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Mechanisms of RNA Mediated Silencing in *S. pombe*

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ABSTRACT

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Heterochromatin of eukaryotic genomes has classically been defined as condensed chromatin that is repressive to transcription and typically resides at highly repetitive regions of the genome. Genetic and molecular analyses have demonstrated that these regions are essential for genome integrity and stability. The fission yeast, *S. pombe* has emerged as a powerful system to understand the mechanisms of heterochromatin assembly. The conserved RNA interference pathway has been shown to silence regions of the genome through post-transcriptional and transcriptional gene silencing mechanisms. RNAi directs transcriptional silencing via the recruitment of heterochromatin formation at the centromeres, telomeres, and mating-type region of *S. pombe*. Assembly of heterochromatin domains has been postulated to occur in discrete nucleation and spreading (from nucleation sites) phases that requires histone modifiers as well as RNAi. However, due to the highly repetitive architecture of heterochromatic regions, defining the mechanisms necessary for initiating heterochromatin nucleation has proven problematic. We therefore aimed to investigate the mechanism of how heterochromatin is targeted to specific loci in order to establish a silent domain. We also sought to determine potential differences in the regulation of the various repeats within the heterochromatin regions of *S. pombe*.

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CHAPTER 1

Introduction

i.

EPIGENETICS AND CHROMATIN

As researchers have gained insights from whole genome sequencing projects, new questions of what additional mechanisms contribute to the vast complexity of an organism beyond the genetic code have become the focus of many investigations. In the beginning of the human genome project scientists estimated that the genome of a human needed approximately 100,000 genes to carry out all the cellular processes needed to make up such a complex organism. However, upon completion, the number of genes in the human genome was more around ~25,000. How then can we have only a few thousand more genes than a worm or a fly and a few thousand less than a weed or a puffer fish? We have moved beyond the study of classical Mendelian genetics, where by allelic differences are caused by mutations or changes in the DNA sequence, and on to the study of "epigenetics". The current working definition of an epigenetic phenomenon would best be described as a heritable change in phenotype that does not involve a change in the DNA sequence. This broad definition covers various cellular processes such as paramutation, imprinting, gene silencing, x-chromosome inactivation, position effect variagation, nucleolar dominance, and even infectious proteins termed prions. We have entered a phase in genome biology were these heritable epigenetic changes are controlled by non-coding transcripts, post-translational modifications to histone tails, DNA methylation, and small RNAs. The expression or repression of a gene is influenced by the architecture of the genome and intricate pathways of modifications and the proteins they recruit contribute to their regulation. The field of epigenetics and even the definition is ever changing and new levels to the puzzle are discovered everyday.

The study of epigenetics began with work done by embryologists studying the evolution and development of eukaryotic organisms. The scientist C. H. Waddington first coined the term "epigenetics" in 1942 (Waddington 1942b; Waddington 1942a). He first described the phenomenon as "the study of casual mechanisms by which genes of the genotype bring about phenotypic effects". He was describing a mechanism by which genes give rise to phenotypes during development and how the interaction of genes with their surroundings may contribute to a particular phenotype.

The fact that DNA is not naked contributes to the multidimentional layers of the genome. The basic organization of eukaryotic chromosomes consists of protein and DNA complexes or chromatin. Chromatin is made up of repeated units of a DNA/protein complex called a nucleosome (Kornberg 1974). Nucleosomes were first described as "beads on a string" when these chains of particles where seen by electron micrograph (Thoma et al. 1979). In 1984 Aaron Klug along with a member of his lab, Timothy Richmond, solved the nucleosome core structure to 7 angstroms resolution (Richmond et al. 1984). Timothy Richmond's group would later solve the structure to 2.8 angstroms resolution in 1997 (Luger et al. 1997). Nucleosomes consist of 147 bases pairs of DNA wrapped around a complex of proteins called histone proteins. The histone octamer includes two of each of the class H2A, H2B, H3, H4 core histones. The linker histone H1 binds the nucleosome at the entry and exit sites of the DNA. The structure of the nucleosome revealed that 30% of the mass of histones could be attributed to their highly flexible N-terminal tails, which protrude from the DNA (Luger et al. 1997; Richmond and Davey 2003). Post-translational modifications of the histone tails contribute to the regulation of gene expression and chromatin organization and will be discussed in detail later in this chapter

(Jenuwein and Allis 2001). The structure of the nucleosome continues to contribute to the understanding of how the chromatin template potentiates genetic information.

In 1950 Stedman and Stedman would suggest that histones act as general repressors of gene expression (Stedman 1950). However, they explained a mechanism by which different cells contain different histones, and histones were stripped off of promoters for gene activation and their subsequent expression. They were not to far off considering we now know that histones are remodeled for certain functions. Although, it was not until 1964 when Vincent Allfrey proposed that histone acetylation was correlated to gene activation that chromatin moved beyond a simple role in compacting the genome, to a role in the regulation of gene expression (Allfrey et al. 1964).

Although nucleosomes consist of a histone core octamer, researchers have also uncovered histone variant proteins, which provide an additional layer of transcriptional regulation independent of DNA replication. Some think that replacement of existing histones with histones with a specialized function allows the genome a way to clear the existing modifications on the histone tails in order to provide immediate responses to change in environment or DNA damage. Each histone variant has been linked to specific functions. For example, histone H3 is replaced with H3.3 in order to activate transcription (Ahmad and Henikoff 2002). However, the histone H2A replacement, H2A.Z has been associated with both transcriptional activation and repression (Allis et al. 1986; Meneghini et al. 2003; Rangasamy et al. 2003; Rangasamy et al. 2004). CENP-A is a centromere specific H3 variant and is required for proper centromere function (Palmer et al. 1987; Howman et al. 2000). H2A replacement with H2A.X has been linked to a

response to DNA damage (Howman et al. 2000). In all, histone variants provide a way for an organism to quickly respond to the specialized needs of a cell, independent of the cell cycle in order to regulate gene expression and/or repression, to respond to changes in the environment, and to distinguish distinct regions of the genome.

DNA methylation is the addition of a methyl group to a cytosine within the DNA. DNA methylation was the first epigenetic mechanism known to correlate with gene repression and is present in all eukaryotes except for yeast (Bird 2002). DNA methylation is present at regions of non-coding DNA, repetitive elements, and promoters in order to induce or maintain silencing. However, since this particular modification does not pertain to this thesis (*S. pombe* does not methylate DNA), it will not be discussed further.

The study of epigenetics and chromatin has shifted researchers views on how the genome is controlled and organized. Chromatin is no longer just viewed as a tool to wrap and organize DNA in the nucleus, but a highly dynamic and functional structure. Epigenetic mechanisms provide the genome a way to establish and maintain an "on" or "off" state through cell division as well as provide a mechanism for a cell to respond to developmental cues and environmental factors. In addition, the heritable nature of epigenetic modifications provides a cellular memory from one generation to the next.

Epigenetics studies will continue to impact and change our views on how cellular processes are carried out within the genome.

ii.

HETEROCHROMATIN VS. EUCHROMATIN

Chromatin is usually divided into two classes termed heterochromatin and euchromatin, Emil Heitz first coined the terms while developing a new in situ staining method using moss in 1928. He described the two different nuclear territories he observed as heterochromatin for the dark staining portion of the nucleus that remained condensed during interphase, and euchromatin which was lighter staining and became invisible at late telophase (Heitz 1928). Euchromatin, in the modern sense can be described as part of the genome that is comprised of coding sequences and is generally transcriptionally "active". It is usually described as having an "open" structure that is sensitive to nucleases that allows for a state permissive to transcription. However, the focus of this thesis research is on heterochromatin and silenced regions of the genome.

Heterochromatin is comprised of highly compacted regions of non-coding or "junk" DNA. Due to the limited transcription that occurs in these regions, heterochromatin was once thought to silence regions of the genome that were not needed. However, researchers have discovered that these regions are not only evolutionarily conserved but have an integral role in genome stability and organization. For example, the disruption of centromere silencing factors in *S. pombe* results in a loss of heterochromatin and lagging chromosomes during late anaphase causing a defect in chromosome segregation (Allshire et al. 1995; Ekwall et al. 1996). Centromeric heterochromatin was also shown to be necessary for the recruitment of cohesin in order for proper sister chromatid cohesion and therefore proper chromosome segregation (Bernard et al. 2001). In addition, a loss of the histone H3 lysine 9 methyltransferases, Suv39h1 and Suv39h2, in the

murine system has a dramatic effect on chromosome stability, viability, and results in an increase in tumor risk (Peters et al. 2001). These examples illustrate the importance of maintaining heterochromatin to ensure faithful propagation of the genome thus preserving viability.

The first study of a heritable epigenetic change was performed by Muller in 1930 describing for the first time a phenomenon that what would later be know as PEV or position effect variegation (Muller 1930). While studying the rates of X-ray induced mutations in the *white* gene of *Drosophila*, he noticed that the eye was variegated with patches of white and red. A genetic mutation in the *white* gene should result in a white eye instead of the red pigmentation normally present in a wild-type eye. The variegation in eye color suggested that the *white* gene itself was not mutated because there were still red patches present. However, what would cause the white patches to occur? Muller noticed his mutations were the result of translocations or inversions of the region of the X-chromosome containing the *white* locus and that this was responsible for his variegated phenotypes, although the mechanism escaped him (Muller 1930). Later, staining of the polytene chromosomes would show that the cells containing the inactive gene contained a rearrangement of the chromosome that positioned the gene next to pericentromeric heterochromatin causing silencing (Zhimulev et al. 1988). Barbara McClintock would later describe the same phenomenon in maize (McClintock 1950). PEV occurs in several organisms, including at *S. pombe* centromeres, and we now know that a rearrangement of a gene next to heterochromatin allows the silent state to spread into the adjacent gene (Allshire et al. 1994).

Heterochromatin is found at regions of repetitive DNA, transposons, centromeres, telomeres, rDNA, and imprinted loci such as the mating type region in yeast. Heterochromatin is essential

for genome organization, sister chromatid cohesion, chromosome segregation, differentiation, and dosage compensation (Grewal and Jia 2007). Specific histone tail modifications and effector proteins are associated with heterochromatin such as histone H3 lysine 9 methylation and the effector protein HP1 (heterochromatin protein 1), which will be discussed in detail later in this chapter (Bannister et al. 2001; Lachner et al. 2001). Heterochromatin is usually referred to as either facultative or constitutive (Elgin and Grewal 2003). Facultative heterochromatin is found at genes that are usually repressed but are activated based on times in the cell cycle or a specific developmental stage. Constitutive heterochromatin is permanently silenced. Centromeres are a region of constitutive heterochromatin comprised of highly repetitive elements that are the largest cluster of repeats in the genome (Grewal and Jia 2007). Centromeres not only facilitate structure, but also are essential for chromosome segregation through mitosis and meiosis. Telomeres are another region of constitutive heterochromatin and serve as a way to protect the ends of chromosomes by forming a chromosomal "cap" (Elgin and Grewal 2003)

Heterochromatin and euchromatin were first described based on their staining in the nucleus as described above, however we now know that this spacial organization within the nucleus is non-random and may serve a function in gene expression (Heitz 1928; Mirkovitch et al. 1987; Andrulis et al. 1998). Susan Gasser's group performed the majority of experiments investigating this hypothesis in *S. cerevisiae*. They have demonstrated that key components of the silencing complex were at the nuclear periphery and when mutants were introduced releasing the telomeres from the periphery, the telomeres would lose silencing (Laroche et al. 1998; Andrulis et al. 2002). However, Gasser's lab would later show that if both the silencing factors and the telomeres were released from the periphery, the telomeres would still be silenced (Gartenberg et

al. 2004). These observations discard the notion that localization to the nuclear periphery is essential for gene silencing, but perhaps the specific localization of heterochromatin ensures a more efficient means of silencing due to the concentration of silencing factors at one site.

The essential function of heterochromatin is clear, however the mechanisms behind how the genome decides what part of chromatin should be compacted into heterochromatic regions and what parts should be maintained as euchromatic, are still unclear. The heterochromatin machinery is thought to target a locus, maintain silencing, and spread to adjacent loci. Due to the highly repetitive nature of heterochromatin, determining the mechanisms involved in targeting are difficult. The main focus of this thesis is to address these mechanisms.

iii.

THE COVALENT POST-TRANSLATIONAL MODIFICATIONS OF HISTONES

As mentioned previously, the first observation linking histone tail modifications and gene expression was done my Allfrey, Faulkner, and Mirsky in 1964 (Allfrey et al. 1964). They used chromatin templates and crude calf thymus RNA polymerase preparations to show that prior acetylation of histone tails relieved transcriptional inhibition. The authors commented: "This raises the possibility that relatively minor modifications of histone structure, taking place on the intact protein molecule, offer a means of switching –on or –off RNA synthesis at different loci along the chromosome" (Allfrey et al. 1964). The experiment itself and the speculations provided were astonishing for the time and would only much later be proven. Eventually the N-

terminal tails, which make up more that a quarter of the nucleosome mass, were shown to contain an array of covalent modifications such as phosphorylation, methylation, acetylation, sumoylation, ADP ribosylation, deimination, proline isomerization, and ubiquitination (Vaquero et al. 2003; Kouzarides 2007). These observations also led to the realization that N-terminal tails are marked in order to recruit modifying proteins and the machinery necessary to enhance or repress transcription (Jenuwein and Allis 2001). The scope of this thesis concentrates on histone tail acetylation and methylation therefore, these will be further described in detail.

Nearly ten years after Allfrey et. al, another example correlating gene activity and histone acetylation came from a study done in the ciliate *Tetrahymena thermophila* (Gorovsky et al. 1973). *Tetrahymena*, like other ciliates, contains a transcriptionally active macronucleus and a transcriptionally inert micronucleus. Hyperacetylated histone H4 was only found in the macronucleus while hypoacetylated histone H4 was only found in the micronucleus, again providing an indirect link between the degree of acetylation and gene activity (Gorovsky et al. 1973).

Experiments performed in yeast and chicken provided the first direct evidence that modifications to histone tails might modulate transcription. Chromatin fragments were directly immunoprecipitated with antibodies recognizing hyperacetylated histones, revealing that hypoacetylated histones were associated with repressed genes and hyperacetylated histones occurred within active genes (Hebbes et al. 1988; Braunstein et al. 1993). Further direct evidence was provided when the first histone acetyltransferase (HAT), p55, was purified in *Tetrahymena* uncovering a direct homology to the co-activator GCN5 (Brownell et al. 1996).

GCN5 was already known to be required for transcriptional activation of several genes in yeast. Since these ground-breaking discoveries, many other co-activators have been shown to have HAT activity such as p300/CBP, hormone receptor co-activators SRC-1 and ACTR, and a component of the RNA polymerse II transcription complex, $TAF_{II}250$, just to name a few (Bannister and Kouzarides 1996; Mizzen et al. 1996; Ogryzko et al. 1996; Chen et al. 1997; Spencer et al. 1997). We also now know that typically multiple lysine residues contain acetylation marks and these modifications are carried out by a variety of HAT complexes.

Around the same time that p55 was purified, a mammalian histone deacetylase (HDAC1) was purified and was shown to be homologous to the yeast co-repressor RPD3 (Taunton et al. 1996). Since then, several evolutionarily conserved classes of histone deacetylases have been found. The first class (class I) consists mammalian HDAC1-3, 8 and the yeast RPD3, while the second class (class II) of mammalian HDAC4-7A,9,10) (Rundlett et al. 1996; Downes et al. 2000). The third class of histone deacetylases is referred to as the SIR2-type or sirtuins in mammals, and is NAD^+-dependent (Imai et al. 2000). These results demonstrate that the reversible acetylaton of histones is a widespread, evolutionarily conserved mechanism that contributes to transcriptional control in all eukaryotes.

After initial observations linked the role of histone acetylases and deacetylases to the control of transcriptional activation and repression, respectively, genetic data conclusively demonstrated their influence on transcription. Mutations that abolished HDAC activity of either RPD3 or HDAC1 inhibited their repressive activity on target genes in vivo (Hassig et al. 1998; Kadosh and Struhl 1998). At the same time mutations eliminating the HAT activity of GCN5 effected

the activation of target genes (Kuo et al. 1998). In addition purification studies of RPD3 and GCN5 demonstrated their recruitment to target promoters along with additional binding proteins in order for efficient repression or activation. For example, RPD3 partners with sequence-specific DNA-binding proteins, such as YY1 and UME6, for repression (Yang et al. 1996; Kadosh and Struhl 1997). Similarly, GCN5 is targeted to promoters by interactions with proteins such as VP16, through an association with an adapter protein, ADA2 or with the basal transcription machinery (Silverman et al. 1994; Barlev et al. 1995; Chiang et al. 1996; Drysdale et al. 1998; Utley et al. 1998). Specifically, recruitment of either RPD3 or GCN5 results in the deacetylation or acetylation of specific lysine residues on the N-terminal tails of histones H3 or H4. Typically, acetylation of lysines 5, 8, 12, and 16 on histone H4 and lysines 9 and 14 on histone H3 are associated with active transcription, however, the removal of these marks correlates with repression (Kuo et al. 1996; Rundlett et al. 1998).

Mechanisms controlling transcriptional activation and repression have also been intricately linked to methylation marks of specific lysines on the histone H3 and H4 tails. Methylation marks on H3 lysine 9 and lysine 4 were extensively mapped over 47 kilobases by the Grewal laboratory at the mating type locus of fission yeast, *S. pombe.* They demonstrated that H3 methylated at lysine 9 was enriched in regions of repressed chromatin, whereas H3 methylated at lysine 4 was enriched in regions of active chromatin (Noma et al. 2001). Similar results were also demonstrated at the chicken β-globin domain indicating an evolutionarily conserved association (Litt et al. 2001). Methylation of histone H3 at lysine 9 has a well-established role in heterochromatin and is often referred to as a "hallmark" of heterochromatin (Lachner et al. 2001). We now know several other methylation marks that occur on the N-terminal tails of

histones, and each mark typically is associated with either transcriptional repression or activation. Histone H3 methylated at lysine 4, 36, and 79 are correlated with active regions of the genome, while histone H3 methylated at lysine 9, 27, and histone H4 lysine 20 are associated with transcriptional repression. However, not all organisms have been shown to contain every mark, for example histone H3 methylated at lysine 27 has never been detected in *S. pombe*.

The first examples of histone methyltransferases (HMTs) were mammalian SUV39H1 proteins, which were shown to be homologous to the *Drosophila* suppressor of position effect variagation, SU(VAR)3-9 (Rea et al. 2000). The mammalian homologs, SUV39H1 and SUV39H2 have been shown to be required for pericentromeric H3 lysine 9 methylation as well as genomic stability and developmental viability in double knock-out mice (Peters et al. 2001). Clr4 is also thought to be the sole H3 lysine 9 methyltransferase in *S. pombe* and is required for heterochromatin formation (Nakayama et al. 2001). H3 lysine 4 methyltransferases have also been identified, including SET1 in S. *cerevisiae* and *S. pombe*, SET7 in human HeLa cells, and murine MLL (Briggs et al. 2001; Wang et al. 2001; Bryk et al. 2002; Milne et al. 2002; Noma and Grewal 2002).

Lysine methylation occurs as mono-, di-, or tri-methylation, which adds an extra layer of regulation and complexity to this specific modification. Importantly, each degree of methylation may correlate with activation or repression and specify distinct chromatin domains. For example, in the budding yeast *S. cerevisiae* (where lysine 9 methylation does not occur) H3 lysine 4 dimethylation is found at both inactive and active loci, however, only H3 lysine 4 trimethylation is detected in active gene promoters (Santos-Rosa et al. 2002). In addition, the

histone methyltransferase SET1 is required for the trimethylation and is targeted to promoters by interacting with the elongating form of RNA polymerase II (Ng et al. 2003). Recently these marks were defined further by showing that at a target gene, monomethylation is enriched at the 3'end, dimethylation peaks in the middle, and trimethylation correlates to the transcription start site. Researchers speculated that a possible explanation for this localization is the fact that H3K4me1 occurs at a basal level and as SET1 associates with Pol II, it converts monomethyl into dimethyl and trimethyl (Pokholok et al. 2005). Furthermore, mammalian cells display a pattern of H3 monomethylation at lysine 9 in euchromatic domains that are repressed, whereas H3 trimethylated at lysine 9 is found at constitutively repressed heterochromatin (Peters et al. 2003; Rice et al. 2003).

The first direct evidence that the post-translational modifications present on histone tails recruit other modifying enzymes was seen when the structure of the bromodomain from the histone acetylatransferase, P/CAF, bound to aceytl-lysine was solved (Owen et al. 2000). This was the first example of the conserved bromodomain having a specific association with a lysine residue. Furthermore the double bromodomain structure of human $TAF_{II}250$ was solved and shown to interact specifically with H4 acetylated tails at either lysines 5/12, lysines 8/16, or only lysine 16 (Jacobson et al. 2000). Moreover, the double bromodomain structure binds most tightly to the H4 tail at lysines 5 and 12 during isothermal titration experiments (Jacobson et al. 2000). About a year later the evolutionarily conserved chromodomain of the heterochromatin protein 1, HP1 was shown to bind to histone H3 methylated at lysine 9, along with the structure of the interaction (Bannister et al. 2001; Jacobs et al. 2001; Lachner et al. 2001; Fischle et al. 2003). HP1 is crucial for heterochromatin formation and also nucleates cohesion of sister centromeres

during cell division by recruiting cohesin (Eissenberg et al. 1990; Bernard et al. 2001; Nonaka et al. 2002; Partridge et al. 2002). Homologues exist throughout higher eukaryotes beginning with S. pombe (Swi6) all the way to the three isoforms present in humans (HP1α, HP1β, and HP1γ) (Lorentz et al. 1994; Ma et al. 2001). Each HP1 protein interacts with additional factors and has distinct localization patterns that contribute to their role in heteochromatin formation.

