin (cf. barriers), they reduce the strength of spreading. Additionally we have determined that heterochromatin can spread over spacer DNA, forming a stronger heterochromatin domain than over genomic DNA. In Chapter 2, we hypothesized that in the genome heterochromatin is restricted by the presence of genes and transcribed sequences. Consistent with that hypothesis, insertion of a gene within spacer DNA

results in the attenuation of spreading. This study sets the stage for a comprehensive analysis of the effect of DNA sequences genome-wide on heterochromatin spreading that will be discussed in the future work section below.

In Chapter 3 of this thesis, I demonstrated that heterochromatin is maintained at the ectopic locus, as has been observed at the mating type locus (Grewal and Klar, 1996). This suggests that maintenance of heterochromatin in *S. pombe* shares similarities with maintenance of silencing in *S. cerevisiae* and Polycomb repression in *Drosophila* (Pillus and Rine, 1989; Ringrose and Paro, 2004) (see Chapter 1). Importantly, however, in fission yeast, unlike these other systems, the heterochromatic state can be uncoupled from the DNA sequences required to establish them. After excision of the heterochromatin nucleating sequence heterochromatin can be maintained for over 550 generations, indicating that heterochromatin can be stably inherited in the absence of the nucleating sequence. Another important finding of this study was that, in the absence of the heterochromatin nucleating sequence, heterochromatin cannot be reestablished once lost. This suggests that maintenance of heterochromatin in fission yeast is more similar to paramutation and X inactivation (see Chapter 1), in that the state is self-propagating in the absence of the signal that established the state (Brown and Willard, 1994; Stam et al., 2002). However, unlike paramutation and X inactivation, sequence-independent inheritance of heterochromatin in *S. pombe* does not utilize DNA methylation (Antequera et al., 1984; Riggs, 1975; Stam et al., 2002). Thus, the work in this thesis identified a novel form of sequence-independent maintenance of heterochromatin. A model for how I think this maintenance may be achieved and implications for the use of the word “epigenetic” will be discussed in the following section.

Maintenance of heterochromatin independent of nucleating-sequence – models and implications

Our data suggest that the heterochromatic state can be maintained in *S. pombe* even in the absence of the DNA sequences required to establish them. Thus, one can conclude that the signals that are required for maintenance exist outside of the nucleating sequence, unlike the models proposed for *S. cerevisiae* and *Polycomb* repression in Chapter 1 (Figure). An important question then becomes: what features of heterochromatin in *S. pombe* allow it to self-propagate in such a fashion? One possibility is that the inheritance of modified histones allows for the maintenance of the heterochromatic state. This hypothesis is supported by our observation that the CAF-1 complex, which plays a role in deposition of histones onto daughter strands at the replication fork (Corpet and Almouzni, 2008), is required for efficient maintenance. However, the domains of silenced chromatin in *S. cerevisiae* and Polycomb repression in *Drosophila* also contain modified histones, which, in those cases, are insufficient to maintain the silenced state (Busturia et al., 1997; Cheng and Gartenberg, 2000; Sengupta et al., 2004). Thus, our work implies the existence of features other than modified histones that are required for maintenance of heterochromatin.

One attractive hypothesis is that inheritance of siRNAs mediates heterochromatin maintenance. This is supported by a number of observations. First, it is known that production of siRNAs can spread into adjacent sequences (Bühler et al., 2008; Irvine et al., 2006; Simmer et al., 2010). Spreading of siRNAs would provide targeting of the RNAi pathway to adjacent sequences in the absence of the heterochromatin nucleating sequence. Second, other studies have demonstrated that siRNAs are heritable in other systems (Blumenstiel and Hartl, 2005; Ha et al., 2009; Slotkin et al., 2009). Additionally, production of synthetic siRNAs is sufficient to

establish heterochromatin in fission yeast (Iida et al., 2008; Simmer et al., 2010). Lastly, our own data suggest that heterochromatin maintenance requires the RNAi pathway, since silencing is lost in the absence of Dcr1 (Chapter 3). Of note, paramutation in maize also requires a component of the RNAi pathway (Alleman et al., 2006), suggesting that RNAi can mediate sequence-independent maintenance of heterochromatin states in other organisms as well.

While many lines of evidence point to an important role of siRNAs in maintenance of the heterochromatin state, I propose that siRNAs are insufficient for maintenance. This hypothesis is based on the observation that, while heterochromatin domains in fission yeast can be established by expressing synthetic siRNAs (Iida et al., 2008; Simmer et al., 2010), these domains are not self-propagating; when the synthetic siRNA construct is no longer expressed, the heterochromatin domains are lost. This suggests that other factors may be required for stable maintenance of heterochromatin. Based on these data, we propose a model in which both histone modifications and siRNAs are required for maintenance of the heterochromatic state.

The precise meaning of the word “epigenetic” is quite controversial, and there is a considerable inconsistency in the field as to what criteria need to be met before a phenomenon is considered to be epigenetic. More than just an argument over semantics, this argument is at the crux of the debate regarding the heritability of chromatin and other non-DNA elements. Here, I will discuss the definition of “epigenetic”, its usage to describe chromatin states, and finally whether I believe heterochromatin in *S. pombe* can be described as epigenetic.