Until recently it was thought that methylation marks were irreversible and that the only way to eliminate or replace these marks was to replace the entire histone. However in 2004, a lysine specific demethylase, LSD1 was described (Shi et al. 2004). LSD1 is an amine oxidase that specifically removes histone H3 lysine 4 methylation by a FAD-dependent oxidative destabalization of the amino-methyl bond (Shi et al. 2004). In addition it was shown to only demethylate mono- and di- methylated forms of lysine 4 and not trimethylation. LSD1 was shown to be in a large complex containing HDACs and additional enzymes in order to collectively modify tails and facilitate transcriptional repression (Lee et al. 2005; Shi et al. 2005). Researchers have also shown that LSD1 demethylates H3K9me2 while in a complex with the androgen receptor to activate transcription of target loci (Metzger et al. 2005). The LSD1 homologues in S. pombe have also been shown to specifically demethylate H3K9me2 and associate in a complex (Lan et al. 2007; Opel et al. 2007). A second class of histone demethylases was also found and described as hydroxylases or dioxygenases that contain an evolutionarily conserved jumonji domain (Tsukada et al. 2006). The mammalian genome contains around thirty known jumonji proteins, which suggest the existence of an array of functional demethylases yet to be described (Fodor et al. 2006; Klose et al. 2006; Whetstine et al. 2006; Chang et al. 2007). Studies have not yet revealed whether every methyl mark can be

removed. S. pombe has seven JmjC domain proteins and at least one, Jmj2, has been shown to have H3 lysine 4 demethylase activity (Huarte et al. 2007). However, Jmj2 only demethylates the trimethyl mark, which in turn results in H3 lysine 4 mono- or di- methylation.

In the end, the post-translational modifications of histone tails have implications for how chromatin and the genome are regulated. Histone tail modifications are the framework for intricate pathways that mediate interactions between transcriptional activators and repressors. They also specify the assembly of higher order heterochromatin structures that are vital to an organism's viability. A controversial "histone code" hypothesis has also been proposed suggesting an evolutionarily conserved code that can predict histone modifications and in turn a regions transcriptional state (Jenuwein and Allis 2001). However, unlike the predictable and universal genetic code, histone modifications vary from one organism to the next. Furthermore, a specific modification does not always result in the same biological response inducing a limit on what would be defined as a code. Additional modifications and regulatory mechanisms are consistently being uncovered, so it may be some time before anyone can actually prove a "histone code" exists. **

** This thesis does not aim to provide a description of every histone modification, as such an explanation would be extravagant due to the development of the field. Therefore, a discussion of additional modifications has been intentionally omitted. A background has been provided that pertains to the data presented herein.

iv.

RNAi

RNA interference (RNAi) can be described in a broad sense as a gene silencing mechanism triggered by double stranded RNA (dsRNA). The exogenous or endogenous dsRNA trigger is then processed into small interfering RNAs (siRNAs), which are then loaded into an effector complex in order to act as guides to target complementary mRNAs for degradation or post-transcriptional gene silencing (PTGS). RNAi has also been shown to target heterochromatin to regions in order to induce transcriptional gene silencing (TGS), which will be discussed in detail in the next section. In addition, RNAi is also involved in translational inhibition through the involvement of a micro RNA (miRNA) pathway, however this will not be discussed as it does not pertain to the work described in this thesis. RNAi pathways have been found in higher eukaryotes ranging from fission yeast to humans (Lee and Ambros 2001). Although the phenomenon of RNAi has a relatively short history, the impacts it has made on the understanding of endogenous silencing mechanisms, in addition to the impacts it has made on the field of functional genomics are immeasurable. Furthermore, the use of RNAi as a tool will undoubtedly shape future therapeutics for a wide range of diseases as well as contribute to advances in biotechnology.

The first reports of an RNA intermediate being involved in silencing came from a study done in petunias by Napoli and Jorgenson in 1990 (Napoli et al. 1990). They overexpressed the pigment-producing gene, chalcone synthase, which gives petunias their dark violet color. However, when they transformed in the transgene the resulting progeny were variegated or even

white. The level of chalcone synthase RNA was fifty fold lower than in wild-type petunias. Napoli and Jorgenson used the term "cosupression" to describe this phenomenom because presumably the introduced transgene was "cosupressing" the endogenous copy of the chalcone synthase gene (Napoli et al. 1990). Cosupression would later be described in a number of plant species as well as in *Drosophila*. Around the same time a similar discovery was made in *Neurospora crassa* by Romano and Macino, however they described the insertion of a homologous RNA sequence as "quelling" the expression of the endogenous gene (Romano and Macino 1992). A key experiment examining the silencing of the par-1 gene in *C. elegans* would further add to the discovery of RNAi (Guo and Kemphues 1995). Researchers at the time were using antisense RNA in an attempt to silence a homologous mRNA with the thinking that the antisense strand would hybridize to the sense strand and create double stranded RNA in order for targeting and degradation by cellular ribonucleases. Interestingly, when Guo and Kemphues introduced a control sense RNA, which would not hybridize to the par-1 transcript, they still observed silencing of the par-1 gene regardless of the addition of sense or antisense RNA (Guo and Kemphues 1995). Furthermore, experiments performed by plant virologists studying viral resistance showed that short non-coding regions of viral RNA sequences integrated into plants made them more resistant to the viruses. Alternatively, short sequences of plant genes introduced into viruses suppressed the target gene of the infected plant (Ratcliff et al. 1997). Collectively, these experiments referred to the same observation of post-transcriptional gene silencing, however the molecular mechanisms of this phenomenon would later be explained.

Mello and Fire in 1998 published a historic paper that would provide the first explanation for the observations described above and also won them the Nobel Prize in 2006 for the discovery of

RNAi (Fire et al. 1998). They hypothesized that the silencing trigger was not single-stranded RNA (ssRNA), but dsRNA. Using the nematode *C. elegans*, they extensively purified sense and antisense ssRNA to avoid any dsRNA contamination, which they predicted was the cause of silencing seen by Guo and Kemphues at the par-1 gene (Guo and Kemphues 1995; Fire et al. 1998). Next a direct comparison was done between the introductions of ssRNA vs dsRNA homologous to the unc-22 gene. They consistently found that ssRNA was 10 to 100 fold less effective at silencing than a dsRNA trigger. Furthermore, the dsRNA had the ability to completely silence the unc-22 gene resulting in a null phenotype with only a few dsRNA molecules. Sense RNA was able to silence if the antisense RNA was subsequently introduced or vice versa indicating their ability to hybridize *in vivo* (Fire et al. 1998). While, this work established an entirely new way of thinking regarding the ability of RNA to affect gene silencing, the collective work of several labs using biochemistry as well as classical genetics would determine the molecular mechanism behind their discovery.

Hamilton and Baulcombe were the first to describe the short interfering RNA (siRNA) intermediates necessary for targeting of a homologous mRNA (Hamilton and Baulcombe 1999). They hypothesized that since a full length antisense transcript was never detected perhaps a stable intermediate existed that would serve as a guide that would bind to the target resulting in its degradation. Indeed, they detected 25 nucleotide (nt) RNAs that were only present in plants undergoing cosuppression and not in wild-type plants (Hamilton and Baulcombe 1999). Soon after, two independent labs purified 21-23nt RNAs from *Drosophila* cell extracts that copurified with RNAi (Hammond et al. 2000a; Zamore et al. 2000). These experiments determined that dsRNA was converted into a short RNA intermediates that are capable of binding the target

homologous mRNA and cleaving the transcript (Hammond et al. 2000a; Zamore et al. 2000). Furthermore, the Tuschl lab incubated *Drosophila* cell extracts with synthesized 21-22nt siRNAs homologous to the firefly luciferase transcript to definitively demonstrate that silencing does occur via small effector molecules (Elbashir et al. 2001b). In addition, a 2-3nt overhang on the 3' end of the siRNA was shown to silence the target mRNA more efficiently (Elbashir et al. 2001b). The Tuschl lab also showed efficient silencing using small RNAs in mammalian cells (Elbashir et al. 2001a). This finding was a massive breakthrough for functional genomic assays because up until then, long dsRNA induced an interferon response, inhibiting general translation. RNAi could now be used to knockdown genes in mammalian cells for loss of function assays.

The focus next turned to identifying the enzyme or enzymes responsible for processing the dsRNA trigger into siRNAs as well as the cleavage of the mRNA target. The Hannon lab first determined that these two steps were separable by high-speed centrifugation of *Drosophila* cell extracts (Hammond et al. 2000a; Bernstein et al. 2001). They observed that the activity that produces the siRNA remained in the supernatant where as the activity that cleaved the mRNA target was cleared. The separable cleavage activity of the mRNA target was termed RNA-induced Silencing Complex (RISC), which was referred to as the "effector" phase of RNAi-mediated silencing (Hammond et al. 2000a). In order to determine the enzyme responsible for the "initiator" phase, or cleavage of dsRNA into siRNAs, a candidate gene approach was taken. After several classes of dsRNA ribonucleases were tested for the ability to produce 21-23nt products, a RNase III type enzyme was identified and termed Dicer (Bernstein et al. 2001). Deletion of Dicer eliminates dsRNA silencing, and the enzyme is found in all organisms that have the RNAi machinery (Bernstein et al. 2001).

The RISC complex was purified from human HeLa cells by biotin labeling the 3' termini of siRNAs (Martinez et al. 2002). The siRNAs coimmunoprecipitated with two proteins with molecular weights around 100 kDa identified as Argonaute 1 and Argonaute 2. Argonaute proteins are a highly conserved family that were first named in plants and contain the evolutionarily conserved PAZ and PIWI domains (Bohmert et al. 1998). Genomic screens for proteins involved in RNAi revealed the involvement of the Argonaute proteins in several organisms including rde-1 in *C. elegans*, Ago1 in *Arabidopsis*, and QDE2 in *Neurospora* (Cogoni and Macino 1997; Tabara et al. 1999; Fagard et al. 2000). The identification of slicer activity came from two labs. First, the Joshua-Tor lab crystallized an Argonaute protein from *Pyrococcus furiosus* where they noticed a resemblance between the PIWI domain and the conserved catalytic domain of RNase H enzymes (Song et al. 2004). Similar to an RNase H enzyme, the slicer activity required divalent cations and left a 3' OH and 5' phosphate termini on the cleaved product. Also they determined, based on the structure, that the 3' end of the single-stranded siRNA would sit in the PAZ domain; while the rest of the siRNA could bind the target mRNA transcript and stimulate cleavage by the PIWI domain (Song et al. 2004). In collaboration, the Hannon lab was purifying Ago1-4 from human 293 cells and assaying for their cleavage activity (Liu et al. 2004a). Interestingly, only Ago2 cleaved a target mRNA, and when its catalytic domain was mutated, slicer activity was eliminated (Liu et al. 2004a). In addition, two other labs determined that Ago2 was also responsible for the cleavage of the non-incorporated strand of the double-stranded siRNA and there was no involvement of a helicase for unwinding the duplex (Matranga et al. 2005; Rand et al. 2005). Several different RNAi effector

complexes have now been identified in various organisms; however, they all contain an Argonaute protein.

Another component of the RNAi silencing machinery is an RNA-dependent RNA polymerase or RdRP, which catalyzes the synthesis of RNA using an RNA template. RdRP proteins have only been identified in plants, worms, and fission yeast (Schiebel et al. 1993; Cogoni and Macino 1999; Smardon et al. 2000; Sijen et al. 2001; Volpe et al. 2002). RdRP proteins are thought to function in amplifying the RNAi response by synthesizing additional dsRNA for dicer cleavage using existing siRNAs as templates. In *C. elegans*, RdRP was shown to produce a distinct class of "secondary" siRNAs with specific structural features that use dicer produced "primary" siRNAs as templates for amplification of the initiation pathway (Pak and Fire 2007; Sijen et al. 2007). The RdRP homologue in *S. pombe* is thought to be required for the initiation of Dicer produced siRNAs, however chapter four in this thesis is devoted to showing that this is not the case (Motamedi et al. 2004).

Ultimately, RNAi pathways have far-reaching effects. The RNAi pathway is now the leading tool for studying loss of gene function in most organisms due to the ease and robustness of the silencing assay. The continuing excitement about the possible use of the RNAi pathway in clinical applications has also sparked interest in the study of the molecular mechanisms controlling silencing. Future studies of RNAi will undoubtedly continue to shape the way we study molecular biology and potentially change the framework for how we treat disease.

v.

THE INTERFACE BETWEEN POST-TRANSLATIONAL MODIFICATIONS AND RNAi

RNAi has also been implicated in transcriptional gene silencing (TGS) via the recruitment of heterochromatin to specific loci. Several studies alluded to the fact that an RNAi intermediate was involved in the recruitment of heterochromatic silencing. The introduction of the potato spindle tuber viroid (PSTV) into a tobacco plant induced DNA methylation at transgenic regions of homologous nuclear sequences (Wassenegger et al. 1994). The PSTV genome is 359 nt RNA genome and replicates by an RNA-RNA pathway. Interestingly, what suggested the requirement of an RNA intermediate was the fact that the integrated copies of PSTV DNA were only methylated when the host plant supported transcription of the viroid RNA (Wassenegger et al. 1994). In addition, the observation in *Arabidopsis* that the production of aberrant transcripts induced the recruitment of DNA methylation followed by transcriptional gene silencing of all homologous promoters led to the idea that RNA was somehow involved in the nucleation of silencing throughout the genome (Mette et al. 1999). However, it was not until studies done in S. pombe showed a direct connection between the loss of RNAi and a loss of heterochromatin that the RNAi pathway was established as a pathway involved in the recruitment of heterochromatin and the organization of the genome (Volpe et al. 2002). Plants and fission yeast are the primary systems used to investigate RNAi-directed heterochromatin formation and therefore will be discussed in detail, while examples and correlative studies performed in other eukaryotes will be discussed briefly.

Mechanisms of RNAi induced transcriptional silencing in plants compared to fission yeast are similar with one significant difference: plants contain methylated DNA at heterochromatic regions. The process in plants by which siRNAs produced by the RNAi pathway guide *de novo* DNA methylation to a homologous DNA region is termed RNA-directed DNA methylation (RdDM) (Wassenegger et al. 1994). The major targets of RdDM are transposons and repeats that are present in both constitutive and facultative regions of heterochromatin (Matzke et al. 2007). The RdDM silencing pathway has not only been implicated in silencing transposons and centromeric repeats but also in the regulation of genes required for plant development (Matzke et al. 2007). The initial components required for silencing were identified in genetic screens done by three separate labs (Chan et al. 2004; Mathieu and Bender 2004; Matzke et al. 2004). The silencing screens performed in *Arabidopsis*, utilized inverted repeats introduced *in trans* to induce silencing of endogenous promoters or transgenic reporter genes. Therefore a defect in silencing would indicate a gene required for the RdDM pathway. Initial screens only recovered one component of the RNAi pathway, the argonaute protein AGO4 that has subsequently been shown to be required for *de novo* DNA methylation of a target locus (Zilberman et al. 2003). However, two DNA methyltransferases were identified, MET1 and CMT3 and were shown to be required for RdRM silencing pathway (Bartee et al. 2001; Lindroth et al. 2001). The main problem with identifying components required for silencing using a forward genetic screen is that frequently genes have redundant functions. This may be why only one component of the RNAi pathway was initially identified. For example, DCL3 (DICER-LIKE 3) and RDR2 (RNA-DEPENDENT RNA POLYMERASE 2 are required for production of 24-nt siRNAs originating from transposons and repeats, however, two other DCL genes are partially redundant (Xie et al. 2004). The de novo DNA methyltransferase DRM1 and DRM2 are also redundant genes

required for the RdRM pathway that were identified in reverse genetic screens (Gasciolli et al. 2005).

Other mutants identified in the above screens were the H3K9 methyltransferase SUVH4, along with chromatin remodelers belonging to the SWI/SNF family presumably acting to alter the chromatin structure to facilitate transcription (Jackson et al. 2002; Malagnac et al. 2002). However, loss of the DNA methyltransferase CMT3 or H3K9me does not result in a loss of siRNA accumulation (Lippman et al. 2003). In contrast, a loss of the DNA methyltransferase, MET1 or DRM1 does result in a loss of siRNA accumulation and H3K9me2 at a subset of transposons and repeats (Cao et al. 2003; Lippman et al. 2003). The loss of the chromatin remodeler, DDM1 abolishes siRNA production and H3K9me in some cases as well (Cao et al. 2003). In *Arabidopsis*, the centromeric satellite repeats are composed of arrays of tandem repeats on either side of the centromere that are transcribed and processed by the RNAi machinery. Silencing at the centromeres requires DCL3, RDR2, DDM1, H3K9me, and CMT3. However, if you insert a retrotransposon into the centromere repeats additional machinery is required for silencing including MET1 and HDA6 (Histone Deacetylase 6) (May et al. 2005). Recently, another key component to the RdRM pathway has been identified, RNA polymerase IV (Herr et al. 2005; Onodera et al. 2005). Pol IV was identified by genome sequence analysis of the *Arabidopsis* genome, and contains two separate isoforms, Pol IVa and Pol IVb (Kanno et al. 2005; Onodera et al. 2005; Pontier et al. 2005). The two isoforms act specifically at different stages of the RdRM pathway in conjuction with specific chromatin remodelers.

Collectively, from the data obtained, one can group the components of the RdRM pathway into three stages, including: 1) proteins required for producing, stabilizing, and interacting with the RNAi trigger, 2) proteins involved in the de novo and maintenance DNA methylation, and finally 3) components involved in the histone modifications required for maintenance of silencing at the target loci. The isoform Pol IVa along with the chromatin remodeler, CLSY1 are required to produce the initial transcript from the trigger, followed by amplification by RDR2 and processing by DCL3 (Xie et al. 2004; Smith et al. 2007). siRNAs then bind to AGO4 to guide them to the target locus (Zilberman et al. 2003; Qi et al. 2006). AGO4 interacts with the Pol IVb isoform along with the chromatin remodeler DRD1 to facilitate de novo DNA methylation at the target (Li et al. 2006). At present, it is not clear whether Pol IVb actually transcribes at the target site or simply acts to open the chromatin structure to expose DNA to methytransferases. DRM1 is then required for *de novo* DNA methylation at the target followed by the maintenance methyltransferases, CMT3 and MET1 (Cao et al. 2003). The histone methyltransferase SUVH4 and the histone deacetylase HDA6 are subsequently required for the maintenance of DNA methylation and silencing at the target (Aufsatz et al. 2002; Malagnac et al. 2002). The roles for additional components of the RdRM pathway, specifically proteins with redundant functions in silencing, are still under investigation.

In addition to silencing transposons and repeat elements, the RdRM pathway is also required for silencing developmentally regulated genes. For example, the *Arabidopsis FWA* (Flowering Wageningen) gene is a transcription factor only expressed by the maternal genome in endosperm (Kinoshita et al. 2004). *FWA* contains a pair of transposon-associated tandem repeats in its promoter that give rise to siRNAs that leads to DNA methylation and silencing (Kinoshita et al.

2004; Lippman et al. 2004). The loss of the DNA methyltransferase MET1 eliminates siRNA production and results in the upregulation of *FWA* and a late flowering phenotype. Interestingly, this phenotype is not observed in mutants of the RNAi machinery. However, *FWA* transgenes are silenced when introduced into the genome and this silencing depends on DCL3, RDR2, and AGO4 suggesting a role for RNAi in initiating silencing via the RdRM pathway (Chan et al. 2004). This would also suggest a role for MET1 in maintaining silencing at this locus.

The ciliate *Tetrahymena thermophila* has also been used to study the mechanism of RNAi-mediated heterochromatin formation. *Tetrahymena* contains two distinct nuclei, a micronucleus that contains the germ-line and is transcriptionally inactive, and a macronucleus that contains the transcriptionally active somatic genome. Initial studies distinguishing areas of the genome associated with histone acetylation marks were done in *Tetrahymena* comparing marks associated with the macronucleus compared to the micronucleus (Gorovsky et al. 1973). Remarkably, during macronuclear development approximately 6,000 IES (Internal Eliminated Sequences) elements, which consist of non-coding and repetitive DNA, are rearranged and deleted from the genome. Of course, the question remained how does the genome recognize and target these sequences for elimination? The initial observation that the IES elements were bi-directionally transcribed early in conjugation suggested an RNA component might be involved in DNA elimination (Chalker and Yao 2001). Next, TWI1, a homologue of the Argonaute protein PIWI, was shown to be required for DNA rearrangements (Mochizuki et al. 2002). At the same time, the purification of ~28 nt small RNAs associated with germ-line limited sequences suggested a role for the RNAi machinery (Mochizuki et al. 2002). Moreover, a loss of TWI1 resulted in the destabalization of siRNAs and loss of H3K9me in the developing macronucleus

(Mochizuki et al. 2002; Liu et al. 2004b). The dicer homologue, DCL1, was also shown to be expressed at high levels early in conjugation and localized to the micronuclei. Loss of DCL1 results in high transcript levels of germ-line sequences and loss of siRNAs, ultimately leading to a defect in IES elimination during development (Malone et al. 2005). Interestingly, the loss of siRNAs did not eliminate H3K9me, but the mark was no longer enriched at IES elements indicating that the RNAi pathway is required for the correct targeting of H3K9me to sequences eliminated from the developing macronucleus (Malone et al. 2005). Collectively this data led to the "scan" (scn) RNA model for RNA-mediated DNA elimination (Mochizuki et al. 2002). According to this model, siRNAs are made in the micronucleus where they assemble with the TWI1 complex and then go to the maternal macronucleus. There they scan for homology, and any siRNAs that match with the macronuclei sequences are degraded. Next the remaining TWI1 associated siRNAs are transported to the developing macronucleus where by H3K9me is recruited and sequences are marked for excision. Supporting this model, H3K9me has only been found in *Tetrahymena* at IES sequences in the developing macronucleus immediately before DNA rearrangements occur (Taverna et al. 2002). In addition, TWI1 localizes to the maternal macronucleus early in development after the majority of siRNAs are produced (Mochizuki et al. 2002). *Tetrahymena* utilizes an elegant pathway involving the RNAi machinery along with histone modifications to eliminate non-coding regions of the genome.

RNAi-mediated mechanisms that target heterochromatin formation have also been investigated in *Drosophila*, however limited direct evidence involving the mechanism exists. The first direct evidence of an affect of RNAi on heterochromatin and transcriptional gene silencing came from the Elgin lab where she showed that mutations in the Argonaute family protein, *piwi* and the

RNA helicase, *homeless* suppressed PEV of tandem transgene arrays next to the *white* gene (Pal-Bhadra et al. 2004). In addition, they showed mutants in HP1, *aubergine*, *homeless*, and *piwi* also suppressed silencing of the *white* transgene integrated in pericentromeric heterochromatin or on the fourth chromosome. The loss of silencing was also correlated with a decrease in H3K9me suggesting a role for RNAi in recruiting histone modifications (Pal-Bhadra et al. 2004). More recently, the Elgin lab showed an interaction through a yeast-two hybrid between PIWI and HP1 that is required for transgene silencing, further suggesting a link between RNAi and histone modifying enzymes (Brower-Toland et al. 2007). A class of small RNAs mapping to forty percent of known transposable elements have also been identified in *Drosophila* and are called rasiRNAs or repeat-associated RNAs (Aravin et al. 2003). Another example of transcriptional gene silencing involving RNAi comes from a study done in the germ-line of adult fly ovaries (Klenov et al. 2007). Mutations in *homeless* resulted in the transcription of three transposable elements. Moreover, ChIP analysis showed decreases in H3K9me2, H3K9me3, and HP1, but an increase in H3K4me2 (Klenov et al. 2007). Although, the same observation was not detected in adult somatic cells, a connection between RNAi and chromatin structure was still seen (Klenov et al. 2007). Further evidence may arise from a newly identified class of PIWI associated siRNAs (piRNAs), which have been mapped to transposable elements located within regions of heterochromatin in the germ-line (Brennecke et al. 2007). However, no direct evidence has been provided linking piRNAs to the recruitment of heterochromatin formation.

Several examples have also come from mammalian systems linking RNAi to transcriptional gene silencing. For example introduction of siRNAs into cancer cell lines can induce H3K9me at homologous promoters (Ting et al. 2005). In addition, studies showing that the introduction of

siRNAs into human cell lines inhibited transcription of a targeted reporter gene and induced DNA methylation implicate RNAi in the recruitment of transcriptional gene silencing (Morris et al. 2004). Recent evidence has also shown the association of piRNAs with *de novo* DNA methylation of retrotransposons in the murine germ-line linking the RNAi silencing pathway with heterochromatin silencing (Aravin and Bourc'his 2008; Aravin et al. 2008; Kuramochi-Miyagawa et al. 2008).

The first direct evidence of RNAi-directed transcriptional gene silencing was shown in the fission yeast *S. pombe* (Volpe et al. 2002). The *S. pombe* genome contains areas of constitutive heterochromatin at the telomeres, centromeres, and the imprinted mating type locus. The centromeres of *S. pombe* resemble those of higher eukaryotes and are composed of a central core where the kinetichore forms. This central core is flanked by large inverted repeats (the innermost repeats) that are then flanked by a tandem array of outer centromere *dg* and *dh* repeats (outermost repeats) (Pidoux and Allshire 2004). Centromere transcripts synthesized from both DNA strands of outer *dg* and *dh* sequences (termed forward and reverse centromere transcripts) have been identified (Volpe et al. 2002). In addition, putative forward and reverse strand centromere promoters have been determined from these mapping experiments (Volpe et al. 2002). *S. pombe* only contains a single Dicer, Argonaute, and RdRP gene for each of the RNAi pathway proteins, and a mutation in *dcr1*$^+$, *ago1*$^+$, and *rdp1*$^+$ causes transcript accumulation and a loss of H3K9me (Volpe et al. 2002). These mutants also cause a defect in chromosome segregation and therefore suggest a direct role for RNAi in heterochromatin formation and maintenance at centromeres (Allshire et al. 1995; Volpe et al. 2002; Volpe et al. 2003). Several species of 21-24nt siRNAs have been mapped throughout the *dg* and *dh* repeats of the

centromeres (Reinhart and Bartel 2002; Cam et al. 2005). Interestingly, both transcriptional and post-transcriptional silencing mechanisms were shown to contribute to silencing at the centromeres. The forward transcript is silenced by transcriptional silencing mechanisms whereas the reverse transcript is silenced post-transcriptionally(Volpe et al. 2002).

A centromere homology region containing portions of the *dg* and *dh* repeats is also present within the mating-type region of *S. pombe* and referred to as the *cenH* region. Bi-directional transcripts have also been detected at this region, and RNAi has been shown to be required for the establishment of heterochromatin at this loci (Hall et al. 2002; Noma et al. 2004). However, an additional silencing pathway involving the DNA binding proteins Atf1 and Pcr1 is capable of recruiting heterochromatin independent of RNAi (Jia et al. 2004). RNAi has also been implicated in silencing the centromere homology regions at the telomeres, however, an RNAi-independent silencing mechanism also exists(Kanoh et al. 2005; Mandell et al. 2005; Hansen et al. 2006).

Additional components required for *S. pombe* RNAi-mediated heterochromatin formation are the histone H3 lysine 9 methyltransferase, Clr4, the HP1homolog, Swi6, and the chromodomain protein Chp1 (Allshire et al. 1995; Ekwall et al. 1995; Sadaie et al. 2004). Swi6 and Chp1 have both been shown to bind H3K9me via their chromodomain (Partridge et al. 2002; Yamada et al. 2005). The purification of Chp1 identified a RNAi effector complex termed RITS (RNA-induced transcriptional silencing complex). RITS consists of the Argonaute protein, Ago1, a chromodomain containing protein, Chp1, and the adaptor protein, Tas3 (Verdel et al. 2004). The RITS complex has been shown to bind siRNAs and is required for heterochromatin assembly at

S. pombe centromeres (Verdel et al. 2004). RITS also recruits another complex, RDRC which includes the RNA-dependent RNA polymerase Rdp1, Cid12, a poly(A) polymerase, and Hrr1, a helicase and is also required for heterochromatin assembly (Motamedi et al. 2004). Mutants in the catalytic domain of Rdp1 abolish siRNA production by Dicer as well as RNAi-dependent silencing (Sugiyama et al. 2005). Additional data suggests a requirement for RNA polymerase II transcription in mediating RNAi-dependent heterochromatin formation (Djupedal et al. 2005; Kato et al. 2005). Defects in siRNA generation and heterochromatin assembly are observed when mutants in two different subunits of the RNA polymerase II, Rpb2 or Rpb7 are present (Djupedal et al. 2005; Kato et al. 2005). Although, the requirement for transcription has never directly been tested.

Clr4 forms a complex with Rik1, a protein with homology to the DNA damage protein Ddb1 as well as CPSF (cleavage and polyadenylation specificity factor) (Horn et al. 2005). This complex also contains the cullin ubiquitin ligase Pcu4 and two proteins of unknown function Raf1 and Raf2 (Horn et al. 2005). The Rik1 complex presumably is recruited to centromere repeats through an interaction with RITS and Clr4 (Zhang et al. 2008). However, RITS is thought to load siRNAs in order to recruit silencing to homologous target sequences via the binding of Chp1 to H3K9me (Verdel et al. 2004). If Clr4 is required for the recruitment of RITS and RITS is required for H3K9me, what comes first? The initial trigger for RNAi-dependent nucleation of heterochromatin still remains unclear.

Swi6/HP1 has been shown to recruit several chromatin-modifying enzymes and has been proposed to act as a platform in heterochromatin for the recruitment of an array of effector

proteins. Swi6 recently was shown to recruit the SHREC complex (SNF2-and histone deacetylase containing repressor complex) to the centromeres, mating type locus, and telomeres in *S. pombe* (Sugiyama et al. 2007). Presumably, the activity of proteins in the complex such as the histone deacetylase Clr3 and the nucleosome remodeler Snf2 are required to assemble the higher-order chromatin structure necessary to establish a silenced domain. Swi6 also recruits cohesin for sister chromatid cohesin, thus ensuring faithful segregation of chromosomes in mitosis (Bernard et al. 2001).

Insights into RNA-mediated heterochromatin formation have also come from cell cycle studies from two separate labs (Chen et al. 2008; Kloc et al. 2008). They detected a peak of bi-directional transcripts being made form centromeric repeats during S phase of the cell cycle correlating with increased enrichment of RNA polymerase II (Kloc et al. 2008). This peak of transcription is also associated with a loss of Swi6 localization and H3K9me. Following the initial peak of transcription, components of the RNAi pathway, such as Ago1, increased concentrations of siRNAs, and Rik1, a component of the Clr4 complex, are detected (Kloc et al. 2008). In addition the loss of Clr4 results in increased levels of transcription from the centromeric repeats thoughout the cell cycle (Kloc et al. 2008). Taken together these results suggest a mechanism for heterochromatin maintenance throughout the cell cycle by the recruitment of histone modifying enzymes in order to limit Pol II activity.

vi.

A PERSPECTIVE OF THE EXPERIMENTS DESCRIBED HEREIN

The experiments described in this thesis are performed using the fission yeast, *S. pombe.* *S. pombe* is a unicellular eukaryote, commonly used to study many biological mechanisms including RNAi-mediated heterochromatin formation. The preference to be in a haploid state and the fast and easy nature of manipulating the genome makes *S. pombe* a powerful model organism. We mainly focused our studies on RNAi-mediated heterochromatin formation at the centromeres, which have a similar architecture to centromeres in higher eukaryotes.

Recent studies in *S. pombe* have led to the proposal that RNAi-directed heterochromatin assembly is cis restricted (referred to as cis-PTGS or co-transcriptional gene silencing) (Buhler et al. 2006)**.** In other words, RNAi can only initiate heterochromatin at the locus the double stranded transcripts originated from. Tethering of the RITS subunit, Tas3, to the euchromatic *ura4*$^{+}$ mRNA is sufficient to recruit H3K9 methylation and concomitant Swi6/HP1 binding (Buhler et al. 2006). However, siRNAs generated either by tethering of RITS or via a dsRNA trigger are not sufficient to target heterochromatin nucleation *in trans*, leading to the "cis-restricted" model (Buhler et al. 2006). Additional studies demonstrate that RNAi effects heterochromatin assembly by means of a "self-enforcing loop" *in cis* whereby RNAi directs H3K9 methylation and H3K9 methylation directs RNAi (Sugiyama et al. 2005). Although it is clear that RNAi can direct H3K9 methylation, the mechanisms that initiate the loop of RNAi-directed heterochromatin assembly have been enigmatic. We therefore aimed to investigate the mechanism of how heterochromatin is targeted to specific loci in order to establish a silent

domain. We wanted to determine whether an endogenous *trans* targeting mechanism was present in *S. pombe,* and furthermore what role the RNAi pathway plays in nucleation as well as what specific histone modifications are necessary in establishing the transcriptional silencing of a target locus.

In addition, a key question that has not yet been addressed is whether all heterochromatic centromere repeats are regulated by similar mechanisms or whether individual repeats are under the control of different transcriptional and post-transcriptional repression pathways. A comprehensive analysis was performed in order to assay for differences in the regulation of the *dg* and *dh* forward and reverse promoters located within the centromere repeats. It has been suggested that the right arm of centromere three contains up to seven *dg*/*dh* repeats while the left arm contains three (Wood et al. 2002). However, whether differences exist in the regulation of each repeat relative to the distance from the centromere core has not previously been investigated. Therefore, we sought to investigate differences in the regulation of each repeat as well as differences in the regulation of repeats on different centomeres in order to gain a better understanding of what components contribute to silencing at the centomeres.

The requirement for Rdp1 (RNA-dependent RNA polymerase) for the production of dicer dependent siRNAs in the intiation of the RNAi pathway is an apparent contradiction (Motamedi et al. 2004). RNA dependent RNA polymerases in other organisms have been shown to be required for the amplification of "secondary" siRNAs that perpetuate RNAi silencing (Pak and Fire 2007; Sijen et al. 2007). We also explored the possibility that similar "secondary" siRNAs exist in *S. pombe* in order to amplify Dicer dependent "primary" siRNAs and spread

heterochromatin.

The experiments described in this thesis aim to provide insight into the mechanism of RNAi-mediated nucleation of heterochromatin in *S. pombe*. The goal of this research is to dissect the various pathways required for the nucleation, maintenance, and spreading phases of heterochromatin assembly at silenced regions of the genome.

CHAPTER II

A Comprehensive Analysis of Centromere Transcript Regulation

Abstract

Heterochromatin of eukaryotic genomes has classically been defined as condensed chromatin that is repressive to transcription and typically resides at highly repetitive regions of the genome. In contrast, recent data has revealed bi-directional transcription within heterochromatic outer centromere repeats sequences in *S. pombe*. A key question that has not yet been addressed is whether all heterochromatic centromere repeats are regulated by similar mechanisms or whether individual repeats are under the control of different transcriptional and post-transcriptional repression pathways. To investigate this question, we integrated *ura4*$^{+}$ reporter genes into various repeats within *S. pombe* centromeres, whereby reporter expression was under the regulation of endogenous centromere promoters. The resulting strains allowed the first comprehensive analysis of centromere transcript regulation by RNAi, RNA-turnover, and histone modification pathways. Our results reveal both strand-specific and locus dependent differential regulation of heterochromatic repeats and reveal for the first time that homologous centromere repeat sequences are subject to distinct repression pathways.

Introduction

Heterochromatin, which is critical for maintaining genome integrity, is typically described as condensed chromosomal regions that are gene poor and repressive to transcription whereas euchromatin is gene rich and permissive to transcription (Lippman and Martienssen 2004). Intriguingly, heterochromatic centromere repeats in *S. pombe* are expressed. These centromere transcripts are processed by RNA interference (RNAi) resulting in sequence specific targeting of heterochromatin to centromeres (Volpe et al. 2002). In addition to RNAi, recruitment of effector proteins and specific histone modifications aid in maintaining heterochromatin at centromeres (Richards and Elgin 2002; Cam and Grewal 2004).

RNAi is a process whereby double stranded RNA is degraded by the RNase III-like enzyme, Dcr1 into siRNA (small interfering RNAs) that are subsequently loaded into effector complexes involved in targeting homologous RNA sequences (Fire et al. 1998; Hamilton and Baulcombe 1999; Hammond et al. 2000a; Bernstein et al. 2001). In *S. pombe*, loss of RNAi results in loss of centromere heterochromatin (Volpe et al. 2002). RITS (RNA-induced transcriptional silencing complex), the RNAi effector complex in *S. pombe*, is comprised of the argonaute family protein Ago1, the chromodomain containing protein Chp1, and the adaptor Tas3 (Verdel et al. 2004). siRNAs loaded into RITS are believed to target cognate RNA through base pairing resulting in RNA cleavage. RDRC (RNA-directed RNA polymerase complex) contains the RNA-dependent RNA polymerase, Rdp1, as well as an RNA helicase, Hrr1, and a member of the poly (A) polymerase, Cid12 (Motamedi et al. 2004). This complex is thought to be involved in double stranded RNA synthesis through Rdrp activity using centromere transcripts as templates (Motamedi et al. 2004).

Heterochromatin is enriched with di- and tri-methylated histone H3 (H3K9me2/3), which serves as a binding site for the heterochromatin effector protein Swi6/HP1 (heterochromatin protein 1) via its chromodomain (Bannister et al. 2001; Lachner et al. 2001; Richards and Elgin 2002). The sole H3 lysine9 histone methyltransferase in *S. pombe* is Clr4, which forms a complex with Rik1, a protein with homology to the DNA damage protein Ddb1 as well as CPSF (cleavage and polyadenylation specificity factor) (Rea et al. 2000; Horn et al. 2005). This complex also contains the cullin ubiquitin ligase Pcu4 and two proteins of unknown function Raf1 and Raf2 (Horn et al. 2005). The Rik1 complex is thought to be recruited to centromere repeats through an interaction with RITS and Clr4 (Stoica et al. 2006; Zhang et al. 2008).

A recent study also implicates the exosome components Cid14 and Rrp6 in regulation of heterochromatic silencing at *S. pombe* centromeric repeats (Buhler et al. 2007; Wang et al. 2008). Cid14 is a poly(A) polymerase homologous to components of the TRAMP complex in *S. cerevisiae* (LaCava et al. 2005). In *S. pombe*, loss of Cid14 results in centromere silencing defects but has no effect on H3K9me2, Swi6, or Chp1 binding suggesting RNA degradation via Cid14 occurs downstream of recruitment of these proteins (Buhler et al. 2007).

S. pombe centromeres resemble those of higher eukaryotes and are composed of a central core where the kinetichore forms. This central core is flanked by large inverted repeats (the innermost repeats) that are then flanked by a tandem array of outer centromere *dg* and *dh* repeats (outermost repeats). Centromere transcripts synthesized from both DNA strands of outer *dg* and *dh* sequences (termed forward and reverse centromere transcripts) have been identified and siRNAs have been mapped to these regions (Reinhart and Bartel 2002; Volpe et al. 2002; Cam et al. 2005). In addition, putative forward and reverse strand centromere promoters have been determined from these mapping experiments (Volpe et al. 2002). Previous studies in *S. pombe*

have utilized reporter gene insertions into centromere heterochromatin to assay centromere silencing (Allshire et al. 1995). These reporter genes contained their own promoter as well as 3'UTR sequences and were extremely useful for studying heterochromatin nucleation and spreading. We sought to use a similar strategy to gain insight into the regulation of centromere promoters. We therefore integrated only the coding sequence of *ura4*$^{+}$ within either *dg* or *dh* repeat elements of centromeres one and three in either a forward or reverse orientation. Study of the resulting *ura4*$^{+}$ reporter constructs that are under the control of endogenous centromere promoters allowed the first comprehensive analysis of centromere repeat transcript regulation.

Results

Reporter genes integrated into *dg* and *dh* outer repeats of centromere three are differentially regulated

We explored whether transcripts originating from forward and reverse promoters within individual centromere repeats are regulated by similar or distinct mechanisms. In order to fully understand the regulation of the various centromere transcripts we integrated the coding sequence of *ura4*$^+$ (lacking 3' transcriptional termination sequences and endogenous promoters) into *dg* and *dh* regions of centromere three (*cen3*) (Figure 1A). Expression of these *ura4*$^+$ reporters, were therefore under the control of the endogenous forward and reverse centromere promoters.

The *ura4*$^+$ reporters were first integrated into a *ura4*Δ *ago1Δ* strain in order to facilitate homologous recombination into heterochromatic centromere repeats. Figure 1 indicates the direction of integration and the *cen3* repeat each *ura4*$^+$ reporter was integrated into (*dh* or *dh* centromere repeat). *Cen3* specific integration was confirmed by PCR (data not shown). Stable integrants were then backcrossed to a *ura4*Δ background in order to assay for reporter gene expression. *ura4*$^+$ silencing can be monitored by cell growth on medium supplemented with the cytotoxin 5-FOA, which selects for *ura4*$^+$ repression. Each *ura4*$^+$ reporter in the *dg* repeat of *cen3* was silenced in a wild-type background as shown by growth on 5-FOA. Silencing defects were observed to varying degrees in an *ago1Δ* background. These defects ranged from severe (*dh* forward construct, Figure 1D) to mild (*dh* reverse construct, Figure 1E). Our results suggest that repression of these *ura4*$^+$ reporters, at least in part, are regulated by RNAi (Figure 1B-E). Consistent with this finding, RT-PCR analysis of *ura4*$^+$ transcripts demonstrated increases

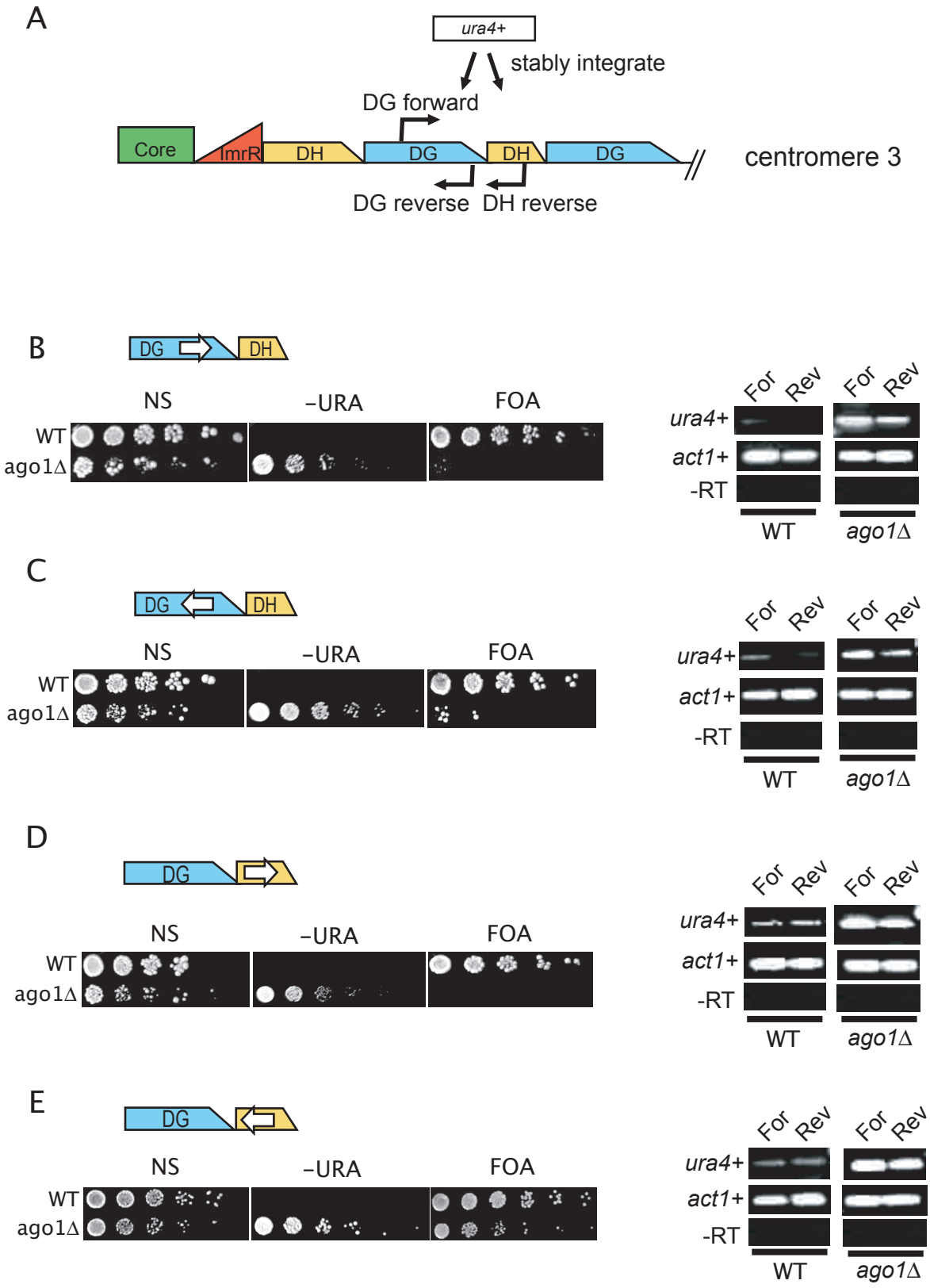

A
ura4+
stably integrate
DG forward
Core
ImrR
DH
DG
DH
DG
centromere 3
DG reverse
DH reverse
B
DG
DH
NS
-URA
FOA
WT
ago1Δ
For
Rev
For
Rev
ura4+
act1+
-RT
WT
ago1Δ
C
DG
DH
NS
-URA
FOA
WT
ago1Δ
For
Rev
For
Rev
ura4+
act1+
-RT
WT
ago1Δ
D
DG
NS
-URA
FOA
WT
ago1Δ
For
Rev
For
Rev
ura4+
act1+
-RT
WT
ago1Δ
E
DG
NS
-URA
FOA
WT
ago1Δ
For
Rev
For
Rev
ura4+
act1+
-RT
WT
ago1Δ