There are two major elements to the most rigorous definitions of “epigenetic”. As the term is commonly used, an epigenetic state must represent a phenotype that arises without a concomitant change in DNA sequence. Second, in the most rigorous usage of the term, the state must be heritable even in the absence of the signal that initiated the epigenetic change (Berger

et al., 2009; Gottschling, 2004; Ptashne, 2007). There is evidence that active chromatin modifications turn over rapidly and are lost upon removal of activators (Katan-Khaykovich and Struhl, 2002); this represents a case in which chromatin modifications are *not* epigenetic. Thus, by this strict measure, the existence of chromatin states by themselves is insufficient to establish that a trait is "epigenetic", and thus it is incorrect to assume that all chromatin states are specified epigenetically.

Silencing in *S. cerevisiae* and Polycomb repression in *Drosophila* are both often called epigenetic states. Indeed, there is significant evidence in both cases that silenced cells are more likely to give rise to silenced progeny (Chan et al., 1994; Pillus and Rine, 1989), and this provides evidence that the tendency to be silenced is inherited. However, in both cases silencing is lost upon excision of the silencer (Busturia et al., 1997; Cheng and Gartenberg, 2000; Sengupta et al., 2004). These data suggest that the chromatin states that contribute to silencing are *not* epigenetic, as they lack the ability to self-propagate. There may be other heritable marks that contribute to the parental expression state bias, but the chromatin modifications appear not to be heritable by themselves. In contrast to these examples, the work described in this thesis demonstrates that heterochromatin in fission yeast *is* self-propagating and can persist in the absence of the initiating signal, the heterochromatin-nucleating sequence. Therefore, we conclude that heterochromatin in *S. pombe* meets the most rigorous predictions of a state that is epigenetic in origin. Thus, *S. pombe* is a highly tractable model organism in which the parameters that govern epigenetic inheritance can now be studied further. In the following section, I suggest experiments to address this.

4.2 Future studies

Enhancing the resolution of silencing in *S. pombe*

Both Chapters 2 and 3 of this thesis relied on two different methods to monitor heterochromatin spreading. The first method was ChIP, which is the most direct test for the presence and extent of heterochromatin at specific sites in the genome. However, the results from these experiments represent the enrichment profile averaged over greater than one million cells. Thus, ChIP cannot capture heterogeneity between cells in the population. We are confident that there is significant heterogeneity in the population based on the outcome of the second test for heterochromatin, monitoring the phenotypic readout of *ade6*$^{+}$ expression. Here, the presence of heterochromatin spreading can be inferred by the silencing of *ade6*$^{+}$, which results in the accumulation of a visible red pigment. This allows the extent to of silencing to be resolved on the level of the colony. However, I believe that there is much insight to be gained in developing a system that allows for silencing to be resolved on the level of single cells, as there is significant heterogeneity even within colonies. I will describe one method to achieve this resolution and how this system could be used to enhance our knowledge of silencing in *S. pombe*. In later sections I will discuss how this system could be applied to refine our understanding of heterochromatin spreading and maintenance.

One means to increase the resolution of gene expression in fission yeast is by using a reporter gene that can be visualized at the single-cell level. This can be accomplished by switching the *ade6*$^{+}$ reporter gene to a fluorescent reporter. This technique has been successfully applied to monitoring silencing in *S. cerevisiae* (Xu et al., 2006). In developing this system, it is of critical importance to use a fluorescent reporter with a low half-life, as long-lived fluorescent molecules belie the actual transcriptional status in *S. cerevisiae* (Zhang et al., 2006a).

This could be accomplished by using destabilized fluorescent proteins (e.g. Mateus and Avery, 2000) or, failing that, monitoring recovery after photobleaching (e.g. Cheutin et al., 2004).

Assuming appropriate half-life conditions could be achieved, this system could be used o address long-standing biological questions about the nature of silencing and variegation in fission yeast. Our work, in addition to classical studies of PEV in *S. pombe*, has demonstrated significant heterogeneity of expression when a reporter is embedded within heterochromatin (Allshire et al., 1995; Ayoub et al., 1999; Partridge et al., 2002), including the presence of what appear to be intermediate transcriptional states. Interestingly, in *Drosophila,* PEV is observed as a binary switch between expressed or silent states, without much evidence for intermediate states (Eissenberg, 1989). Thus, *S. pombe* may be distinct from *Drosophila* in that heterochromatin silencing can result in an intermediate level of gene expression. Alternatively, it is possible that a colony with an intermediate phenotype represents a mixture of cells that are completely silent and completely expressed. These two hypotheses could be tested by monitoring the distribution of the fluorescent protein within a population of cells. In *S. cerevisiae*, such analysis has resulted in the conclusion that silencing does result in the formation of an intermediate silencing state (Xu et al., 2006). It will be interesting to determine whether *S. pombe* behaves more like *S. cerevisiae* or *Drosophila* in this respect.

In addition to searching for intermediate silencing states, the fluorescent assay will be particularly important in assessing whether silencing states are concordant at different loci within the same cell. This could be achieved inserting constructs containing L5 upstream of two different fluorescent proteins at different sites in the genome. By measuring whether or not, for example, the two loci showed more concordance in silencing than expected by chance, we would make strides in understanding the chromosomal or genome-wide nature of PEV. If

silencing at the two loci is concomitant it would suggest that PEV is a property shared within the nucleus. Alternatively, if the two loci behave independently it would suggest that silencing state reflects locus-specific properties.

Finally, another advantage of the use of a fluorescently labeled reporter is that cells can then be sorted based on their expression state. This technique could be useful in many scenarios, some of which are described in future sections, including enhancing our ChIP procedure. In this thesis, our ChIP experiments have been conducted on heterogeneous populations of cells. This precludes us from rigorously addressing questions such as whether silencing state correlates with the presence of H3K9me. However, using a fluorescent activated cell sorter (FACS) to sort cells into groups based on expression level would provide us with a large, homogenous, population of cells that could then be ChIPped. The results of these experiments would provide insight into the fundamental nature of gene silencing, and whether H3K9me correlates with expression output.