Figure 1

Figure 1. *ura4*$^+$ reporters integrated into the *dg* and *dh* repeats of *Cen3* in a forward and reverse orientation

(A) Schematic of the right arm of *cen3,* which contains a core surrounded by an inner repeat (*Imr*). Flanking outer repeats are termed *dg* and *dh* and contain forward and reverse promoters as indicated. *ura4*$^+$ reporters were stably integrated between the convergent promoters in either the *dg* or *dh* repeat. (B) *ura4*$^+$ in the *dg* repeat in a forward orientation (*cen3dg::ura4*$^+$) in a *ura4Δ* background (CK833) and *ago1Δ* (CK190). Strand-specific RT-PCR of the forward and reverse strands of *ura4*$^+$ in the indicated strains. (C) *ura4*$^+$ in the *dg* repeat in a reverse orientation (*cen3dg::ura4*$^+_{inv}$) in a *ura4Δ* background (CK836) and *ago1Δ* (CK423). Strand-specific RT-PCR of the forward and reverse strands of *ura4*$^+$ in the indicated strains. (D *ura4*$^+$ in the *dh* repeat in a forward orientation (*cen3dh::ura4*$^+$) in a *ura4Δ* background (CK326) and *ago1Δ* (CK302). Strand-specific RT-PCR of the forward and reverse strands of *ura4*$^+$ in the indicated strains. (E) *ura4*$^+$ in the *dh* repeat in a reverse orientation (*cen3dh::ura4*$^+_{inv}$) in a *ura4Δ* background (CK833) and *ago1Δ* (CK190). Strand-specific RT-PCR of the forward and reverse strands of *ura4*$^+$ in the indicated strains. . Serial dilution assays were performed on non-selective medium (NS), medium lacking uracil (-ura), or medium supplemented with 5-FOA (FOA).

in steady-state *ura4*$^+$ transcript levels in *ago1Δ*. Intriguingly, *ura4*$^+$ reporters integrated in either forward or reverse orientation within *dg* repeats still exhibited weak silencing in the absence of *ago1Δ* suggesting the existence of an RNAi-independent contribution to *ura4*$^+$ repression at these regions (Figure 1B-C). Interestingly, *ura4*$^+$ reporters under the control of the reverse centromere promoter in the *dh* repeat still exhibited robust silencing in the absence of *ago1Δ* as shown by growth on FOA (Figure 1E). However, *ura4*$^+$ transcript levels increased in *ago1Δ* suggesting the existence of additional silencing mechanisms that function downstream of centromere repeat expression.

Outer centromere repeats of *cen3* are regulated in a similar way

Cen3 of *S. pombe* has a unique arrangement of the *dg* and *dh* elements compared to centromere one (*cen1*) and centromere two (*cen2*). A full-length *dg* element and a truncated *dh* repeat comprise the most frequent arrangement of these elements at any centromere. It has been suggested that the right arm of centromere three contains up to seven *dg*/*dh* repeats while the left arm contains three. The exact number of repeats, however, is unclear. Furthermore, whether differences exist in the regulation of each repeat relative to the distance from the centromere core has not previously been investigated.

We integrated *ura4*$^+$ coding sequences at each *dg* and *dh* repeat within *cen3* in either forward or reverse orientations under the regulation of endogenous centromere promoters. Southern blotting was utilized to map the locations of each reporter using *ura4*$^+$ specific probes flanking a Stu1 restriction site (Figure 2). The repeat most proximal to the centromere core was denoted as #1 with the most distal repeat as #4. A representative Southern blot revealing the position of *ura4*$^+$ integrations into each centromere *dg* repeat is shown in Figure 2. Interestingly,

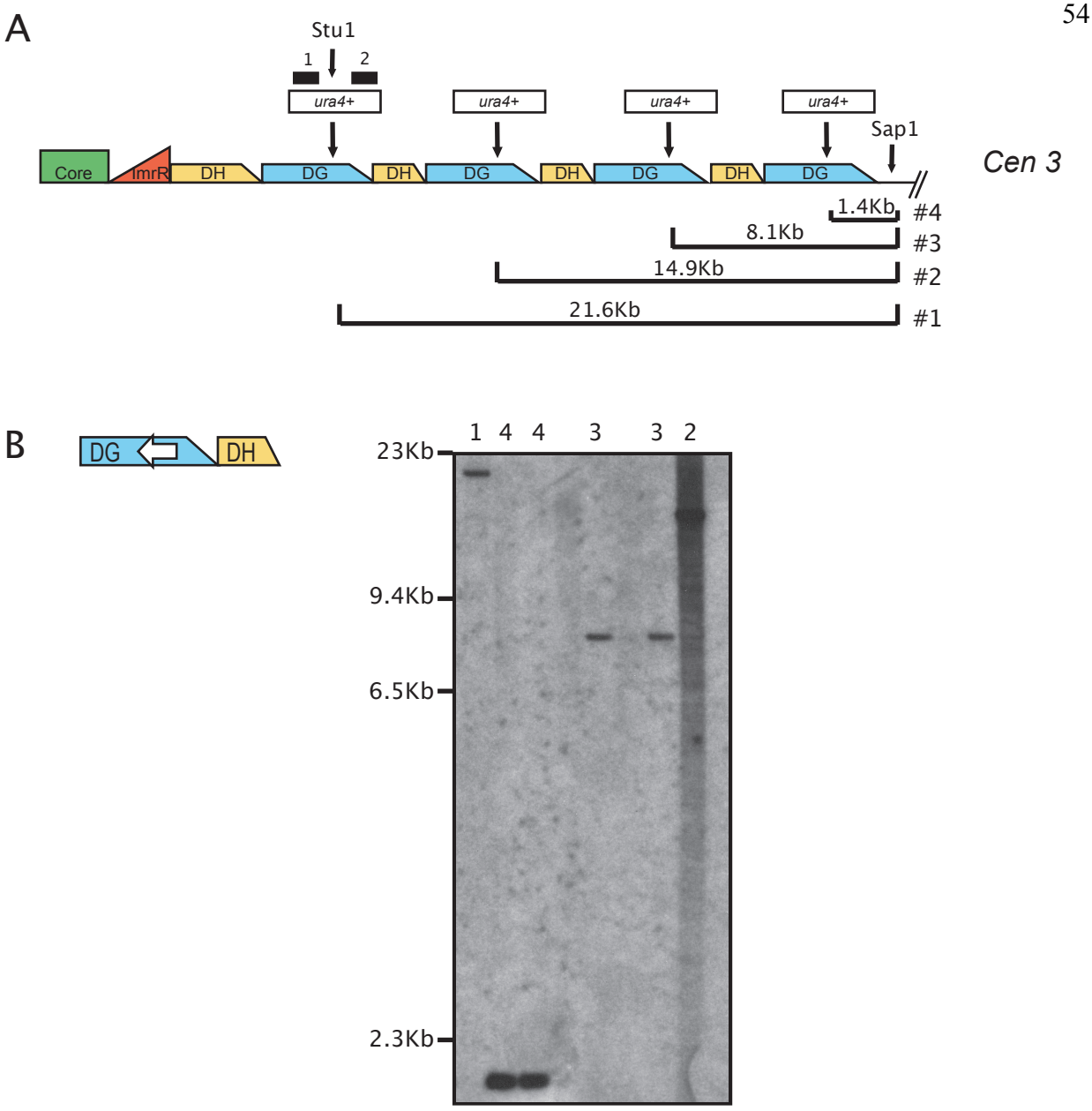


Figure 2: Mapping of *ura4+* reporters integrated into the repeats of *Cen3*

(A) Schematic of mapping strategy for each *ura4+* reporter integrated into *Cen3* repeats. A unique Sap1 restriction site was used outside of the repeat elements at *Cen3* along with a unique Stu1 site within the *ura4+* reporter. *ura4+* specific probes were used for detection depending on the *ura4+* orientation in relation to the Sap1 site. (B) Example of southern blot used to map the *ura4+* integrations located in the *dg* repeat in the reverse orientation. Bands corresponding to a *ura4+* reporter in each repeat using the #1 probe.

we were only able to obtain integrants in repeats #1 through #4 suggesting only four *dg*/*dh* outer repeats may flank the *cen3* central core. Intriguingly, we found that all *dg* integrants, in both the forward and reverse orientation, were efficiently silenced and this silencing was alleviated in *ago1Δ* to a similar extent in all repeats (Figure 3B). The same result was found for *ura4*$^+$ integrants into the *dh* repeat in the forward direction. We were unable to obtain a stable *ura4*$^+$ integrant in the *dh #1* repeat in the forward orientation. Whether integration of *ura4*$^+$ sequences in the forward orientation at *dh* #1 was unstable or resulted in lethality is not known. Furthermore, *ura4*$^+$ integrants in the reverse orientation within *dh* retained robust silencing even in the absence of *ago1* similar to our previous analysis (Figure 1E).

ura4$^+$ reporters integrated into the repeats of _cen1_ are regulated differently than corresponding repeats of _cen3_

The *dg*/*dh* repeats located at *cen1* are arranged differently than the repeats at *cen3*. One full-length *dg* and *dh* repeat are located on either side of the *cen1* central core. Interestingly, the orientation of the *cen1 dg* repeat is inverted relative to the orientation of the *cen3 dg* (compare Figure 4A to 1A). We again integrated the *ura4*$^+$ coding sequence into both *dg* and *dh* repeats of *cen1* in either a forward or reverse orientation. Although we were able to obtain *ura4*$^+$ integrants into the cen1 *dg* repeat in the "forward" orientation in *ago1Δ*, we were unable to maintain these integrants upon backcrossing to wild-type. Therefore, integration of *ura4*$^+$ at *cen1* is highly unstable in the absence of *ago1*$^+$.

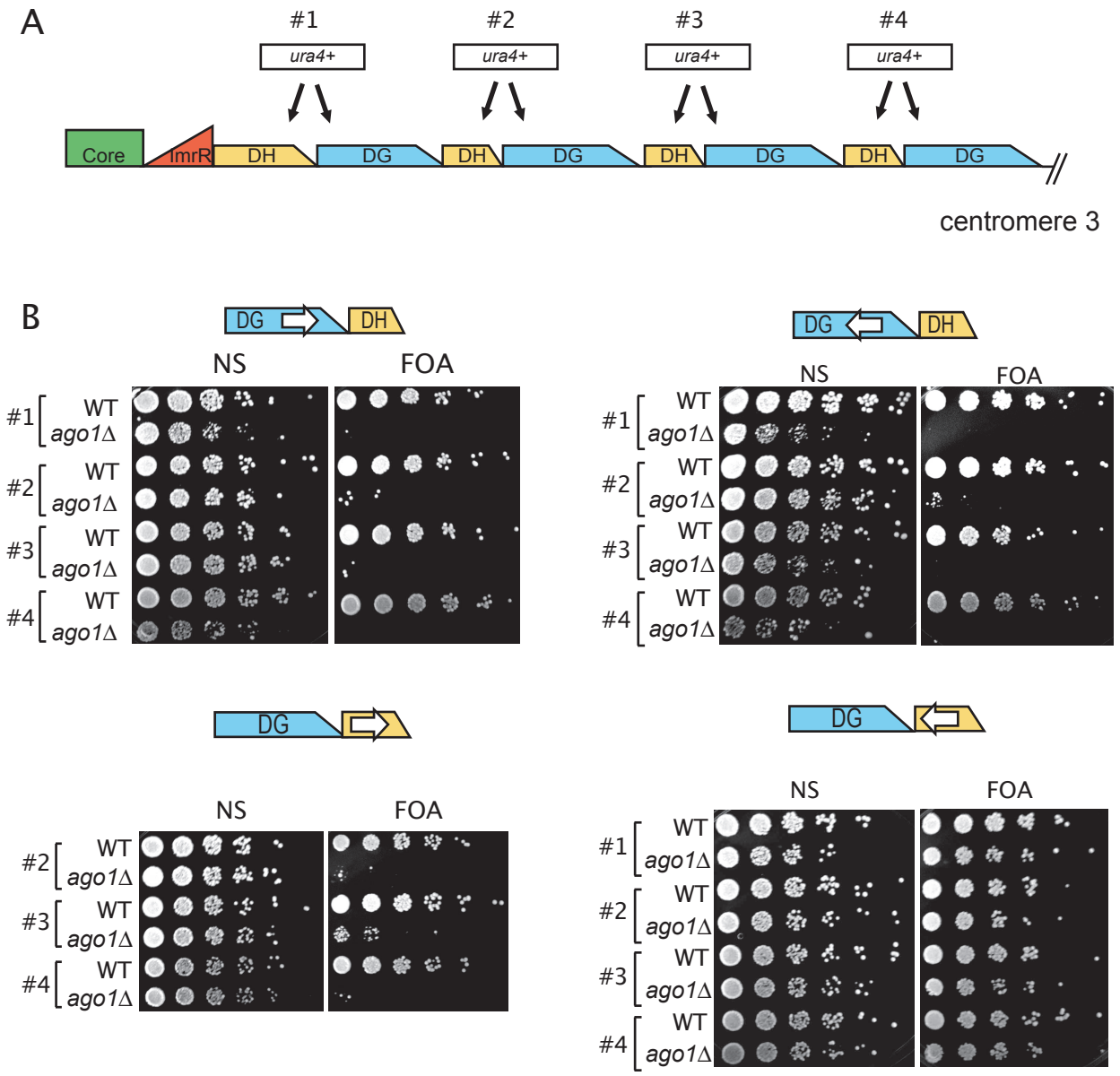


Figure 3. Mapped *ura4+* reporters integrated into each repeat of *Cen3*

(A) Schematic of the right arm of centromere three indicating the arbitrary numbering of each repeat element. (B) Dilution assays of each mapped *ura4+* reporter in each of the *dg* and *dh* repeats of centromere three in the indicated strains. Serial dilution assays were performed on non-selective medium (NS) or medium supplemented with 5-FOA (FOA).

The *ura4*$^+$ reporter integrations into the *cen1 dh* repeat in both orientations and at the *cen1 dg* repeat in the reverse orientation were stable and therefore assayed for silencing. We found that the *dg* integrated *ura4*$^+$ reporters and the *dh ura4*$^+$ insertion were regulated similarly. Expression of each of these *ura4*$^+$ reporter integrations were silenced in a wild-type background and this silencing was alleviated in the absence of *ago1*$^+$ (Figure 4B-D). Interestingly, *ura4*$^+$ integrated into the *dh* at *cen1* is effectively transcribed by a putative forward promoter not yet described (Figure 4C). Again we detect a small population of growth on FOA in *ago1Δ* suggesting an RNAi-independent mechanism contributes to silencing. Interestingly, *ura4*$^+$ reporters integrated in the reverse orientation at *cen1 dh* did not retain robust silencing in the absence of *ago1*$^+$ as was previously observed in *cen3* (Figure 1 and 3). This suggests different silencing pathways act on *dh* repeats at *cen3* compared to *cen1*. Strand-specific RT-PCR demonstrated that although *ura4*$^+$ transcripts were still detectable in wild-type, in *ago1Δ* both strands were elevated indicating a post-transcriptional mechanism for silencing (Figure 4B-D).

Analysis of *ura4*$^+$ reporters at *cen3* in RNAi and transcriptional silencing mutants

We sought to investigate whether silencing of the *ura4*$^+$ reporters at *cen3* were regulated by similar or distinct pathways. Therefore, representatives of the four *cen3 ura4*$^+$ reporter strains (*ura4*$^+$ integrated into *dg* and *dh* in either forward or reverse orientation) were crossed to mutants known to be involved in RNAi and heterochromatin formation.

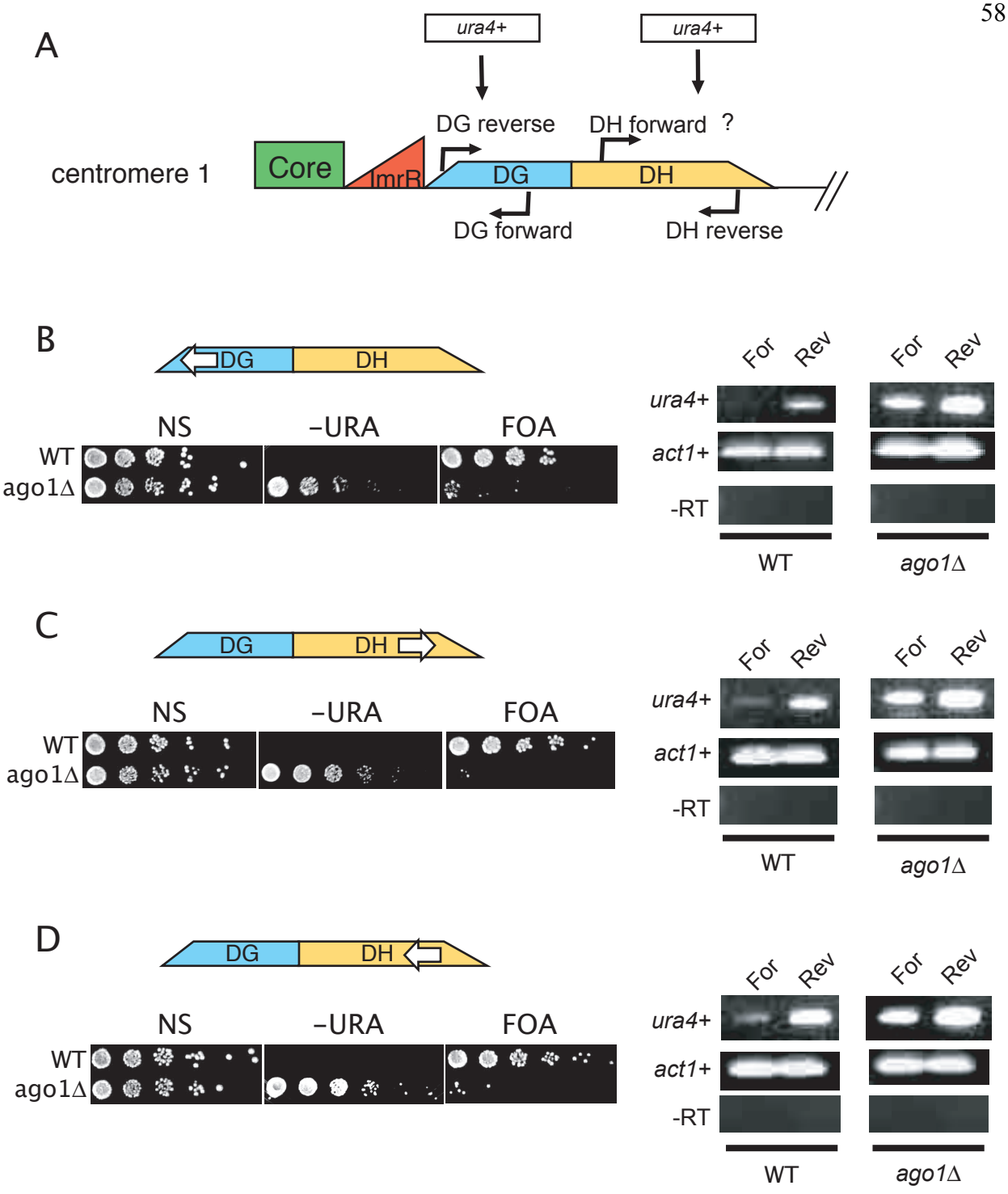

A
ura4+
ura4+
DG reverse
DH forward ?
centromere 1
Core
ImrR
DG
DH
DG forward
DH reverse
B
DG
DH
NS
-URA
FOA
WT
ago1Δ
For
Rev
ura4+
act1+
-RT
WT
ago1Δ
C
DG
DH
NS
-URA
FOA
WT
ago1Δ
For
Rev
ura4+
act1+
-RT
WT
ago1Δ
D
DG
DH
NS
-URA
FOA
WT
ago1Δ
For
Rev
ura4+
act1+
-RT
WT
ago1Δ

Figure 4

Figure 4. *ura4*$^{+}$ reporters integrated into the *dg* and *dh* repeats of *Cen1* in a forward and reverse orientation

(A) Schematic of the right arm of *cen1,* which contains a core surrounded by an inner repeats (*Imr*). Flanking outer repeats are termed *dg* and *dh* and contain forward and reverse promoters as indicated. *Ura4*$^{+}$ reporters were stably integrated between the convergent promoters in either the *dg* or *dh* repeat. (B) *Ura4*$^{+}$ in the *dg* repeat in a reverse orientation (*cen1dg::ura4*$^{+}_{Inv}$) in a *ura4Δ* background (CK900) and *ago1Δ (*CK859). Strand-specific RT-PCR of the forward and reverse strands of *ura4*$^{+}$ in the indicated strains. (C) *ura4*$^{+}$ in the *dh* repeat in a forward orientation (*cen1dh::ura4*$^{+}$) in a ura4Δ background (CK896) and ago1Δ (CK855). Strand-specific RT-PCR of the forward and reverse strands of ura4 in the indicated strains. (D) *ura4*$^{+}$ in the *dh* repeat in a reverse orientation (*cen1dh::ura4*$^{+}_{Inv}$) in a *ura4Δ* background (CK936) and *ago1Δ* (CK921). Strand-specific RT-PCR of the forward and reverse strands of *ura4*$^{+}$ in the indicated strains. Serial dilution assays were performed on non-selective medium (NS), medium lacking uracil (-ura), or medium supplemented with 5-FOA (FOA).