Modifying the current assay system such that silencing can be resolved at the single-cell level will provide a much more sophisticated view of silencing within *S. pombe*. It would help us understand the nature of the intermediate colony phenotypes we observe, the extent to which silencing state is a feature of loci or nuclei, and whether transcriptional state is reflected by histone modifications. Additional uses of the assay will be discussed below.

Genome-wide analysis of sequences that influence heterochromatin spreading

The results of Chapter 2 demonstrate that heterochromatin spreading is influenced by the genomic content over which it spreads. This conclusion was based on monitoring the spread

of heterochromatin at two endogenous loci and over two types of spacer DNA. We have established a framework for more complete classification of sequences genome-wide with respect to their influence on heterochromatin spreading. Here, I propose a method of classifying the effect specific DNA sequences have on heterochromatin spreading.

Insertion of barrier sequences between L5 and a reporter gene results in the increased expression of the reporter gene (Scott et al., 2006). Thus, sequences that negatively effect the propagation of heterochromatin can be identified by the phenotypic readout of an associated reporter gene. Similarly, we hypothesize that when sequences that enhance spreading are inserted between L5 and *ade6*$^{+}$, silencing of the reporter gene would be increased. It would be possible to create strains with a library of genomic fragments inserted between L5 and the reporter, monitor silencing in these strains, and infer how these fragments affect the spread of heterochromatin. This approach would benefit from using a fluorescent reporter coupled with high-throughput FACS to monitor the distribution of silencing within a population containing the same inserted DNA sequences.

If the model proposed in Chapter 2 – that all sequences fall along a continuum with respect to their ability to enhance or impede the spread of heterochromatin – is correct, then I would predict that the results of the screen would identify fragments of the genome spanning the entire range of effects. Furthermore, one could choose to focus on the extremes of the continuum to derive general properties of and classification for DNA sequences that impede or enhance the spread of heterochromatin. For example, our work in Chapter 2 suggests that genes with strong promoters attenuate the spread of heterochromatin. This argument would be strengthened by the identification of a number of strong promoters that negatively effect the propagation of heterochromatin. Additionally, heterochromatin barriers have been shown to

work by diverse and only somewhat understood mechanisms (West et al., 2002). Thus, it would be beneficial to create a complete list of the sequences that exhibit barrier activity and try to elucidate general themes and mechanisms.

Identification of factors that affect the maintenance of heterochromatin

My work has demonstrated the heterochromatin can be maintained independent of the DNA sequence used to establish it. This novel finding sets the stage for future studies that characterize the features that govern this maintenance. Here I will describe different experimental approaches to identify address the role of the genomic "neighborhood", trans-acting factors, and DNA replication on heterochromatin maintenance. The outcome of these proposed studies will provide insight into factors that influence maintenance of chromatin state and will be highly valuable to understanding how these states are maintained throughout development in other organisms.

Genomic neighborhood

Our work in Chapter 3 examined the extent to which heterochromatin was maintained throughout a *de novo* heterochromatin domain. Even within this single neighborhood, there was significant heterogeneity in terms of the extent to which heterochromatin was maintained. These data serve as preliminary evidence that sequence content affects maintenance. Additionally, the finding that maintenance of silencing requires Dcr1 suggests that genomic sequences within the immediate vicinity or neighborhood are being processed into siRNAs, thus allowing the heterochromatic state to persist epigenetically. As the ability of genomic sequences

to support the production of siRNAs and gene silencing is thought to vary (Iida et al., 2008), we anticipate this is another mechanism through which sequence can affect the probability or efficiency of maintenance. There is support for this hypothesis from work in other organisms. In *S. cerevisiae*, for example, protosilencers are required for maintenance of silenced chromatin in the absence of the silencer in G_o-arrested cells (Cheng and Gartenberg, 2000; Cheng et al., 1998). In maize, gene silencing can be initiated by the transient presence of a transposable element, and the stability of this silencing after loss of the transposable element depends on location in the genome (Singh et al., 2008).

Based on these converging lines of evidence that suggest that DNA sequence can alter heterochromatin maintenance, it would be promising to test this hypothesis directly in fission yeast. To accomplish this, I propose that the floxed-L5-*ade6*$^+$ construct could be inserted, using random or targeted methods, throughout the genome. After excision of L5, maintenance of silencing could be monitored at the different loci. However, maintenance of silencing cannot be examined in the same fashion as Chapter 3. Here I will discuss why the old methods are not applicable to this approach and the development of an alternate strategy to assess maintenance.

The formation of heterochromatin at ectopic sites allows us to uncouple the nucleating sequence from the heterochromatic state in a fashion that would complicated by the presence of redundant nucleating sequences at endogenous heterochromatic loci. However, this approach is complicated by the possibility that the formation of heterochromatin at ectopic sites negatively affects the viability of the cell. This could result from partially silencing genes with required, and dosage-sensitive, roles in the cell or from interference with chromosome segregation or other cellular activities. I expect that, if the formation of heterochromatin causes

a reduction in the ability of a cell to grow or divide in liquid culture, the magnitude of this effect will vary at different locations in the genome. Thus, the comparison of the extent of heterochromatin maintenance at different sites in the genome could be heavily influenced by effect of heterochromatin at that site. To bypass this, maintenance of heterochromatin could be assessed via pedigree analysis in which newly divided cells are separated on solid media and allowed to form colonies. Comparison of switching rates on solid media would largely overcome a bias in growth rate that could impede analysis in liquid media.

The results from this pedigree analysis would provide evidence as to whether genomic position influences the maintenance of heterochromatin. Features that could influence this may include proximity to another heterochromatin domain, replication timing (discussed in later sections), nucleosome turnover, transcription, and the propensity of a locus to form siRNAs. Implicating any one of these factors in heterochromatin maintenance would provide insight into the factors that affect the stability and inheritance of chromatin states in fission yeast and could therefore be used to inform studies of chromatin maintenance in other organisms.

At the *de novo* heterochromatin domain discussed in Chapter 3, heterochromatin cannot be reestablished once lost. Thus, this heterochromatin domain is eventually lost from the population. It is possible that there are other genomic loci at which heterochromatin can be reestablished in the absence of the original nucleating sequence. These genomic loci would possess intrinsic characteristics that would allow them to be converted to nucleating sequences by the transient presence of H3K9me. This finding would be important to understanding how domains of heterochromatin arise throughout evolution, suggesting that the transient presence of H3K9me in conjunction with particular genomic characteristics results in the formation of a stable heterochromatin domain.

Trans-acting factors

In Chapter 3 our work demonstrated that the CAF-1 histone chaperone complex plays an important role in the maintenance of heterochromatin. These data demonstrate that trans-acting factors can mediate maintenance and that our system, in which heterochromatin cannot be reestablished, provides a particularly sensitive assay to identify these factors, which have an otherwise subtle effect on heterochromatin. As a screen, silencing could be monitored after excision of the L5 element in a library of *S. pombe* mutant strains. We expect, that like CAF-1, there exist other factors with specific roles in maintenance that have yet to be identified because their mutant phenotype in strains that can reestablish heterochromatin is mild.

DNA replication

As discussed in Chapter 1, after DNA replication old histones, those that would carry the heterochromatic histone modifications, are thought to be diluted by half on the daughter DNA strands (reviewed in Probst et al., 2009). Thus, I propose that the period following DNA replication may be a particularly vulnerable time for heterochromatin. Failure to transmit the appropriate number of modified histones, or other factors involved in the maintenance of heterochromatin, could result in the loss of the heterochromatin state. If this hypothesis were true, one would expect that much of the loss of heterochromatin that we observe would occur immediately after DNA replication, resulting from a failure to replenish the modified state. However, in *S. cerevisiae* silencing can be lost in arrested cells, suggesting that loss of a chromatin state can occur independent of DNA replication (Cheng and Gartenberg, 2000). To

determine the effect of DNA replication on maintenance of the heterochromatin state in *S. pombe*, I propose to test the stability of heterochromatin in conditions in which the doubling time of yeast is increased, including both low nitrogen and low glucose. These experiments would allow me to determine whether loss rates correlate with total time in culture (the same for both slow and normally growing strains) or with the number of divisions (decreased in the nutrient-restricted strains). The latter observation would suggest that progression through the cell cycle is disruptive to the maintenance of heterochromatin. As the results from these experiments could be altered by an unanticipated effect of nutrient starvation on heterochromatin, independent of DNA replication, it will be important to follow these studies with direct tests for the loss of silencing in the absence DNA replication. The fluorescent protein strategy could prove particularly useful, as the loss of silencing could be monitored in single, arrested, cells.

In addition to the dilution of histones during DNA replication, heterochromatin in fission yeast is remodeled during S phase of the cell cycle (Chen et al., 2008; Kloc et al., 2008). In fission yeast, unlike many other eukaryotes, pericentromeric heterochromatin is replicated early in S phase (Kim et al., 2003), and studies support the hypothesis that specific events, including the production of siRNAs, that occur during this window allow heterochromatin to be maintained in the next generation (Chen et al., 2008; Kloc et al., 2008). Thus, it is possible that if DNA sequences were replicated outside of early S phase, the conditions that allow for maintenance of the heterochromatic state would not be met. The *de novo* heterochromatin domain studied in Chapter 3 is located in a region that, like the centromeres, is replicated early in S phase (Hayashi et al., 2007). Moving the L5-construct to late-replicating regions of the genome could disrupt the coordination of heterochromatin remodeling and DNA replication. If the coupling of

these two events is important, then one would expect the loss of heterochromatin to occur much faster in late-replicating regions of the genome.

4.3 Conclusion

If the challenge of this post-genomic era is to understand how the set of instructions contained within the genome are interpreted to enact patterns of gene expression that are both specific and flexible, then critical to this endeavor are studies that address the relationship between genomic code and chromatin state. In this thesis I have chosen to focus on the heterochromatic state, as it can effect gene expression over entire domains. I have demonstrated that the extent of heterochromatin spreading is linked to the genomic sequences over which it spreads. However, in fission yeast, the heterochromatic state is maintained in the absence of the sequences required to establish it. Thus, the work presented here has contributed to understanding how genomic features contribute to the establishment, maintenance, and spreading of heterochromatin and provides evidence that chromatin domains are shaped by both genomic and epigenetic signals.