Silencing of the *ura4*$^+$ coding sequences integrated into the *dg* repeat under the control of the forward or reverse promoters was defective in *rdp1Δ*, *chp1Δ*, *tas3Δ*, *rik1Δ*, and *clr4Δ* but retained in swi6*Δ* (Figure 5A and 5B). Intriguingly, loss of *dcr1*$^+$ strongly de-repressed the *ura4*$^+$ reporter in the forward orientation but very weakly in the reverse orientation. Silencing of *ura4*$^+$ located in the *dh* repeat in a forward orientation was de-repressed in all mutant backgrounds tested with the exception of a *swi6Δ* (Figure 5C).

Remarkably, *ura4*$^+$ integrated in the *dh* element in the reverse direction at *cen3* retained robust silencing in all mutant backgrounds tested (Figure 5D). FOA colonies were picked to verify that the *ura4*$^+$ had not been lost from the strains, but all the strains retained the *ura4*$^+$ integration (data not shown). This result suggests the involvement of another pathway contributing to silencing, such as RNA processing and/or translational inhibition since the steady-state transcript levels were still elevated in *ago1Δ* even though the strain grew on FOA (Figure 1E). We also tested for a loss of silencing in mutants of the nonsense-mediated decay pathway, exosome, and RNA-editing pathway including *upf1Δ*, *nmd2Δ, xrn1Δ, tad1Δ,* and *ddp1Δ*. However, none of the mutants tested had any effect on the silencing of *ura4*$^+$ integrated in the *dh* repeat of *cen3* under the control of the reverse promoter (data not shown). Although *ura4*$^+$ silencing was defective to some degree in all of the mutant backgrounds tested, in most cases we still observe populations of cells with the ability to grow on FOA (Figure 5A-D). This is also consistent with the existence of alternate pathways contributing to centromere silencing.

Interestingly, Swi6 was dispensable for silencing of *ura4*$^+$ transcripts at all four *ura4*$^+$ reporters. Thus, silencing of centromere transcripts can be maintained independently of Swi6. The comprehensive examination of the effect of various silencing mutants on *ura4*$^+$ reporter

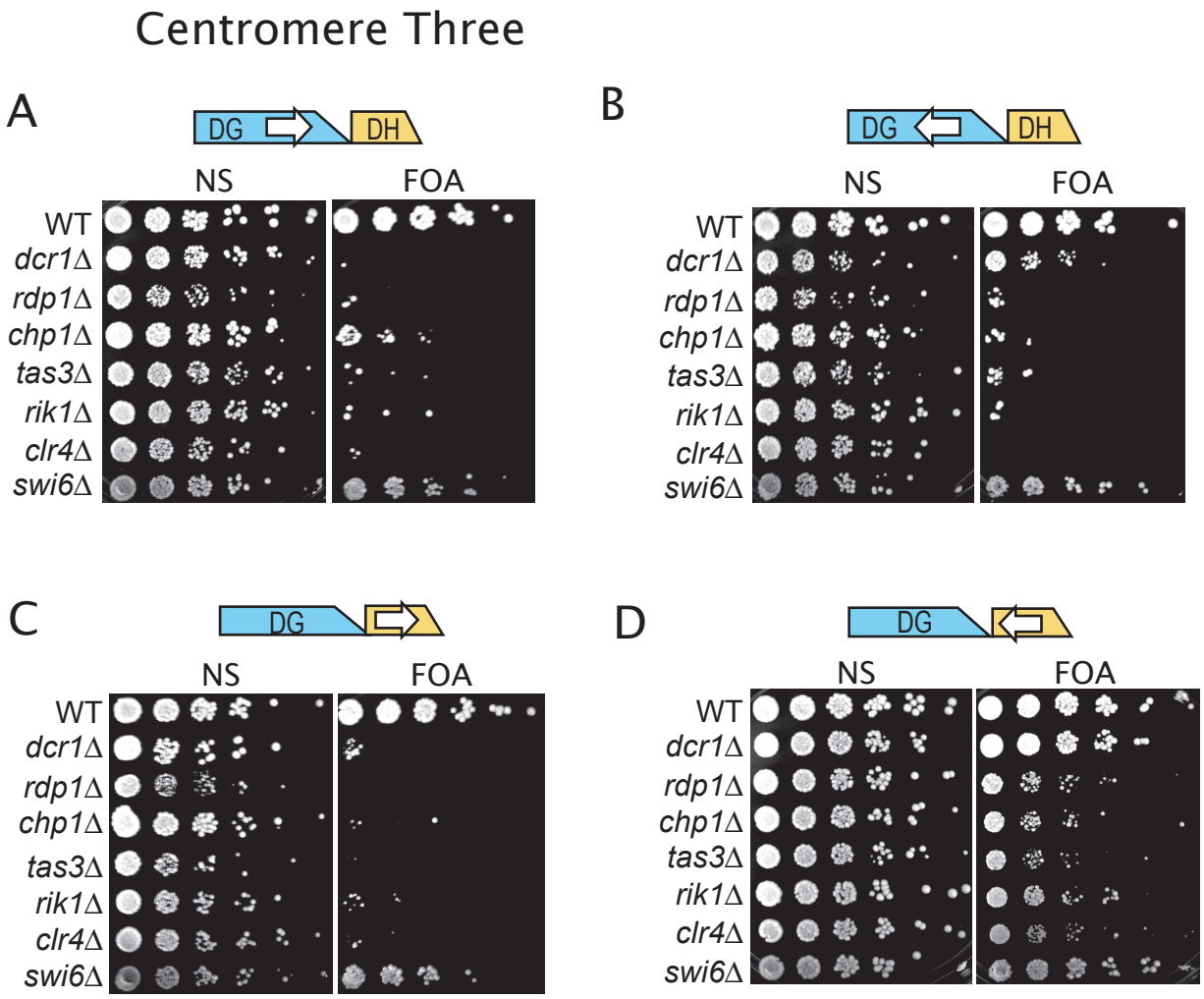


Figure 5. *Cen3 ura4+* reporters in RNAi and histone H3K9 methylation mutants

(A) *ura4+* in the *dg* repeat in a forward orientation (*cen3dg::ura4+*) in the indicated strains. (B) *ura4+* in the *dg* repeat in a reverse orientation (*cen3dg::ura4+inv*) in the indicated strains. (C) *ura4+* in the *dh* repeat in a forward orientation (*cen3dh::ura4+*) in the indicated strains. (D) *ura4+* in the dh repeat in a reverse orientation (*cen3dh::ura4+inv*) in the indicated strains. Serial dilution assays were performed on non-selective medium (NS) or medium supplemented with 5-FOA (FOA).

constructs in both the *dg* and *dh* elements of *cen3* alludes to the complexity of the contribution from different silencing pathways acting on this region to maintain a silenced state.

Analysis of *ura4*$^+$ reporters at *cen1* in RNAi and transcriptional silencing mutants

The observation that *ura4*$^+$ reporter constructs integrated into the *cen3 dg* and *dh* repeats respond differently in various silencing mutant backgrounds led us to investigate the regulation of *ura4*$^+$ reporters integrated at *cen1*.

The *ura4*$^+$ reporter integrated at *cen1 dg* was de-repressed to varying degrees in all silencing mutants backgrounds assayed (Figure 6A). Interestingly, silencing was maintained in a *swi6Δ* mutant background and to a lesser extent in *rdp1Δ* (Figure 6A).

The *ura4*$^+$ reporter integrated into the *dh* repeat at *cen1* in the forward direction was also de-repressed to varying degrees in silencing mutant backgrounds tested, however, silencing was maintained to a greater extent in a *swi6Δ* mutant background (Figure 6B). Interestingly, silencing of *ura4*$^+$ integrated at the *dh* in the reverse direction appeared to be completely de-repressed in every mutant background tested except for *swi6Δ*. This is in contrast to the pattern of *ura4*$^+$ regulation observed at *cen3 dh* when *ura4*$^+$ is integrated in the reverse orientation. This result suggests differential regulation of repeat transcripts depending on the centromere tested. As the *cen1 dh* repeat has a small deletion of sequences present in *cen3* an attractive hypothesis is that there are specific *cis* sequences at *cen3* that are required for the maintenance of silencing in the various mutant backgrounds tested.

Analysis of *ura4*$^+$ reporters integrated at *cen3* in the exosome mutants Rrp6 and Cid14

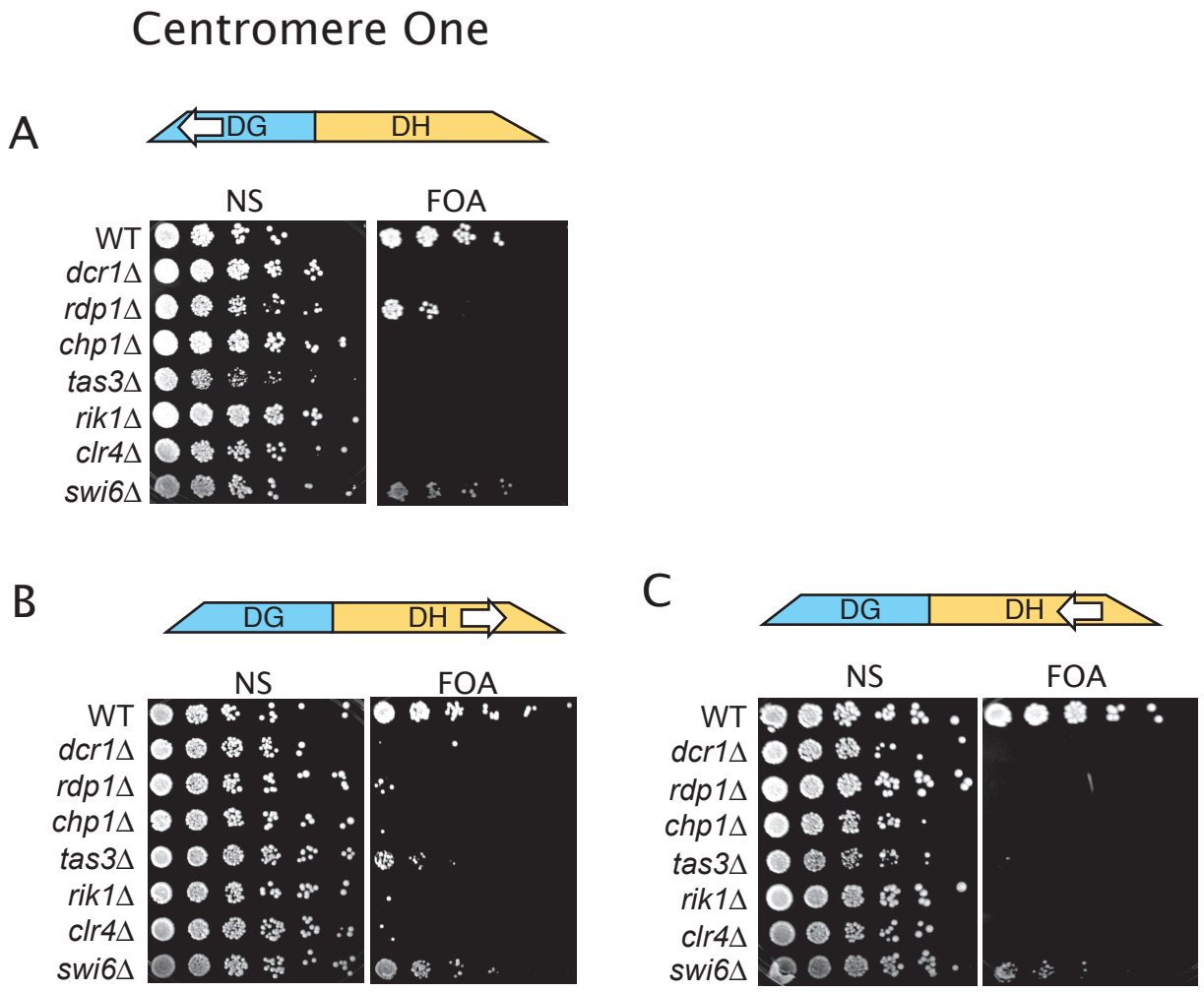


Figure 6. *Cen1 ura4+* reporters in RNAi and histone H3K9 methylation mutants

(A) *ura4+* in the *dg* repeat in a reverse orientation (*cen1dg::ura4+Inv*) in the indicated strains. (B) *ura4+* in the *dh* repeat in a forward orientation (*cen1dh::ura4+*) in the indicated strains. (C) *ura4+* in the *dh* repeat in a reverse orientation (*cen1dh::ura4+Inv*) in the indicated strains. Serial dilution assays were performed on non-selective medium (NS) or medium supplemented with 5-FOA (FOA).

A recent study has implicated the exosome mutants Cid14 and Rrp6 in the regulation of heterochromatic silencing at the centromere repeats in *S. pombe* (Buhler et al. 2007). Cid14 is a poly(A) polymerase homologous to components of the TRAMP complex in *S. cerevisiae* (LaCava et al. 2005). The exosome component Rrp6 degrades aberrant transcripts specifically within the nucleus. Cid14 was shown to be required for silencing at the centromeres, but did not affect H3K9me2, Swi6, or Chp1 binding suggesting that the degradation of RNA occurs downstream of the recruitment of these heterochromatin proteins (Buhler et al. 2007). We introduced mutations in either the spTRAMP component Cid14 or the exosome component Rrp6 to stains carrying the *ura4*$^+$ reporter constructs located at *cen3* in order to determine what contribution the exosome had on regulation of the *dg* and *dh* repeat transcripts.

Loss of *cid14* led to a modest de-repression of *ura4*$^+$ inserted into *cen3 dg* and *dh* repeats in both orientations. Modest de-repression of *ura4*$^+$ inserted at *cen3 dh* in the reverse orientation was also observed in *rrp6Δ* mutants. *ura4*$^+$ de-repression, however, was more pronounced at the *ura4*$^+$ *dh* reverse insertion as well as at the *ura4*$^+$ *dg* forward and reverse insertions in an *rrp6Δ* mutant background (Figure 7A).

We found it curious that repression of *ura4*$^+$ insertions into the *cen3 dh* repeat was largely maintained in various silencing mutant backgrounds (Figure 3B and 5D). We therefore wondered if the exosome could be involved in regulating centromere transcripts

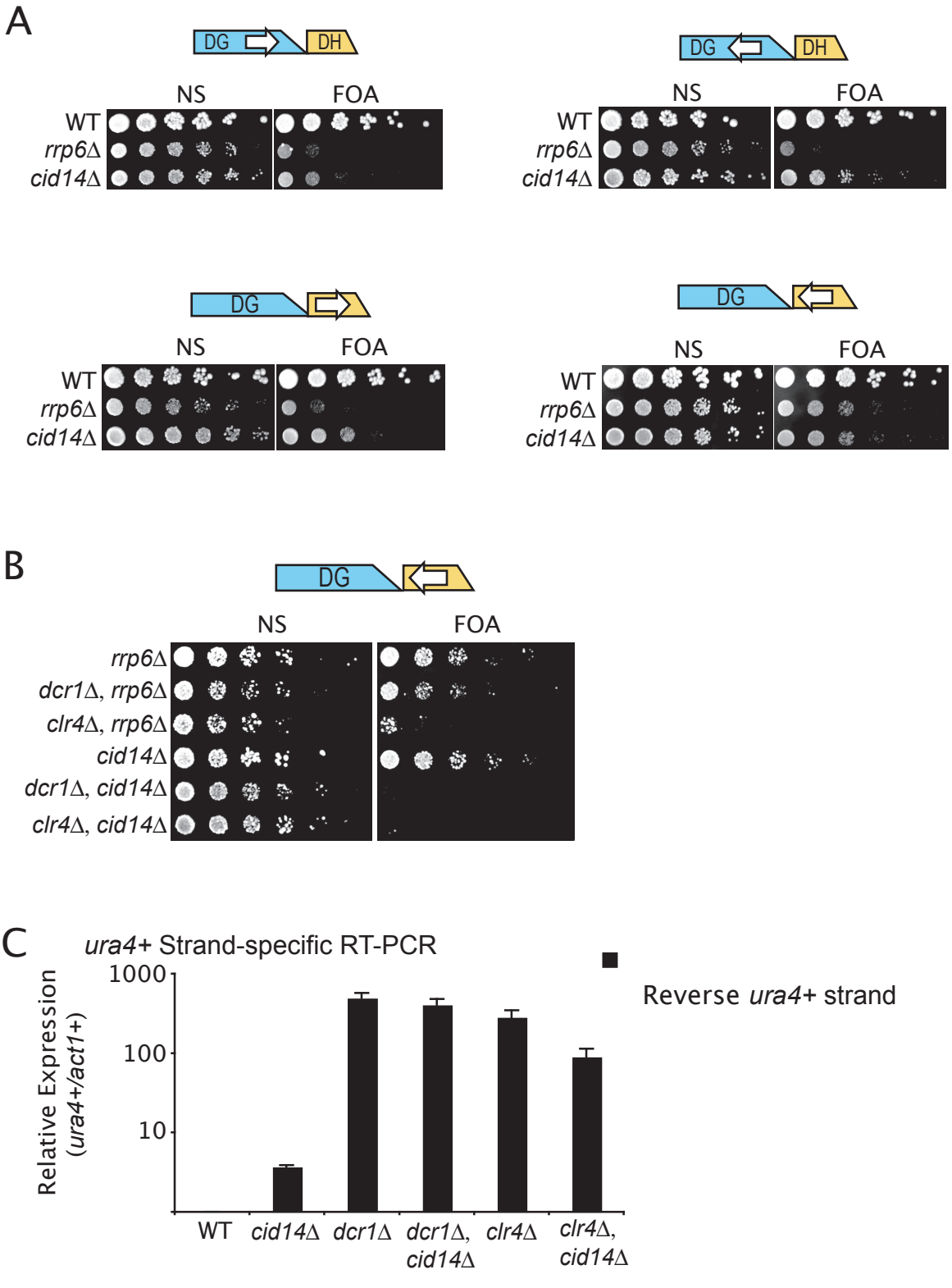

A
DG
DH
NS
FOA
WT
rrp6Δ
cid14Δ
B
dcr1Δ, rrp6Δ
clr4Δ, rrp6Δ
dcr1Δ, cid14Δ
clr4Δ, cid14Δ
C
ura4+ Strand-specific RT-PCR
Relative Expression
(ura4+/act1+)
1000
100
10
Reverse ura4+ strand
cid14Δ
dcr1Δ
dcr1Δ, cid14Δ
clr4Δ
clr4Δ, cid14Δ

Figure 7

Figure 7. *Cen3 ura4*$^{+}$ reporters in *cid14Δ* and *rrp6Δ* strains

(A) Serial dilution assays of the four *ura4*$^{+}$ reporters at centromere three in the indicated strains. Serial dilution assays were performed on non-selective medium (NS) or medium supplemented with 5-FOA (FOA). (B) Serial dilution assays of the *ura4*$^{+}$ reporter integrated in the reverse orientation in the *dh* repeat of centromere three (*cen3dh::ura4*$^{+}_{Inv}$) in the indicated strains. Serial dilution assays were performed on non-selective medium (NS) or medium supplemented with 5-FOA (FOA). (C) Strand-specific RT-PCR with primers specific for the *ura4*$^{+}$ integrated in the reverse orientation in the *dh* repeat of centromere three (*cen3dh::ura4*$^{+}_{Inv}$) strand-specific RT-PCR in the indicated strains. Data is presented using a logarithmic scale.

in the absence of RNAi or the transcriptional silencing machinery. We combined mutations in *clr4Δ* or *dcr1Δ* with *rrp6Δ* or *cid14Δ* to determine if RNAi or H3K9 methylation is redundant with the exosome in maintaining silencing of the *ura4*$^+$ reporter under the control of the reverse *dh* promoter. *ura4*$^+$ repression was still observed in both *dcr1Δ rrp6Δ* and *clr4Δ rrp6Δ* double mutants as seen by growth on FOA (Figure 7B), although silencing in the *clr4Δ rrp6Δ* double mutant exhibited the most pronounced de-repression. Remarkably, *ura4*$^+$ was completely de-repressed when introduced into a *clr4Δ cid14Δ* or *dcr1Δ cid14Δ* double mutant (Figure 7B). We next determined what affect these mutant backgrounds had on *ura4*$^+$ expression. Interestingly, analysis of *ura4*$^+$ transcript levels in these mutants revealed that reverse transcript levels of *ura4*$^+$ increased dramatically in both *clr4Δ* and *dcr1Δ* single mutants (Figure 7C). However, the transcript levels only modestly increased over wild-type levels in the absence of *cid14* (~5 fold, Figure 7C). Since we still observe growth on FOA in *dcr1Δ* and *clr4Δ* (Figure 5D), this suggests that the growth on FOA is due to some form of post-transcriptional silencing that is independent of RNA degradation. Consistent with this hypothesis, *cid14Δ dcr1Δ* or *cid14Δ clr4Δ* double mutants did not show increases of *ura4*$^+$ transcript levels above those observed in *dcr1Δ* or *clr4Δ* single mutants backgrounds (Figure 7C); however, these double mutants showed a loss of growth on FOA. These results suggest that in the absence of H3K9 methylation and/or RNAi the *ura4*$^+$ reporter driven by the *dh* reverse promoter of *cen3* is still post-transcriptionally repressed via a mechanism that involves the exosome component Cid14 resulting in growth on FOA. Possible mechanisms are discussed below.