Appendix A: List of strains used in this thesis

Strain	Genotype	Source
Kfy1	h- ura4D18	R. Allshire (FY106)
Kfy2	h- ade6-210	R. Allshire (FY92)
Kfy3	h+ ura4D18 ade6-210 leu1-32 arg3D his3D	R. Allshire (FY1645)
Kfy4	h- ura4D18 ade6-210 leu1-32 arg3D his3D	R. Allshire (FY1646)
Kfy450	h+ ade6DN/N leu1-32 ura4D18 his3D arg3D	R. Allshire (FY3749)
Kfy687	swi6+333 (leu2+) KΔ::ura4+ leu1-32 ura4DS/E his2 ade6-210	S. Grewal (SPG1232)
Kfy501	h+ ade6DN/N his3D leu1-32 arg3D	This Thesis
Kfy616	h+ ura4::ade6 ade6DN/N his3D leu1-32 arg3D	This Thesis
Kfy617	h+ ura4::ade6 ade6DN/N his3D leu1-32 arg3D	This Thesis
Kfy618	h+ ura4::ade6 ade6DN/N his3D leu1-32 arg3D	This Thesis
Kfy619	h+ ura4::ade6 ade6DN/N his3D leu1-32 arg3D	This Thesis
Kfy620	h+ ura4::L5-ade6 ade6DN/N his3D leu1-32 arg3D	This Thesis
Kfy621	h+ ura4::L5-ade6 ade6DN/N his3D leu1-32 arg3D	This Thesis
Kfy622	h+ ura4::L5-ade6 ade6DN/N his3D leu1-32 arg3D	This Thesis
Kfy623	h+ ura4::L5-ade6 ade6DN/N his3D leu1-32 arg3D	This Thesis
Kfy812	spbc2f12.03::ura4::L5-ade6 ade6DN/N his3D leu1-32	This Thesis
Kfy847	swi6+333 (leu2) ura4::L5-ade6 ade6DN/N his3D leu1-32 arg3D	This Thesis
Kfy901	swi6+333 (leu2) ura4::L5-ade6 ade6DN/N his3D leu1-32 arg3D	This Thesis
Kfy902	swi6+333 (leu2) ura4::L5-ade6 ade6DN/N his3D leu1-32 arg3D	This Thesis
Kfy937	ura4::5kbPombe-ade6 ade6DN/N his3D leu1-32 arg3D	This Thesis
Kfy1011	ura4::5kbPombe-ade6 ade6DN/N his3D leu1-32 arg3D	This Thesis
Kfy1044	ura4::5kbPombe-ade6 ade6DN/N his3D leu1-32 arg3D	This Thesis
Kfy1012	ura4::L5-5kbPombe-ade6 ade6DN/N his3D leu1-32 arg3D	This Thesis
Kfy1013	ura4::L5-5kbPombe-ade6 ade6DN/N his3D leu1-32 arg3D	This Thesis
Kfy1014	ura4::L5-5kbPombe-ade6 ade6DN/N his3D leu1-32 arg3D	This Thesis
Kfy935	ura4::7kb-ade6 ade6DN/N his3D leu1-32 arg3D	This Thesis
Kfy991	ura4::7kb-ade6 ade6DN/N his3D leu1-32 arg3D	This Thesis
Kfy992	ura4::7kb-ade6 ade6DN/N his3D leu1-32 arg3D	This Thesis
Kfy641	ura4::L5-7kb-ade6 ade6DN/N his3D leu1-32 arg3D	This Thesis
Kfy644	ura4::L5-7kb-ade6 ade6DN/N his3D leu1-32 arg3D	This Thesis
Kfy647	ura4::L5-7kb-ade6 ade6DN/N his3D leu1-32 arg3D	This Thesis

Kfy724	h+ ura4::2xL5-ade6 ade6DN/N his3D leu1-32 arg3D	This Thesis
Kfy725	h+ ura4::2xL5-ade6 ade6DN/N his3D leu1-32 arg3D	This Thesis
Kfy726	h+ ura4::2xL5-ade6 ade6DN/N his3D leu1-32 arg3D	This Thesis
Kfy1130	ura4::L5-7kb::(P3nmt1-his3)-ade6 ade6DN/N his3D leu1-32	This Thesis
Kfy1132	ura4::L5-7kb::(P3nmt1-his3)-ade6 ade6DN/N his3D leu1-32	This Thesis
Kfy1133	ura4::L5-7kb::(P3nmt1-his3)-ade6 ade6DN/N his3D leu1-32	This Thesis
Kfy1137	ura4::L5-7kb::(P41nmt1-his3)-ade6 ade6DN/N his3D leu1-32	This Thesis
Kfy1140	ura4::L5-7kb::(P41nmt1-his3)-ade6 ade6DN/N his3D leu1-32	This Thesis
Kfy1141	ura4::L5-7kb::(P41nmt1-his3)-ade6 ade6DN/N his3D leu1-32	This Thesis
Kfy1265	h- ura4::L5flox-ade6 ade6DN/N leu1-32 his3D arg3D4	This Thesis
Kfy1266	h+ ura4::L5flox-ade6 ade6DN/N leu1-32 his3D arg3D4	This Thesis
Kfy1267	h+ ura4::L5flox-ade6 ade6DN/N leu1-32 his3D arg3D4	This Thesis
Kfy1270	swi6::arg ura4::L5flox-ade6 ade6DN/N leu1-32 his3D arg3D4	This Thesis
Kfy1271	swi6::arg ura4::L5flox-ade6 ade6DN/N leu1-32 his3D arg3D4	This Thesis
Kfy1272	swi6::arg ura4::L5flox-ade6 ade6DN/N leu1-32 his3D arg3D4	This Thesis
Kfy1274	h- Δpcf1::KanMX6	Y. Murakami DK 235
Kfy1275	h- Δpcf2::KanMX6	Y. Murakami DK 238
Kfy1297	Δpcf1::KanMX6 ura4::L5flox-ade6 ade6DN/N leu1-32	This Thesis
Kfy1301	Δpcf2::KanMX6 ura4::L5flox-ade6 ade6DN/N leu1-32	This Thesis
Kfy1455	dcr1:;KanMX6 ura4::L5flox-ade6 ade6DN/N leu1-32	This Thesis
Kfy1465	dcr1::KanMX6 ura4::L5flox-ade6 ade6DN/N leu1-32	This Thesis

Appendix B: List of primers used in this thesis

Oligo	Sequence	Purpose
BWP32F	TTCCTACTGCCATCAAAGCA	iPCR
BWP33F	ATCCATCCCAGCTGAACAAA	Confirm L5 excision
BWP33R	ATACTGCACCAGGCTGGATT	Confirm L5 excision
BWP34F	gatatcaggcctactagtgcatgcatcgatagatctTCAACTTGGTGGTGAGGTAAACG	Construction of BW5
BWP34Rb	aggcctcccgggagctcCAGCACATTATTCGGGGGG	Construction of BW5
BWP37F	TGATATGAGCCCAAGAAGCA	iPCR
BWP38F	CCCTACATGGAATGGAATCG	*his3*$^{+}$ qRT-PCR
BWP38R	GCAAACAGCAAGGTTTGGAT	*his3*$^{+}$ qRT-PCR
BWP41F	TTTACCAAGGCCTTTGATGC	*pmp20*$^{+}$ qRT-PCR
BWP41R	CCTTGTCAGCGGAGGAAATA	*pmp20*$^{+}$ qRT-PCR
BWP45F	TTCAACGAGACATGCGAAAC	*mug135*$^{+}$ qRT-PCR
BWP45F	TTCAACGAGACATGCGAAAC	*mug135*$^{+}$ qRT-PCR
BWP45R	TCGAGGCAAATTAGGGTCAG	*mug135*$^{+}$ qRT-PCR
BWP45R	TCGAGGCAAATTAGGGTCAG	*mug135*$^{+}$ qRT-PCR
BWP50F	TGGCCTGAATATGTTAATGAGAGA	7.2kb lambda ChIP
BWP50R	GCAATAGCTGATAATCGATGCAC	7.2kb lambda ChIP
BWP51F	TACGTTGCAGGTTGCTTTCA	7.2kb lambda ChIP
BWP51R	TTAACCGCCCTATTCTCTCG	7.2kb lambda ChIP
BWP53F	TTACTATGTAAACACCAGGCATGA	7.2kb lambda ChIP
BWP53R	GAATGAAAAGTGATATACTGGAATGTT	7.2kb lambda ChIP
BWP54F	GTCATCTGCGAGGCTGTTCT	7.2kb lambda ChIP
BWP54R	TCGATGGAAAAACTTTTCTCTTTAC	7.2kb lambda ChIP
BWP55F	CCGACCAGAACACCTTGC	7.2kb lambda ChIP
BWP55R	CCCACAGTACCCAATGATCC	7.2kb lambda ChIP
BWP56F	ATAAAGCACCAACGCCTGAC	7.2kb lambda ChIP
BWP56R	AACACAAGAGCAGCTTGAGGA	7.2kb lambda ChIP
BWP57F	GCGAGATTACAAAGTTACCTGTCA	7.2kb lambda ChIP
BWP57R	TGCACCATTCTGAGATGTTTTT	7.2kb lambda ChIP
BWP58F	GCGATAAGTGGACCCAACTC	7.2kb lambda ChIP
BWP58R	CATCTGCTCACGGTCAAAGT	7.2kb lambda ChIP
BWP59F	GCGCACTACTGGCTGGTTAC	7.2kb lambda ChIP
BWP59R	GCCCATGACAGGAAGTTGTT	7.2kb lambda ChIP
BWP60F	TTCGATCTCCAGCACATCAG	7.2kb lambda ChIP
BWP60F	AGGCCCATAGCTTCCTGTTT	7.2kb lambda ChIP
BWP61F	CGGTTTGTCAGGGAAGTTGT	7.2kb lambda ChIP
BWP61R	ATGGCTTCAGAACAGGCATT	7.2kb lambda ChIP