Discussion

Our goal was to determine whether individual centromere repeats sequences are regulated by similar or different mechanisms in *S. pombe*. Results from this study reveal differential regulation of centromere transcripts.

One of the most interesting differences in centromere transcript regulation we observed was through analysis of the *ura4* insertion at *cen1* dg in the reverse orientation. Expression of *ura4*$^+$ was de-repressed in *dcr1Δ* and *ago1Δ* but *ura4*$^+$ silencing was maintained in *rdp1Δ* (Figure 4B and 6A). An attractive hypothesis to explain this observation is that siRNAs can be generated independently at this locus through bi-directional transcription followed by post-transcriptional degradation of the resulting centromere transcripts. An intriguing possibility is that Rdp1 independent RNA processing is due to "primary siRNA" synthesis that is dependent on Dcr1 and Ago1. Rdp1 may then be required for amplification of the primary siRNA signal via synthesis of "secondary siRNAs" as has been observed in other organisms. Such secondary siRNAs have not yet been identified in *S. pombe*.

Another interesting observation from this analysis was that silencing was maintained at each *ura4*$^+$ reporter in the absence of *swi6Δ*. This result is likely reflective of RNAi-mediated silencing of the reporters in the absence of Swi6 as RNAi specifies H3K9me2/3 which in turn binds Swi6 (Bannister et al. 2001; Volpe et al. 2002). Thus, in the absence of Swi6, RNAi retains the ability to post-transcriptionally regulate centromere transcripts.

One of the most intriguing differences in centromere transcript regulation was revealed through analysis of the *ura4*$^+$ insertion at *cen3 dh* in the reverse orientation. Robust *ura4*$^+$ repression was maintained in all of the mutant backgrounds we assayed, including mutations in RNAi, H3K9 methylation, and the exosome (Figure 3B and 5D). However transcript levels were

still elevated in the *ago1Δ*, *dcr1Δ*, and *clr4Δ* strains relative to wild-type (Figure 1E and 7C), suggesting the activity of a a post-transcriptional mechanism leads to the ability of these strains to grow on FOA.

Intriguingly, growth on FOA was abrogated in *cid14Δ clr4Δ* and *cid14Δ dcr1Δ* double mutants. The observation that these double mutants did not lead to additional increases in transcript levels compared to *dcr1Δ* or *clr4Δ* single mutants suggests the FOA growth in the plating assay could potentially be due to a translational inhibition mechanism that is controlled by the exosome component Cid14. Possible mechanisms to explain these observations include nuclear retention of mRNA, RNA deadenylation, or decapping of RNA, which could all be mediated by the exosome. We speculate that the *dh* reverse transcript may be nuclear retained in order to provide a template for Rdp1 activity in dsRNA synthesis and siRNA amplification.

The repetitive nature of centromere sequences makes it difficult to study the regulation of individual repeat elements. We therefore used *ura4*$^{+}$ reporter genes under the regulation of endogenous centromere promoters to analyze centromere transcript regulation in *S. pombe*. Our results reveal both strand-specific and locus dependent differential regulation of heterochromatic repeats and reveal for the first time that homologous centromere repeat sequences are subject to distinct repression pathways. While regulation of centromere repeats within each centromere appears to be similar, we do find that the *dg* and *dh* sequences themselves are regulated by different mechanisms. Furthermore, we find centromere transcript regulation varies when comparing repeats from *cen1* and *cen3*. In addition to the work presented here, the *ura4*$^{+}$ reporters created for use in this study will provide a useful tool for future studies involving regulation of the *dg* and *dh* outer repeats of the *S. pombe* centromeres.

Materials and Methods

Growth of Yeast Strains

S. pombe strains were grown in YEA medium (yeast extract supplemented with adenine), medium lacking uracil, or medium supplemented with 850mg/L of 5-FOA at 33^{O}C. Strains used for growth assays were grown overnight in non-selective YEA medium and then plated onto the indicated plates after a serial dilution. All the stains used are listed in the Appendix 1.

Construction of Strains

All *ura4*$^{+}$ reporters were constructed by a two-step PCR method with a vector template as previously described (Bahler et al. 1998). All *ura4*$^{+}$ reporters were stably integrated by standard transformation protocols for *S. pombe*, and then confirmed using primers outside the integration site. *ura4+* reporters were integrated into the *dg* repeat of *cen3* between the following sequences: 5'- atgtactcccaactgcggat -3' and 5- gagaaaagtgaaccgattgga-3 and transformed into *ago1Δ* strains and selected on media lacking uracil. *Ura4+* reporters were integrated into the *dg* repeat of *cen3* between the following sequences: 5'-agcatgggtatagaaagaagacg-3' and 5'-gaatgaacgtagcaatagatacaag-3' and transformed into *ago1Δ* strains and selected on media lacking uracil. The same primers were used to integrate *ura4*$^{+}$ reporters into the dg and dh repeats of *Cen1*, however, they were selected using primers specific to *Cen1*.

RNA extraction and RT-PCR

RNA was extracted from strains according to standard yeast protocols. Briefly, 50ml *S. pombe* cultures were grown to an O.D.$_{600}$ of ~0.5, pelleted, and frozen at -80°C. Frozen pellets were extracted with a solution of 50 mM sodium acetate, 10 mM EDTA, 1% SDS, and three phenol

chloroform extractions. RNA was then ethanol precipitated. cDNA was made with 1ug of RNA, DNase treated with 2 Units of DNAse RQ1 (Promega) at 37°C for 60 minutes. Reactions were then used in a cDNA reaction with 100 units of SuperScript III (Invitrogen), and primed with oligo-dT for 50 minutes at 42°C. cDNA for strand-specific RT-PCR was made using similar methods except the RT reaction was done with a gene specific primer and incubated at 55°C**.** The control *act1*$^{+}$ reverse primer was used in the same reaction wth the gene specific primer for each reaction. All RT-PCR reactions were done in triplicate. Primers for *ura4*$^{+}$ are listed below.

Quantitative PCR

Quantitative PCR was performed on an MJ Research/BioRad Chromo4 Thermocycler using Opticon 3.0 software. The optimal annealing temperature was determined for each primer set. The thermocycling conditions were as follows: an initial denaturation at 95°C for 3 minutes followed by 40 cycles at 95°C for 10 seconds, 50-60°C for 30 seconds, and 72°C for 1 minute. Fold enrichments and relative expressions were calculated using the Pfaffl method on Gene Ex software (Biorad). The reactions done for qRT-PCR were normalized to *act1*+. Primers for *ura4*$^{+}$ are the following: 5'- aattcgcagacattggaaatacc-3' (B86) and 5'-tgtgatatgagcccaagaagc-3' (B87). Reactions were performed in duplicate.

Southern Blottting and Mapping of *Ura4*$^{+}$ Reporters

Southern blotting was performed using standard protocols. 10ug of genomic DNA was digested with Stu1 and Sap1 overnight and then ethanol precipitated. DNA was then loaded onto a 0.7% agarose gel. Probes specific to the *ura4*$^{+}$ coding sequence on either side of the Stu1 site were labeled with ^{32}P labeled ATP and incubated with the blot over night at 65^{O}C. The membrane

was washed two times for twenty minutes each with .2X SSC, .1% SDS at 65^{O}C. The probe used for the reverse *ura4*$^{+}$ reporters was between the following sequences: 5'-caaagttatggatgctagagt-3' and 5'-tgtgatatgagcccaagaagc-3'. The probe used for the forward *ura4*$^{+}$reporters was between the following sequences: 5'-tatagctggtcgtcgatttcc-3' and 5'-gcaaacaaggcatcgacttt-3'.

CHAPTER III

RNAi Directed Targeting of Heterochromatin *in trans* Requires CENP-B Proteins

Abstract

RNA interference is a conserved mechanism observed in most eukaryotes that regulates gene expression by one of two mechanisms. RNAi effects post-transcriptional silencing by a well studied mechanism; RNAi also directs transcriptional silencing via heterochromatin assembly, however this process is poorly understood. A common feature of RNAi-mediated posttranscriptional silencing is the ability to target and silence homologous sequences *in trans*. However, whether an endogenous *trans* silencing pathway for RNAi-mediated heterochromatin assembly functions in wild-type *S. pombe* is unclear. This has proven problematic for defining the mechanisms necessary for initiating heterochromatin nucleation. Here we uncover a novel assembly pathway of RNAi-mediated targeting of heterochromatin that occurs *in trans* within centromeres and the mating-type locus. Remarkably, this process requires the CENP-B homolog, Abp1. Ultimately these studies will provide a basis for future investigations on the mechanism of RNAi-mediated heterochromatin assembly.

Introduction

Heterochromatin was first described as domains in the nucleus that remain condensed throughout interphase (Heitz 1928). Genetic and molecular analyses have demonstrated that these regions are essential for genome integrity and stability (Lippman and Martienssen 2004). The fission yeast, *S. pombe* has emerged as a powerful system to understand the mechanisms of heterochromatin assembly. Like metazoans, *S. pombe* heterochromatin is found at repetitive loci such as centromeres and telomeres (Cam and Grewal 2004). Heterochromatin is typically gene poor and genes juxtaposed with or inserted into these regions are silenced. In *S. pombe*, heterochromatin formation occurs by an RNAi-mediated mechanism that involves post-transcriptional gene silencing and the post-translational modification of histones (Hall et al. 2002; Volpe et al. 2002). Typically, deacetylated histones and histone H3 methylated at lysine 9 (H3K9me) define silenced regions associated with heterochromatin whereas euchromatic regions contain acetylated histones and histone H3 methylated at lysine 4 (Richards and Elgin 2002). A conserved class of SET domain containing histone methyltransferases of the Su(var)3-9 family mediate H3K9 di- or tri-methylation (H3K9me2/3); in *S. pombe*, the enzyme responsible for H3K9 methlytransferase activity is Clr4 (or Kmt1) (Nakayama et al. 2001). Proteins such as heterochromatin protein 1 (HP1, Swi6 in fission yeast) bind H3K9me2/3 via chromodomains to assemble higher order chromatin structures (Bannister et al. 2001; Lachner et al. 2001).

The RNAi pathway has been implicated in the formation of heterochromatin in several eukaryotes. RNAi is an evolutionarily conserved mechanism whereby double stranded RNA is processed by the RNaseIII enzyme Dicer into 21-25nt short interfering RNAs, which are subsequently loaded into an effector complex called RISC (RNAi Induced Silencing Complex) (Hamilton and Baulcombe 1999; Hammond et al. 2000b; Bernstein et al. 2001). The minimal

component of the RISC complex is Argonaute, an endonuclease that uses siRNAs to guide endonucleolytic cleavage of cognate RNAs (Liu et al. 2004a; Irvine et al. 2006). The RNAi effector complex implicated in transcriptional regulation in *S. pombe* is called RITS (RNAi-induced transcriptional silencing complex) (Verdel et al. 2004). RITS consists of the Argonaute protein, Ago1, a chromodomain containing protein, Chp1, and an adaptor protein, Tas3. The RITS complex has been shown to bind siRNAs and is required for heterochromatin assembly at *S. pombe* centromeres (Verdel et al. 2004). Another complex, RDRC which includes the RNA-dependent RNA polymerase Rdp1, a poly(A) polymerase, Cid12, and an RNA helicase, Hrr1, is also required for heterochromatin assembly (Motamedi et al. 2004). Due to the repetitive nature of the centromeres and the multiple silencing pathways that contribute to heterochromatic regions in *S. pombe*, the mechanism by which RNAi nucleates assembly of heterochromatin remains unclear.

Swi6/HP1 has been shown to recruit several chromatin-modifying enzymes and has been proposed to act as a platform within heterochromatin for the recruitment of an array of effector proteins. Swi6 recently was shown to recruit the SHREC complex (SNF2-and histone deacetylase containing repressor complex) to centromeres, the mating-type locus, and telomeres in *S. pombe* (Sugiyama et al. 2007). Presumably, the activity of proteins in the complex such as the histone deacetylase Clr3 and the nucleosome remodeler Mit1 are required for the assembly of the higher-order chromatin structure necessary to establish a silenced domain. Swi6 also recruits cohesin that functions in sister chromatid cohesion, thus ensuring faithful segregation of chromosomes in mitosis (Bernard et al. 2001). While it is clear that Swi6 is recruited to heterochromatin by binding histone H3 methylated at lysine 9 via its chromodomain, Swi6 can

also be recruited directly by DNA binding proteins at the mating-type locus (Atf1/Pcr1) and telomeres (Taz1/Ccq1) (Grewal and Elgin 2007).

A class of centromere DNA binding proteins called CENP-B's, have also been implicated in heterochromatin assembly in *S. pombe* (Nakagawa et al. 2002). Although defects in the CENP-B proteins of fission yeast have been shown to affect chromosome segregation and silencing at centromeres, the mechanism by which they contribute to heterochromatin assembly is unclear. These proteins are of particular interest since mammalian CENP-B proteins have been shown to be involved in *de novo* centromere formation (Masumoto et al. 2004; Okada et al. 2007).

Recent studies in *S. pombe* have led to the proposal that RNAi-directed heterochromatin assembly is *cis* restricted (referred to as *cis*-PTGS or co-transcriptional gene silencing)**.** In other words, RNAi can only assemble heterochromatin at the loci synthesizing double stranded RNA. Tethering of the RITS subunit, Tas3, to the euchromatic *ura4*$^{+}$ mRNA has recently been demonstrated to be sufficient for recruitment of H3K9 methylation and concomitant Swi6/HP1 binding. However, siRNAs generated either by tethering of RITS to mRNA or via a dsRNA trigger are not sufficient to target heterochromatin nucleation to unlinked *ura4*$^{+}$ reporters *in trans*, leading to the "*cis*-restricted" model (Buhler et al. 2006). Additional studies demonstrate that RNAi effects heterochromatin assembly by means of a "self-enforcing loop" *in cis* whereby RNAi directs H3K9 methylation and H3K9 methylation directs RNAi (Grewal and Elgin 2007). Although it is clear that RNAi can direct H3K9 methylation, the mechanisms that initiate this cycle have been enigmatic. In addition, whereas heterochromatin is restricted to the centromeres, telomeres, and the mating-type locus in *S. pombe*, it has recently been demonstrated that heterochromatin can form transiently during G1 of the cell cycle at genomic regions

containing bi-directionally transcribed convergent genes (Gullerova and Proudfoot 2008). Thus, the mechanisms that determine a cell's decision to stably assemble heterochromatin at certain parts of the genome and not others remain unclear.

Here we characterize the mechanism of RNAi mediated initiation of heterochromatin assembly *in trans*. Remarkably, we find this mechanism to be dependent on the CENP-B protein, Abp1. Thus, this work provides insight into the initiation step of RNAi-directed heterochromatin assembly in *S. pombe* and describes a novel role for CENP-B proteins in this process.

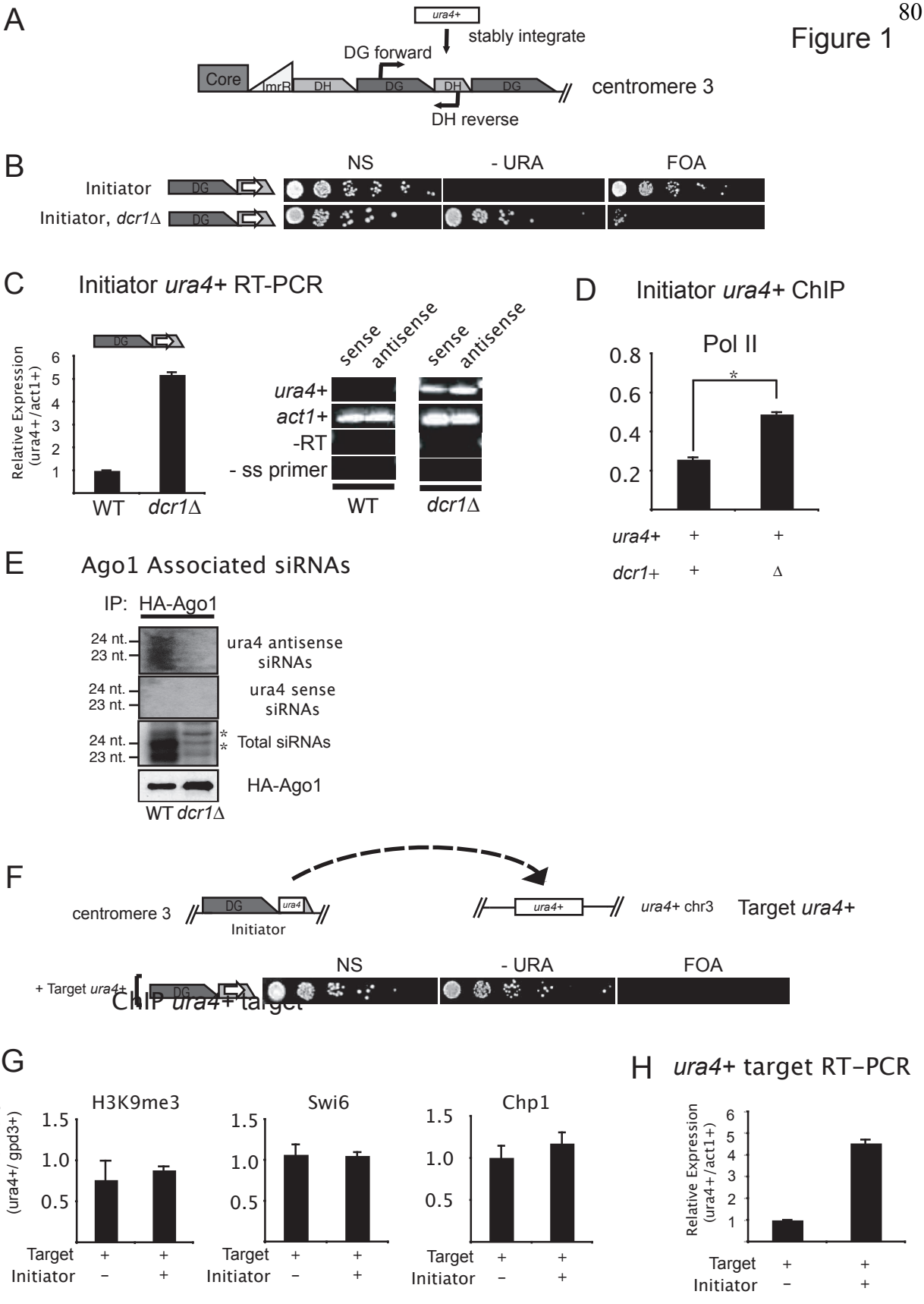
Results

RNAi dependent nucleation of heterochromatin *in trans*

While it has been proposed that RNAi mediated heterochromatin assembly in *S. pombe* is *cis* restricted we found it curious that RNAi directed epigenetic silencing in plants and animals does not appear to be *cis* restricted (Morris et al. 2004; Buhler et al. 2006; Kim et al. 2006; Matzke et al. 2007). However, a possible reason for this apparent dissimilarity between systems could be that *trans* targeting in *S. pombe* has not been fully investigated. We hypothesized that if RNAi-directed heterochromatin assembly occurs *in trans*, then such a mechanism might likely occur within or between the heterochromatic repeats of centromeres, as abundant siRNAs have been mapped to these regions (Reinhart and Bartel 2002; Cam et al. 2005). The centromeres of *S. pombe* resemble those of higher eukaryotes and are composed of a functional kinetochore that assembles at a core region flanked by innermost repeats (ImrR/L). These sequences are themselves flanked by outer repeats termed *dg* and *dh* where RNAi-mediated heterochromatin formation is known to occur (Volpe et al. 2002).

Centromeres are highly repetitive, which necessitates the integration of heterologous sequences within the repeats to distinguish specific loci. Therefore, we stably integrated the coding sequence of *ura4*$^{+}$ into a *dh* repeat of centromere three (*cen3*) in either a forward or reverse orientation (Fig. 1A, Fig. 2A). This region of the centromere was chosen because previous mapping studies identified it as a region of bi-directional transcription and siRNA synthesis (Volpe et al. 2002). Therefore, the *ura4*$^{+}$ reporter sequences (hereafter referred to as initiator *ura4*$^{+}$) should be transcribed

Figure 1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ura4+
stably integrate
DG forward
Core
imrR
DH
DG
DH
DG
centromere 3
DH reverse
B
NS
- URA
FOA
Initiator
Initiator, dcr1Δ
C
Initiator ura4+ RT-PCR
Relative Expression (ura4+/act1+)
sense
antisense
sense
antisense
ura4+
act1+
-RT
- ss primer
WT
dcr1Δ
D
Initiator ura4+ ChIP
Pol II
ura4+
dcr1+
E
Ago1 Associated siRNAs
IP: HA-Ago1
24 nt.
23 nt.
ura4 antisense siRNAs
ura4 sense siRNAs
Total siRNAs
HA-Ago1
WT dcr1Δ
F
centromere 3
Initiator
ura4+ chr3
Target ura4+
+ Target ura4+
ChIP ura4+ target
G
H3K9me3
Swi6
Chp1
Fold enrichment (ura4+/ gpd3+)
Target
Initiator
H
ura4+ target RT-PCR
Relative Expression (ura4+/act1+)

Figure 1. Integration and targeting of *ura4*$^+$ at *cen3* to the endogenous *ura4*$^+$ at chromosome three

(A) Schematic of the right arm of *cen3,* which contains a core surrounded by innermost repeats (ImrR). Flanking outer repeats are termed *dg* and *dh* and contain a forward and reverse promoter. A *ura4*$^+$ coding sequence was stably integrated between the convergent promoters in a forward (CK326, *cendh::ura4*$^+$) orientation into the *dh* repeat. (B) Schematic of the *ura4*$^+$ initiator at *cen3* targeting the endogenous *ura4*$^+$ on chromosome three. Serial dilution assays of strains including the initiating *ura4*$^+$ but lacking the endogenous target *ura4*$^+$ in wild-type (CK326) and *dcr1Δ* (CK715) on non-selective medium (NS), medium lacking uracil (-URA), or medium supplemented with 5-FOA (FOA). (C) Expression of the *ura4*$^+$ reporter at *cen3* in wild-type and *dcr1Δ* cultured in non-selective medium. Quantitive RT-PCR for *ura4*$^+$ was performed and normalized to the level of expression of the the wild-type strain. Error bars reflect SE. Expression of the sense (B87) and antisense (B86) strands of the *ura4*$^+$ reporter at *cen3* in wild-type and *dcr1Δ* cultured in non-selective medium. Controls include actin expression levels (*act1*$^+$*),* exclusion of reverse transcriptase (-RT), and exclustion of the strand specific primer (-ssprimer). (D) Quantitative chromatin immunoprecipitation (qChIP) analysis of RNA polymerase II enrichment at the initiator *ura4*$^+$ with or without *dcr1Δ*. Primers were specific to the *ura4*$^+$ (B86-B87). (E) Northern blot analysis of Ago1 associated *ura4*$^+$ siRNAs in wild-type (CK1237) and *dcr1Δ* (CK1249) strains. DNA oligos spanning the *ura4*$^+$ coding sequence were end labeled and hybridized to detect sense or antisense siRNAs. A fraction of the total siRNAs purified were 3' end labeled with γ-P32 ATP and run on a 12% polyacrylamide gel as a control. Astericks indicate degradation products from the Ago1 purification. Immunoprecipitated fractions from the indicated strains were analyzed by Western blotting with anti-HA antibody to

detect HA-Ago1. (F) Serial dilution assays with the initiating *ura4*$^{+}$ with the endogenous target *ura4*$^{+}$ (CK327) on non-selective medium (NS) or medium lacking uracil (-URA) or supplemented with 5-FOA (FOA). (G) Quantitative chromatin immunoprecipitation (qChIP) analysis of H3K9me, Swi6, and Chp1 enrichment in the indicated strains. Error bars reflect SE. (H) Quantitive RT-PCR for *ura4*$^{+}$ was performed and normalized to the level of expression of the the target *ura4*$^{+}$.

(* $P < 0.05$)

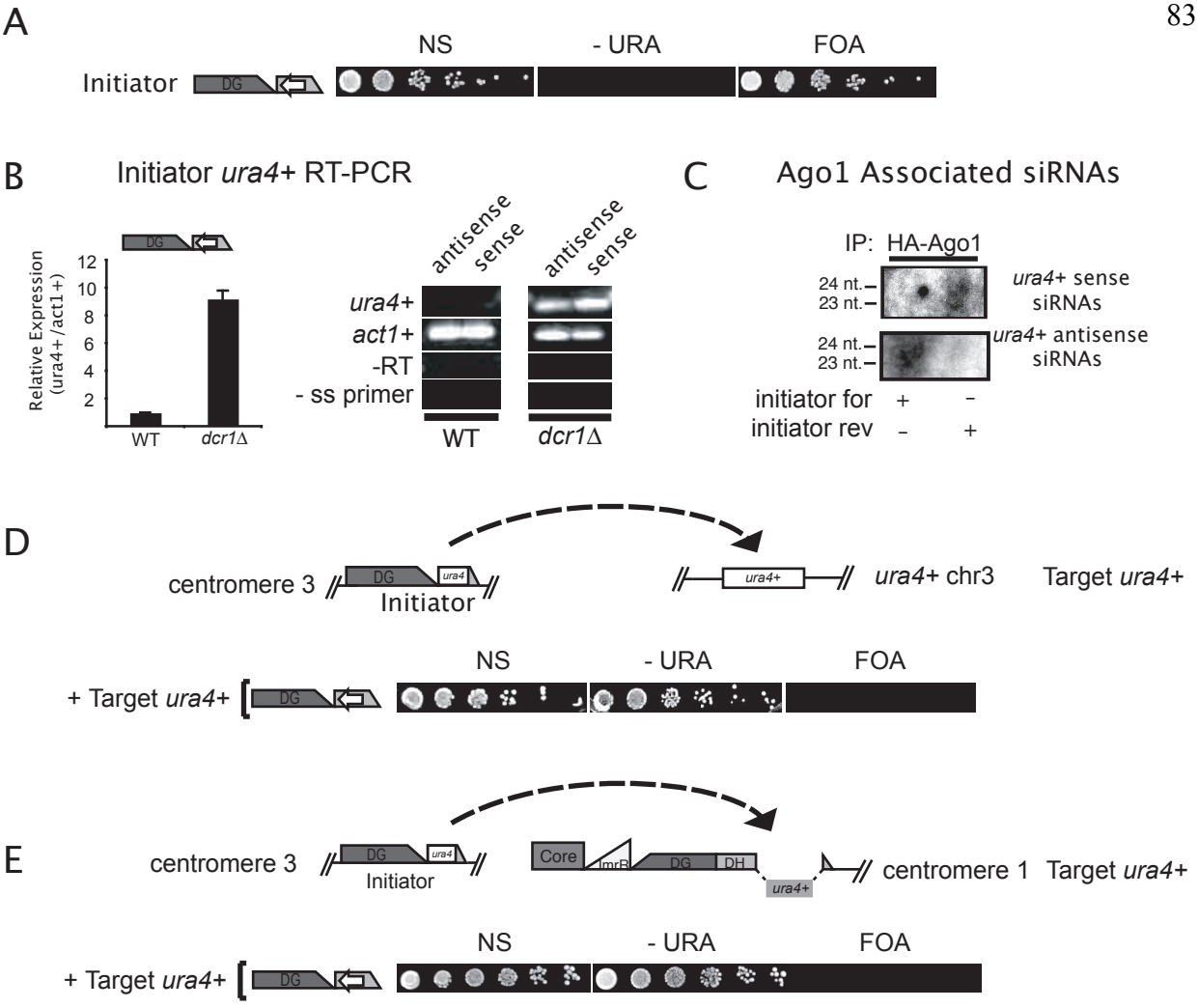


Figure 2. *Trans* Targeting of silencing from an initiating *ura4+* integrated at *cen3* in a reverse orientation to a target *ura4+*

(A) Serial dilution assay with the initiator *ura4+* (CK918) on non-selective (NS), medium lacking uracil (-URA), or supplemented with 5-FOA (FOA). (B) Expression of the *ura4+* initiator in WT (CK918) and *dcr1*Δ (CK714) cultured in non-selective medium. Quantitative RT-PCR for *ura4+* was performed and normalized to the level of expression of the wild-type (WT) strain. Error bars reflect SE. Expression of the *ura4+* sense and antisense transcripts in WT and *dcr1*Δ. (C) Northern blot analysis of Ago1 associated *ura4+* siRNAs in the forward (for) initiator (CK1237), shown previously in Fig. 1, and the reverse (rev) initiator (CK1223) strains. DNA oligos spanning the *ura4+* coding sequence were end labeled and hybridized to detect sense or anisense siRNAs. (D) Schematic of a *ura4+* initiator in the reverse orientation (*cendh::ura4+inv*) at *cen3* targeting the endogenous *ura4+* on chromosome three (chr3). Serial dilution assay of the intiator *ura4+* with the endogenous target *ura4+*. (E) Schematic *ura4+* intiator at *cen3* targeting a *ura4+* reporter at *cen1* (*dh1RindHNE*Δ::*ura4+*) (RL699).

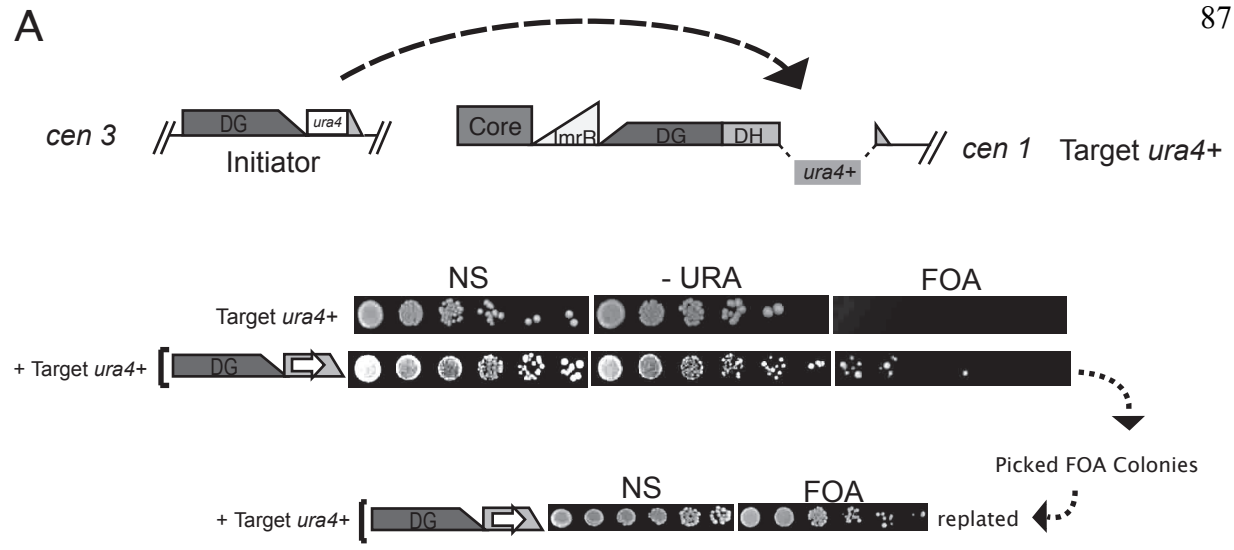
and processed into siRNAs. *ura4*$^+$ initiators introduced into a *ura4Δ* background were stably silenced as assayed by growth of these strains on 5-FOA media, and silencing was alleviated in a *dcr1Δ* background suggesting regulation by the RNAi machinery (Fig. 1B). 5-FOA is a counter selective media commonly used to select cells not expressing *ura4*$^+$*;* thus cells in which *ura4*$^+$ is silenced are able to grow on media containing FOA. RT-PCR analysis confirmed that both the forward and reverse *ura4*$^+$ initiators were effectively silenced in wild-type cells (Fig. 1C, Fig. 2B). Moreover, both *ura4*$^+$ sense and antisense mRNA accumulate in *dcr1Δ* indicating that expression of *ura4*$^+$ is being regulated by the RNAi machinery (Fig. 1C, Fig. 2B). In addition, we assayed for enrichment of RNA polymerase II at the initiator *ura4*$^+$. We detect an increase of RNA polymerase II at *ura4*$^+$ in *dcr1Δ* suggesting that regulation of *ura4*$^+$ expression, at least in part, is mediated by transcriptional silencing mechanisms (Fig. 1D). Since the *ura4*$^+$ initiators are bi-directionally transcribed it is likely that *ura4*$^+$ transcripts are also processed by the RNAi machinery. We hypothesized that an RNAi mediated *trans* silencing mechanism would require a homology based targeting mechanism including loading of *ura4*$^+$ siRNAs into the RITS complex. We tested this possibility by purifying epitope-tagged HA-Ago1 and assaying for RITS associated *ura4*$^+$ siRNAs in either a wild-type or *dcr1Δ* background. This analysis revealed association of antisense *ura4*$^+$ siRNAs with RITS in wild-type but not in *dcr1Δ* (Fig. 1E). The absence of detectable *ura4*$^+$ sense siRNAs suggests preferential loading or biogenesis in agreement with recent reports of preferential siRNA loading into Ago1 when assaying a previously described *ura4*$^+$ reporter at *cen1* (Buhler et al. 2008). Consistent with this idea, the *ura4*$^+$ reporter in the reverse orientation revealed association of sense *ura4*$^+$ siRNAs with RITS in wild-type (Fig. 2C). However, the mechanism involved in strand specific siRNA loading into RITS is not known.

We assayed the ability of *ura4*$^+$ initiators to target heterochromatin assembly *in trans* by introducing them into strains containing a wild-type *ura4*$^+$ gene at the endogenous locus (on the left arm of chromosome three). No obvious *trans* silencing effects were observed by either forward or reverse *ura4*$^+$ initiators as indicated by the inability of these strains to grow on medium containing 5-FOA (Fig. 1F, Fig. 2D). Although stable silencing was not targeted *in trans* to the endogenous *ura4*$^+$, we used chromatin immunoprecipitation (ChIP) to test whether heterochromatin is targeted transiently to the target *ura4*$^+$ when the initiator is present. However, no significant changes were detected in H3K9me3, Swi6 or Chp1 levels at the target *ura4*$^+$ with or without the initiator *ura4*$^+$ present (Fig. 1G). Interestingly, the endogenous *ura4*$^+$ steady-state mRNA levels are elevated when the initiator is present, however, the reason for this increase is unknown (Fig. 1H).

These results suggest *ura4*$^+$ initiators were unable to target heterochromatic silencing to the endogenous *ura4*$^+$ on chromosome 3. Next, we investigated whether *trans* targeting occurs within centromeres by utilizing a *ura4*$^+$ reporter that has been integrated within *dh* centromere sequences (Lawrence, R.J. et. al, submitted). This *ura4*$^+$ reporter insertion (*dh1RindHNEΔ::ura4*$^+$) replaces an RNAi-independent nucleation element in centromere one (*cen1*) and results in elimination of heterochromatin assembly at this region as well as heterochromatin spreading into sequences flanking the insertion (Fig. 3A). Due to the fact that the *ura4*$^+$ in *cen1* is within a heterochromatic domain, we investigated whether there was a low level of silencing present by comparing its expression to *ura4*$^+$at its endogenous locus on chromosome 3. Steady-state *ura4*$^+$ reporter mRNA levels were similar to endogenous *ura4*$^+$ transcript levels (Fig. 4A). In addition, loss of *dcr1* did not cause increased *ura4*$^+$ reporter mRNA levels suggesting the RNAi machinery has no influence on expression of the *ura4*$^+$

reporter integrated at *cen1* (Fig. 4A). Furthermore, neither bi-directional transcription (we only detect *ura4*$^+$ sense strand by RT-PCR) nor siRNAs originating from the target *ura4*$^+$were detected (Fig 4A-B). Chromatin immuno-precipitation assays revealed similar enrichments of H3K9me3, H3K9me2, Swi6, Chp1, H3K9ac, H3K14ac, and RNA polymerase II at the target *ura4*$^+$ reporter compared to endogenous *ura4*$^+$ (Fig. 4C). Taken together, these data strongly suggest that no basal level heterochromatin exists at *dh1RindHNEΔ::ura4*$^+$. This construct, therefore, provides a unique tool to assay nucleation of heterochromatin within centromeres.

We introduced *ura4*$^+$ initiator constructs to strains containing *dh1RindHNEΔ::ura4*$^+$ in order to assay for *trans* silencing. Strikingly, the "forward" initiator *ura4*$^+$ reporter located at *cen3* when introduced into a *dh1RindHNEΔ::ura4*$^+$ background resulted in *trans* silencing of the *ura4*$^+$ at *cen1* (referred to as the target *ura4*$^+$), as shown by growth on FOA. Presence of the "reverse" initiator *ura4*$^+$, however, had no effect on target *ura4*$^+$ expression (Fig. 3A, Fig. 2E). The preferential loading of sense siRNAs from the "reverse" initiator *ura4*$^+$ construct into RITS could explain the inability of the reverse construct to target the *ura4*$^+$ reporter at *cen1*. It is possible that sense *ura4*$^+$ siRNAs loaded into RITS are unable to target the nascent sense mRNA at the target locus whereas antisense *ura4*$^+$ siRNAs from the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cen 3
DG
ura4
Initiator
Core
ImrR
DG
DH
ura4+
cen 1
Target ura4+
NS
- URA
FOA
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+ Target ura4+
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Picked FOA Colonies
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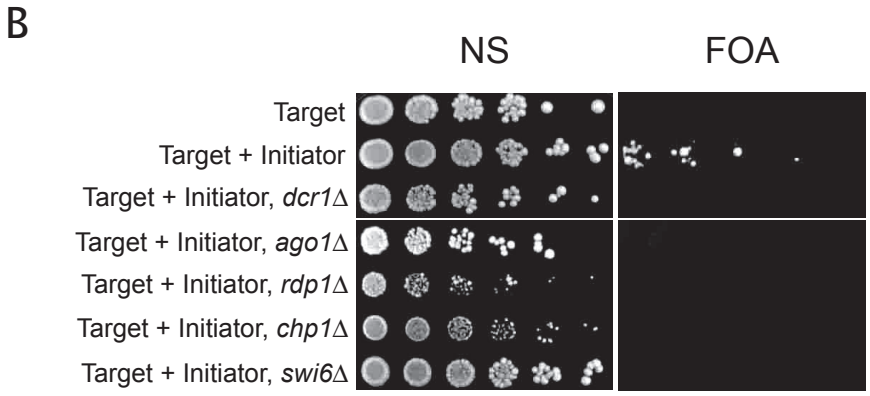

B
NS
FOA
Target
Target + Initiator
Target + Initiator, dcr1Δ
Target + Initiator, ago1Δ
Target + Initiator, rdp1Δ
Target + Initiator, chp1Δ
Target + Initiator, swi6Δ

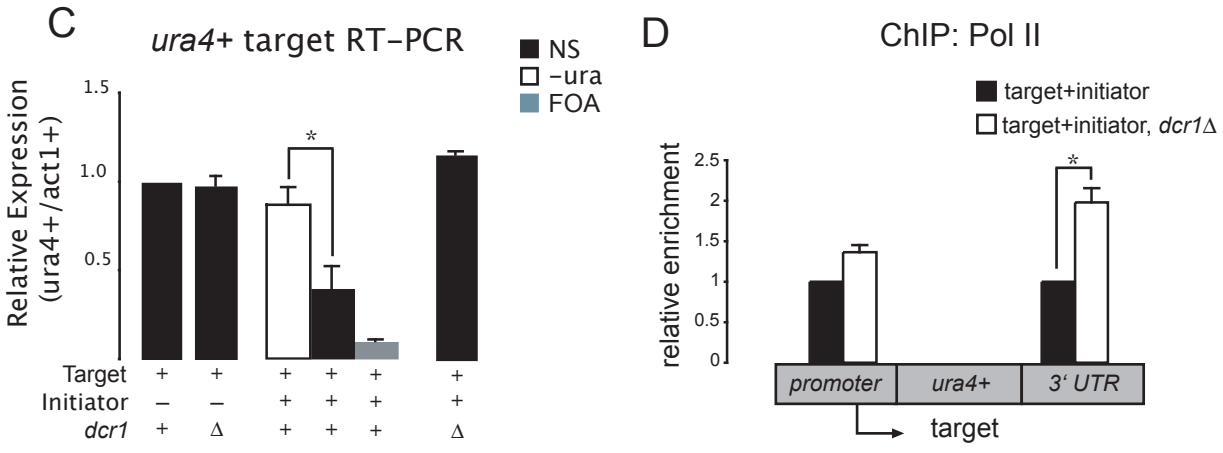

C
ura4+ target RT-PCR
NS
-ura
FOA
Relative Expression
(ura4+/act1+)
1.5
1.0
0.5
*
Target + + + + + +
Initiator – – + + + +
dcr1 + Δ + + + Δ
D
ChIP: Pol II
target+initiator
target+initiator, dcr1Δ
relative enrichment
2.5
2
1.5
1
0.5
0
*
promoter
ura4+
3' UTR
target

Figure 3

Figure 3. *Trans* targeting of silencing is dependent on RNAi.

(A) Schematic of *ura4*$^+$ reporter at *cen3* targeting a *ura4*$^+$ reporter at *cen1* (*dh1RindHNEΔ::ura4*$^+$). Serial dilution assays with both initiating *ura4*$^+$ with (CK1101) or without (RL699) the *cen1* target *ura4*$^+$ on non-selective medium (NS) or medium lacking uracil (-URA) or supplemented with 5-FOA (FOA). Serial dilution of cells picked from FOA were cultured in non-selective medium and then re-plated on either non-selective medium or FOA. (B) Serial dilution assay with the target *ura4*$^+$ (RL699), or the target *ura4*$^+$ plus the initiator *ura4*$^+$ with (CK1186) or without *dcr1Δ* (CK1101), *ago1Δ* (CK1265), *rdp1Δ* (CK1188), *chp1Δ* (CK1263), or *swi6Δ* (CK1266) on non-selective (NS) medium or supplemented with 5-FOA (FOA). (C) Expression of the target *ura4*$^+$ in the target only strain, target only with *dcr1Δ* (CK1315) and the target plus initiator with or without *dcr1Δ*. RNA was extracted from strains grown in non-selective (black bars), medium lacking uracil (-URA) (white bar), or medium supplemented with 5-FOA (FOA) (gray bar). Quantitive RT-PCR for *ura4*$^+$ was performed and normalized to the target *ura4*$^+$ alone. (D) Quantitative chromatin immunoprecipitation (qChIP) analysis of RNA polymerase II enrichment at the target *ura4*$^+$ in a strain with the initiator *ura4*$^+$ plus the target *ura4*$^+$ with or without *dcr1Δ*. Primers were either specific to the *ura4*$^+$ promoter (B66-B67) or 3'UTR (B68-B69). (* $P < 0.05$)

"forward" initiator can. Moreover, silencing was stable when FOA resistant colonies were selected and re-cultured in non-selective media. Furthermore, silencing of the target *ura4*$^+$ was abrogated in *dcr1Δ*, *ago1Δ*, *chp1Δ*, *rdp1Δ*, and *swi6Δ* mutant backgrounds suggesting that *trans* silencing is dependent on RNAi (Fig. 3B).