BWP71F	TCAGGAAGCGAATTCTAAGGA	*spcc330.03*$^{+}$ qRT-PCR
BWP71R	AAAGCATCCCAATCTTGTTGA	*spcc330.03*$^{+}$ qRT-PCR
BWP74F	ACTCTGTCTGGATTGGTGGA	*act1*$^{+}$ qRT-PCR
BWP74R	AACGATACCAGGTCCGCTCT	*act1*$^{+}$ qRT-PCR
BWP77F	GGCTGACAAAAGCGAAAAAG	*330.07*$^{+}$ qRT-PCR
BWP77R	AATGCCACTTCCGCTAAAAA	*330.07*$^{+}$ qRT-PCR
BWP84F	CGGCATCGCTTGTACTTTTT	ChIP qPCR centromere
BWP84R	GACGGAACCAAATGATGTGA	ChIP qPCR centromere
BWP85F	CATGGAAATTGCAGTGATGG	*ade6*$^{+}$ qRT-PCR
BWP85R	CGAGCAGGGGCATATACTAAA	*ade6*$^{+}$ qRT-PCR
BWP86F	TTGATGCCAGACCGTAATGA	*ura4* locus ChIP
BWP86R	TCTGACATGGCATTCCTCAA	*ura4* locus ChIP
BWP89F	CTGCGGTAGCGATATCATCA	iPCR
BWP91F	TAAGGGGAAGGGAAAGGAAA	5kb IG ChIP
BWP91R	GCTAGCCGTTTCTCATCTCG	5kb IG ChIP
BWP92F	TTGAACGATGGAAAACAGTGA	5kb IG ChIP
BWP92R	TTTCAGACTCCCTACCCTTTTT	5kb IG ChIP
BWP93F	CAAACGATAGCAACTATGTCAGC	5kb IG ChIP
BWP93R	GCCTGCGAAAACTTCAATCT	5kb IG ChIP
BWP94F	AAAAACTGCAAGCACGAGGT	5kb IG ChIP
BWP94R	AAACGGCCGACAGATTAAAA	5kb IG ChIP
BWP95F	TTGCATCTAATGAATGTTTTTGC	5kb IG ChIP
BWP95R	TAGGGGGATAAGTGGAACGA	5kb IG ChIP
BWP96F	GGATAGGAATTTCCCGGTGT	5kb IG ChIP
BWP96R	TGCATAGGCCGATGAAAAGT	5kb IG ChIP
BWP98F	TGCATCATGAGAAAGGGAGA	*ura4* locus ChIP
BWP98R	GATTTGGGAAAACGGATGTG	*ura4* locus ChIP
BWP99F	TTTTGAATACGCCGTTACTCC	*ura4* locus ChIP
BWP99R	GATCCGACAGCAACCATTTT	*ura4* locus ChIP
BWP100F	GGCCTTAGGTAAAAAGCATCG	*ura4* locus ChIP
BWP100R	TGAGCCCAAGAAGCAATTTT	*ura4* locus ChIP
BWP102F	CACAATATCGGTGCAAATAGG	*ura4* locus ChIP
BWP102R	TCAGGAGGAATTATTTTGGGAAT	*ura4* locus ChIP
BWP103F	AAGATGCATTTCCATTATAATCCTC	*ura4* locus ChIP
BWP103R	TCATTTTTGCTTATCGATCACTG	*ura4* locus ChIP
BWP104F	CGTATATGGGGTTGGCTTGT	*ura4* locus ChIP
BWP104R	CGACAAGGTACAGTTTAGCAATG	*ura4* locus ChIP
BWP107F	TTTATTCAACACAGCCAAGGAA	*ura4* locus ChIP
BWP107R	CATACAAATGAATATTCGACAATGTG	*ura4* locus ChIP
BWP108F	TGACCGTAGTCGTAAACTCGAA	*spcc330.19*$^{+}$ qRT-PCR

BWP108R	AAAGCAAGACAAGCGGTATGA	*spcc330.19*$^{+}$ qRT-PCR
BWP111F	GCCTGGTGATTCAATCTTCAAA	*rhp7*$^{+}$ qRT-PCR
BWP111R	TTGCCCAAATTTGATTAGCC	*rhp7*$^{+}$ qRT-PCR
BWP114F	AAACAACCTTGAAAATGAATCAAA	*ura4* locus ChIP
BWP114R	AACGAAGTGATGATGTTTTTCTTG	*ura4* locus ChIP
BWP118F	ACGCTGAGAGGGAGTTTGAA	*ura4* locus ChIP
BWP118R	CCAACATGCCTGTAAGTAGTTTT	*ura4* locus ChIP
BWP120F	CCCGTATTTCTCAAAATAAATCTCA	*ura4* locus ChIP
BWP120R	CACCAAAGCCGATTTATTCC	*ura4* locus ChIP
BWP121F	GCTAGAAAGGAAAGCATTGGAA	*ura4* locus ChIP
BWP121R	TCAAAAGCCCTTTTCTTGGA	*ura4* locus ChIP
BWP128F	AGCATCTACAACAAATTTGACTTTT	*alg11*$^{+}$ qRT-PCR
BWP128R	GCGTTCGAGGTGAAGAAGAC	*alg11*$^{+}$ qRT-PCR
BWP135F	AACCAACGACATCATGGGTAG	L5-*ade6*$^{+}$ junction
BWP135R	CAGGCTGGATTTCGTTTACC	L5-*ade6*$^{+}$ junction
BWP159F	GTATTCTGGGCCTCCATGTC	lambda-Pnmt1 junction
BWP159R	TGAAACAACCGCTGATGACT	lambda-Pnmt1 junction
BWP160F	TTTCAATCTCATTCTCACTTTCTGA	Pnmt1-*his3*$^{+}$ junction
BWP160R	GCACAGCGATAAGGCTGAAG	Pnmt1-*his3*$^{+}$ junction
BWP165F	TTACGCACGTTTCATTTCCA	*spbc2f12.03*$^{+}$ locus ChIP
BWP165R	GGCTCATCTAGGTCAAGTTCG	*spbc2f12.03*$^{+}$ locus ChIP
BWP166F	GATGCCTATTATCCGTGAAGC	*ceg1*$^{+}$ qRT-PCR
BWP166R	TTGATTGCAGGAGCATCATC	*ceg1*$^{+}$ qRT-PCR
BWP167F	AACCATCAACACGTTGGTCA	*rpl802*$^{+}$ qRT-PCR
BWP167R	AAAGACCGGTTCTACGAGCA	*rpl802*$^{+}$ qRT-PCR
BWP168F	CAAATATGCGGGCTCTCAA	*spbc2f12.03*$^{+}$ locus ChIP
BWP168R	TTCGTGGCTCATTCTCATTTT	*spbc2f12.03*$^{+}$ locus ChIP
BWP169F	TGCCCGTGAATTTAAGAAGG	*spbc2f12.03*$^{+}$ locus ChIP
BWP169R	TTCATTCAAATCAATAGCATCATT	*spbc2f12.03*$^{+}$ locus ChIP
BWP170F	AACCATGCAAACAAAGCAGA	*spbc2f12.03*$^{+}$ locus ChIP
BWP170R	GAAGGTTGTGGCTTATGTTCAG	*spbc2f12.03*$^{+}$ locus ChIP
BWP171F	CGATCGTCCGAAAATCAGTC	*spbc2f12.05*$^{+}$ qRT-PCR
BWP171R	TTCATTGAAGCTTACTGGCAAA	*spbc2f12.05*$^{+}$ qRT-PCR
BWP172F	TTTGCAAAAAGGCAAGGAAT	*spbc2f12.03*$^{+}$ locus ChIP
BWP172R	TAAATAAACTTTACTCAGTGTTGTTGG	*spbc2f12.03*$^{+}$ locus ChIP
BWP173F	CTGAATAGCGGACCATTGCT	*spbc2f12.03*$^{+}$ locus ChIP
BWP173R	AAGTGTCCAAACCAGTCACAA	*spbc2f12.03*$^{+}$ locus ChIP
BWP174F	CAAGGCTTGCTGCTCTTTTT	*spbc2f12.03*$^{+}$ locus ChIP
BWP174R	GGATGATGGAGAGCCTGAAA	*spbc2f12.03*$^{+}$ locus ChIP
BWP175F	TGCCAATGCTTCTACCTTTG	*spbc2f12.03*$^{+}$ locus ChIP

BWP175R	TCTGTGAAAGTCCAGTTCGAATA	*spbc2f12.03*$^{+}$ locus ChIP
BWP176F	TTGTTTACATAGGACTGCGTTGA	*spbc2f12.03*$^{+}$ locus ChIP
BWP176R	AATCAATTGGCAAGCTGAGG	*spbc2f12.03*$^{+}$ locus ChIP
BWP177F	CGGATTTCCTTACTTGGCACT	*spbc2f12.03*$^{+}$ locus ChIP
BWP177R	GGATCTTAGGTACTAGGGCTGGA	*spbc2f12.03*$^{+}$ locus ChIP
BWP178F	CATGCCTTTATTTCCCCAAA	*mrpl7*$^{+}$ qRT-PCR
BWP178R	CCAAACGGGTATCTTTGGAA	*mrpl7*$^{+}$ qRT-PCR
BWP179F	AAATGATGGGGATGAACCAG	*byr2*$^{+}$ qRT-PCR
BWP179R	CCAACGGAAATGAAAAATCG	*byr2*$^{+}$ qRT-PCR
BWP180F	TCCATAAATGCAGGAAAGTGG	*spbc2f12.03*$^{+}$ locus ChIP
BWP180R	CGAGACTAAGGGAGGCAGGT	*spbc2f12.03*$^{+}$ locus ChIP
BWP181F	TGGTTCAGCTCACTTCTCCA	*spbc2f12.03*$^{+}$ locus ChIP
BWP181R	TTGGCAATATAATGCTGTTTCG	*spbc2f12.03*$^{+}$ locus ChIP
BWP232F	GTGCCAGGCGAGGGTATTAT	ChIP qPCR
BWP232R	TTTCGTTTACCTCACCACCA	ChIP qPCR
BWP246F	ACTATGCTTCGTCGGCATCT	Confirm L5 excision
BWP248F	GCCACCCTTTCTCTGAATTG	ChIP qPCR
BWP248R	TAAAGCAAGGGAGCATACGG	ChIP qPCR
BWP249F	CAAATGGGGACGTCATGTAA	ChIP qPCR
BWP249R	TGAAGCATTCCCCTTTGAAT	ChIP qPCR
BWP251F	CGGCCATAAACCAATGAGTC	ChIP qPCR
BWP251R	CTAGCTTTCCTGGACCTTCG	ChIP qPCR
BWP254F	TTATCGAAAATCTTTCTTTGAAAAC	ChIP qPCR
BWP254R	GGACTGTGAATTTGAGTAATGAAG	ChIP qPCR
BWP255F	TCCTGTTTCCTCCACTAGATATG	ChIP qPCR
BWP255R	TTGTTATTCAGAGTATGGCAACTAAAA	ChIP qPCR
BWP256F	CCTCAGGAAACTCAAAGACGA	ChIP qPCR
BWP256R	AAAAGCAGTTTTTGAGAAACCA	ChIP qPCR
BWP258F	AATTTGGACCGGTAAACAGTG	ChIP qPCR
BWP258R	GGCAATCTTCATATGCTCGTC	ChIP qPCR
BWP260F	GCTTAGACCCGAAACTCTTAATG	ChIP qPCR
BWP260R	TTGTTAACGGCATGTTTTCAAG	ChIP qPCR
BWP261F	CCCTGATTTGCCTCCAATTA	ChIP qPCR
BWP261R	AAAGTTACCCCATAGCCCTGT	ChIP qPCR
E367	CCATAAAGATGCAAAGTTGCACC	iPCR

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Biography

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Wheeler, B. S., Blau, J. A., Willard, H. F., and Scott, K. C. (2009). The impact of local genome sequence on defining heterochromatin domains. PLoS Genet *5*, e1000453.