In order to confirm that *ura4*$^+$ target transcripts were effectively silenced, we assayed the levels of the *ura4*$^+$ target transcript by qRT-PCR. Target *ura4*$^+$ transcripts can be specifically assayed since they, unlike the initiating *ura4*$^+$, contain 3'UTR sequences (Fig. 3C). Fig. 3C reveals steady state transcript levels of target *ura4*$^+$ mRNA were decreased ~2.5 fold when the forward initiating *ura4*$^+$ was present. However, this experiment assays a population of cells in non-selective culture, presumably only a fraction of which have initiated silencing. In order to determine the transcript levels of the target *ura4*$^+$ in the "on" (expressed) or "off" (silenced) state, we cultured cells in either medium lacking uracil to select for the "on" state or FOA to select for the "off" state. The transcript levels of target *ura4*$^+$ mRNA when selected in medium lacking uracil were similar to those observed in cells grown in non-selective media without the initiating *ura4*$^+$ present, however *ura4*$^+$ transcript levels of cells selected in the FOA media were reduced by ten fold (Fig. 3C). Furthermore, *ura4*$^+$ target silencing was lost in *dcr1Δ*. In addition, we assayed for enrichment of RNA polymerase II throughout the target *ura4*$^+$. Consistent with an increase in transcript levels in *dcr1Δ*, we detect a significant increase of RNA polymerase II at the 3'UTR of the target *ura4*$^+$ in *dcr1Δ* (Fig. 3D). These data demonstrate a bona fide RNAi dependent *trans* silencing mechanism leading to transcriptional gene silencing.

Effective targeting of heterochromatin and silencing to *ura4*$^+$ *in trans*

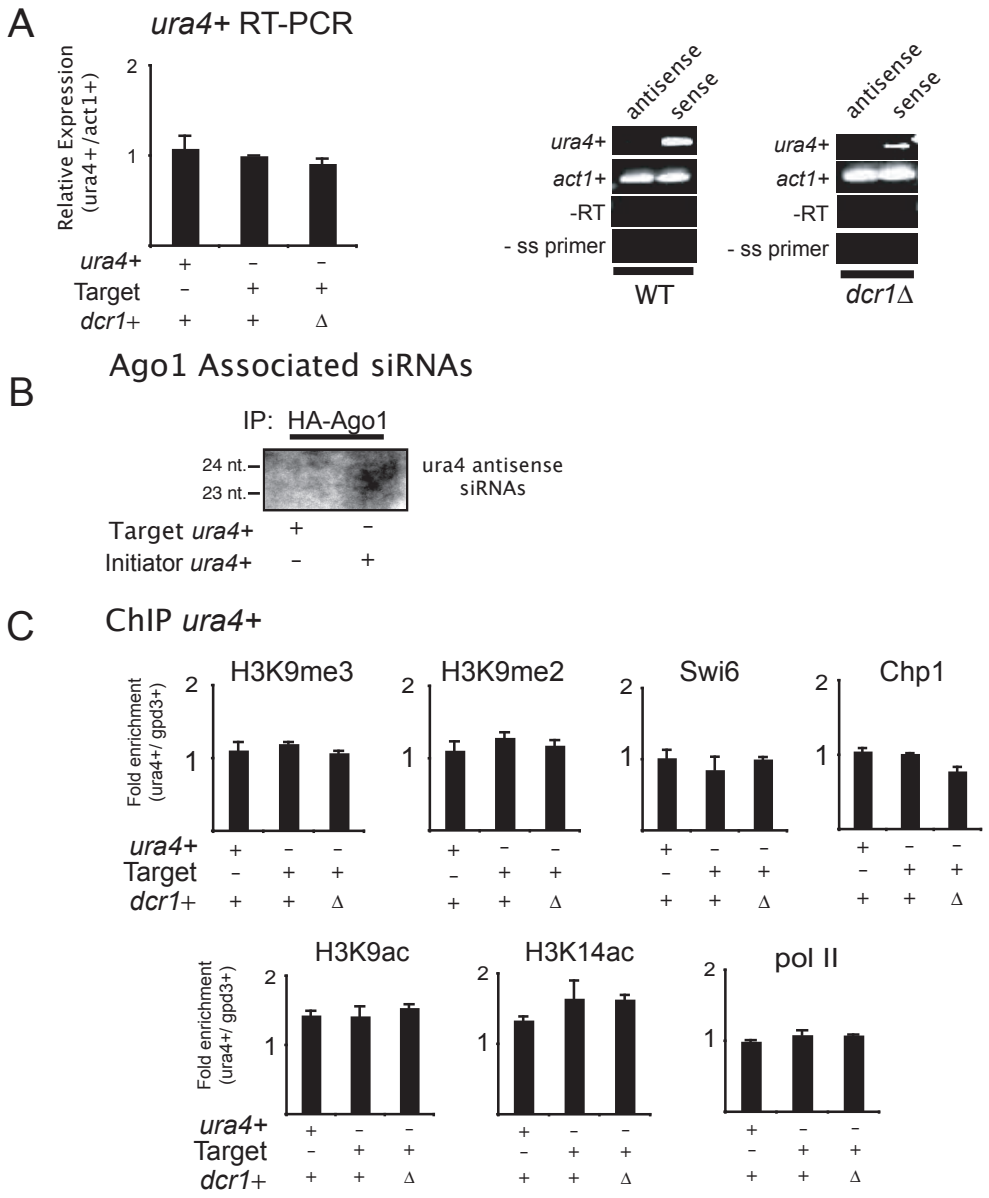


Figure 4: The target *ura+* at *cen1* is transcribed and does not have a basil level of silencing present

(A) Expression of the endogenous *ura4+* (CK306), the *cen1* target *ura4+* (CK1180), and the *cen1* target *ura4+*, *dcr1*Δ (CK1315). Quantitative RT-PCR for *ura4+* was performed and normalized to the target *ura4+*. Error bars reflect SE. Expression of the *ura4+* sense and antisense transcripts in WT and *dcr1*Δ. (B) Northern blot analysis of Ago1 associated *ura4+* siRNAs in the target *ura4+* (CK1247) and the initaitor *ura4+* (CK1237) strains. DNA oligos spanning the *ura4+* coding sequence were end labeled and hybridized to detect anisense siRNAs. (C) Quantitative chromatin immunoprecipitation (ChIP) analysis of H3K9me3, H3K9me2, Swi6, Chp1, H3K9ac, H3K14ac, and Pol II enrichment at endogenous *ura4+* (CK306), the *cen1* target *ura4+* (CK1180), and the *cen1* target *ura4+*, *dcr1*Δ (CK1315). Error bars reflect SE.

We used ChIP to test whether heterochromatin is targeted *in trans* to the target *ura4*$^+$ construct integrated at *cen1*. Not only did this analysis demonstrate increased enrichment of H3K9me3 and Swi6, but it also revealed an increased enrichment of the RITS component Chp1 specifically when the target is in the presence of the initiator *ura4*$^+$ (Fig. 5B). This suggests a mechanism whereby *ura4*$^+$ siRNAs are loaded into RITS from *cen3* and targeted *in trans* to the homologous target *ura4*$^+$ via RITS.

Replacement of the RNAi-independent nucleation locus at the *cen1 dh* with the *ura4*$^+$ reporter (target *ura4*$^+$) results in a loss of heterochromatin spreading into flanking regions. We assayed by ChIP whether targeting of heterochromatin to *ura4*$^+$ *in trans* restores heterochromatin spreading into flanking regions. ChIP analysis demonstrated an increased enrichment of H3K9me3, Swi6, and Chp1 in regions flanking the *ura4*$^+$ reporter at *cen1* when compared to the target only strain (Fig. 5C). Furthermore this increased enrichment was lost in *dcr1Δ*. These data demonstrate the effective RNAi dependent nucleation of heterochromatin occurring *in trans* that subsequently results in spreading of heterochromatin divergently to sequences flanking the target *ura4*$^+$ reporter.

Targeting of heterochromatin *in trans* is dependent on CENP-B proteins

Since the initiating *ura4*$^+$ was able to target nucleation of heterochromatin to a target *ura4*$^+$ reporter inserted within *cen1* yet unable to target the endogenous *ura4*$^+$ locus on chromosome three; we hypothesized that factors specifically enriched at centromeres might play a role *in trans* targeting of heterochromatin. Potential candidates were the centromere binding proteins (CENP-Bs). *S. pombe* has three CENP-B homologs, Abp1, Cbh1, and Cbh2, which have been shown to play a role in heterochromatin assembly (Nakagawa et al. 2002). CENP-Bs

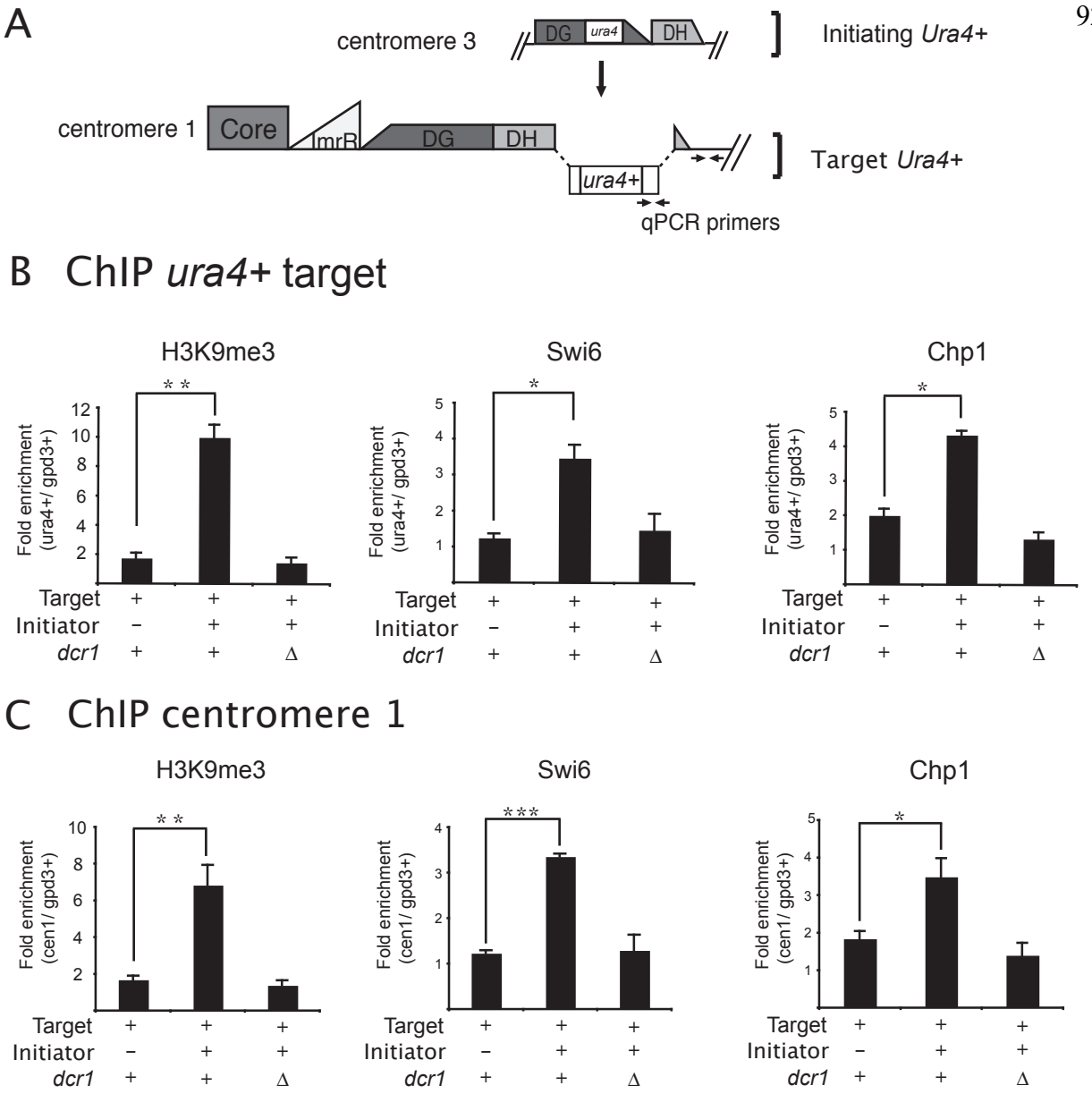


Figure 5. RNAi-mediated targeting of heterochromatin in *trans*

(A) Schematic of the initiating *ura4+* at *cen3* targeting in trans to the target *ura4+* at *cen1*. qPCR primers are indicated by arrows in the 3'UTR (B68-B69) of the target *ura4+* and primers specific for sequences in cen1 (D29-D30) outside of the *ura4+* integration site. (B) Quantitative chromatin immunoprecipitation (qChIP) analysis of H3K9me, Swi6, and Chp1 enrichment at the target *ura4+* in *cen1* in the target alone, or the target plus initiator with or without dcr1Δ. (C) Quantitative chromatin immunoprecipitation (qChIP) analysis of H3K9me, Swi6, and Chp1 enrichment at the target *ura4+* in *cen1* in the target alone, or the target plus initiator with or without *dcr1*Δ. Error bars reflect SE. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)

have also recently been shown to recruit HDAC proteins to retro-transposable elements throughout the genome (Cam et al. 2008).

Remarkably, as shown by the growth assays in Fig. 6A, in all three CENP-B mutants, *trans* silencing was lost or reduced. An *abp1Δ* mutant displayed a complete loss of *trans* silencing, whereas loss of *cbh1* or *cbh2* resulted in milder effects on target *ura4*$^+$silencing. However, to confirm that lack of growth on FOA in CENP-B mutant strains was not due to a loss of silencing at the initiating *ura4*$^+$ locus, we performed silencing assays on strains containing the initiating *ura4*$^+$ alone in each CENP-B mutant. Loss of *abp1*, *cbh1*, or *cbh2* had no effect on silencing at the initiating *ura4*$^+$ suggesting the effect of the CENP-B proteins was specific to initiation of heterochromatin assembly at the target *ura4*$^+$ reporter at *cen1* (Fig. 6B). Since Abp1 had the most dramatic effect on *trans* silencing, we investigated its role in heterochromatin assembly further.

We assayed for the effects of *abp1Δ* on recruitment of H3K9me3, Swi6, and Chp1 *in trans*. Interestingly, H3K9me3 and Swi6 recruitment to the target *ura4*$^+$ were dramatically decreased, but enrichment of Chp1 remained similar to wild-type levels (Fig. 6C). These results suggest that Abp1 is not required for recruitment of RITS *in trans*, but does affect H3K9me3 and Swi6 enrichment.

Because RITS is still recruited to the target *ura4*$^+$ in the absence of Abp1, we hypothesized that *ura4*$^+$ siRNAs originating from the initiating *ura4*$^+$ are still loaded into RITS. Consistent with this proposal, we detect *ura4*$^+$ siRNAs in the absence of Abp1 (Figure 6D). These results suggest *ura4*$^+$siRNAs are able to target RITS to the *ura4*$^+$ reporter at *cen1*. This targeting, however, appears to be insufficient to result in methylation of H3K9.

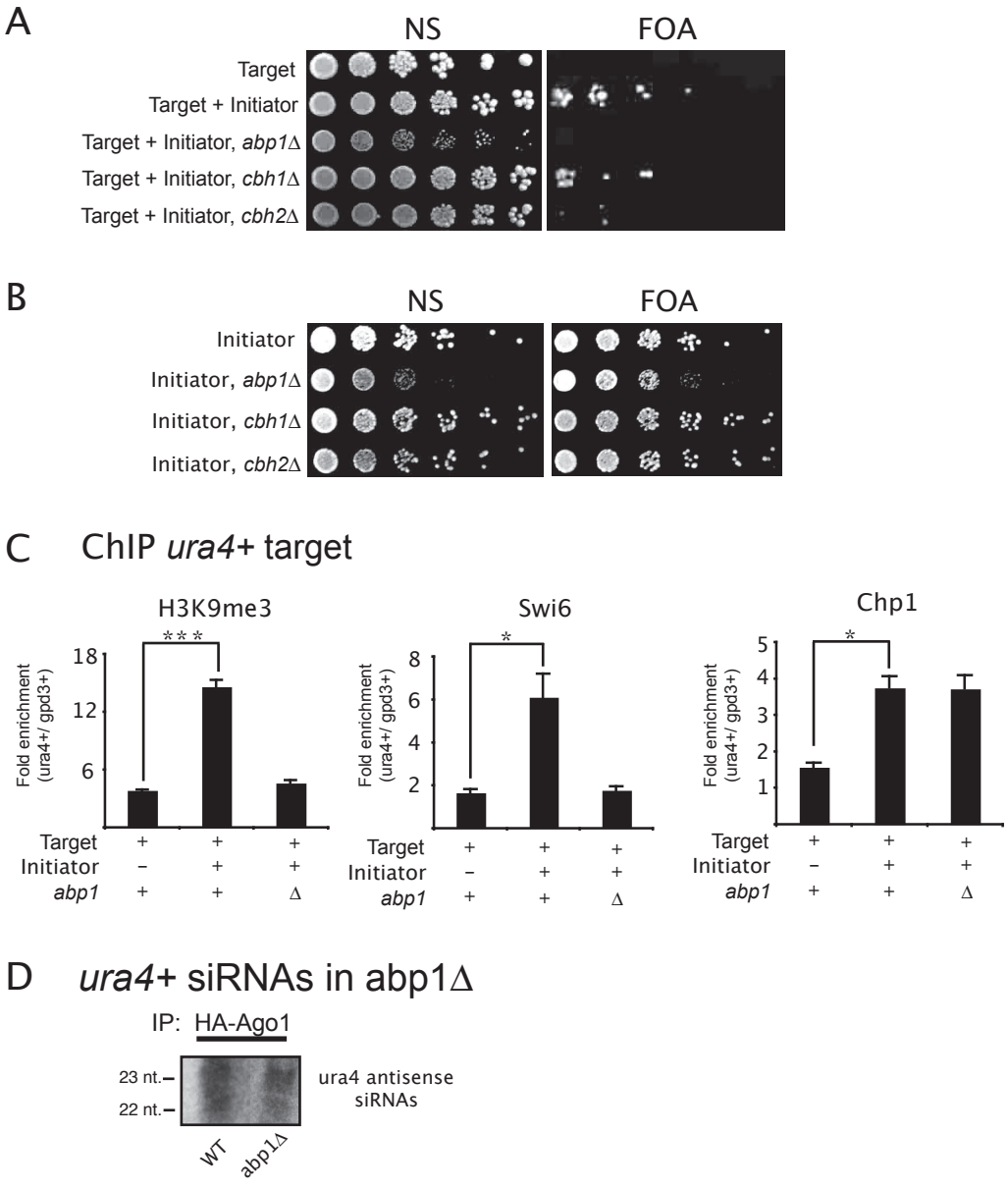


Figure 6. Trans targeting is dependent on CENP-B proteins.

(A) Serial dilution assay with the target *ura4+*, the target plus the initiator, or the target plus the initiator with *abp1*Δ (CK1222), *cbh1*Δ (CK1259), or *cbh2*Δ (CK1258) on non-selective (NS) medium or supplemented with 5-FOA (FOA). (B) Serial dilution assay with the initiator *ura4+* alone (CK326) or with *abp1*Δ (CK1145), *cbh1*Δ (CK1146), or *cbh2*Δ (CK1149) on non-selective (NS) medium or supplemented with 5-FOA (FOA). (C) Quantitative chromatin immunoprecipitation (qChIP) analysis of H3K9me, Swi6, and Chp1 enrichment at the target *ura4+* at *cen1* in the target *ura4+*, target plus initiator with or without *abp1*Δ. Error bars reflect SE. (D) Northern blot analysis of Ago1 associated ura4+ siRNAs in wild-type and abp1Δ (CK1262) strains. siRNA blot was performed as described in Fig. 2C. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)

Trans targeting of heterochromatin is dependent on the HDAC Clr3 and is recruited by Swi6

Recent data indicate that CENP-B proteins recruit the histone deacetylase Clr3 to silence retro-transposable elements (Cam et al. 2008). If Abp1 functions to recruit HDACs to centromeres then this would provide a potential explanation as to why H3K9me3 is not recruited in the absence of Abp1, as deacetylation would be required before lysine 9 methylation can occur. We investigated whether this same pathway contributed to establishment of heterochromatin *in trans*. Indeed, we find that silencing *in trans* to the target *ura4*$^{+}$ at *cen1* is dependent on Clr3 as shown by a lack of growth on FOA (Fig. 7A).

Next we performed ChIP to assay for enrichment of H3K9me3, Swi6, and Chp1 at the target *ura4*$^{+}$ in a *clr3Δ* strain. Interestingly, whereas H3K9me3 enrichment is lost in *clr3Δ*, Swi6 levels remain the same as wild-type suggesting that the histone deacetylase activity of Clr3 is necessary for H3K9me3 in establishment of heterochromatin (Fig. 7B). These data also suggest that Clr3 recruitment is downstream of Swi6 recruitment; which is in agreement with a previous study showing that Swi6 recruits Clr3 (Yamada et al. 2005). This suggests then that Swi6 is recruited upstream of Clr3 via an interaction with an effector protein such as Abp1 in addition to H3K9me (see below). Similar to the results of *abp1Δ* from Fig. 6C, levels of Chp1 enrichment in *clr3Δ* were similar to wild-type (Fig. 7B), again consistent with RITS being targeted in a *clr3Δ* strain. H3K9, however, was not methylated in *clr3Δ* suggesting RITS recruitment is independent of H3K9me3.

Clr3 has been shown to deacetylate K9 and K14, thus we used ChIP to assay for these modifications at the target *ura4*$^{+}$ in *clr3Δ*. We observed H3K9ac and H3K14ac enrichment decreases in a wild-type strain with the initiator actively silencing the target relative to the target

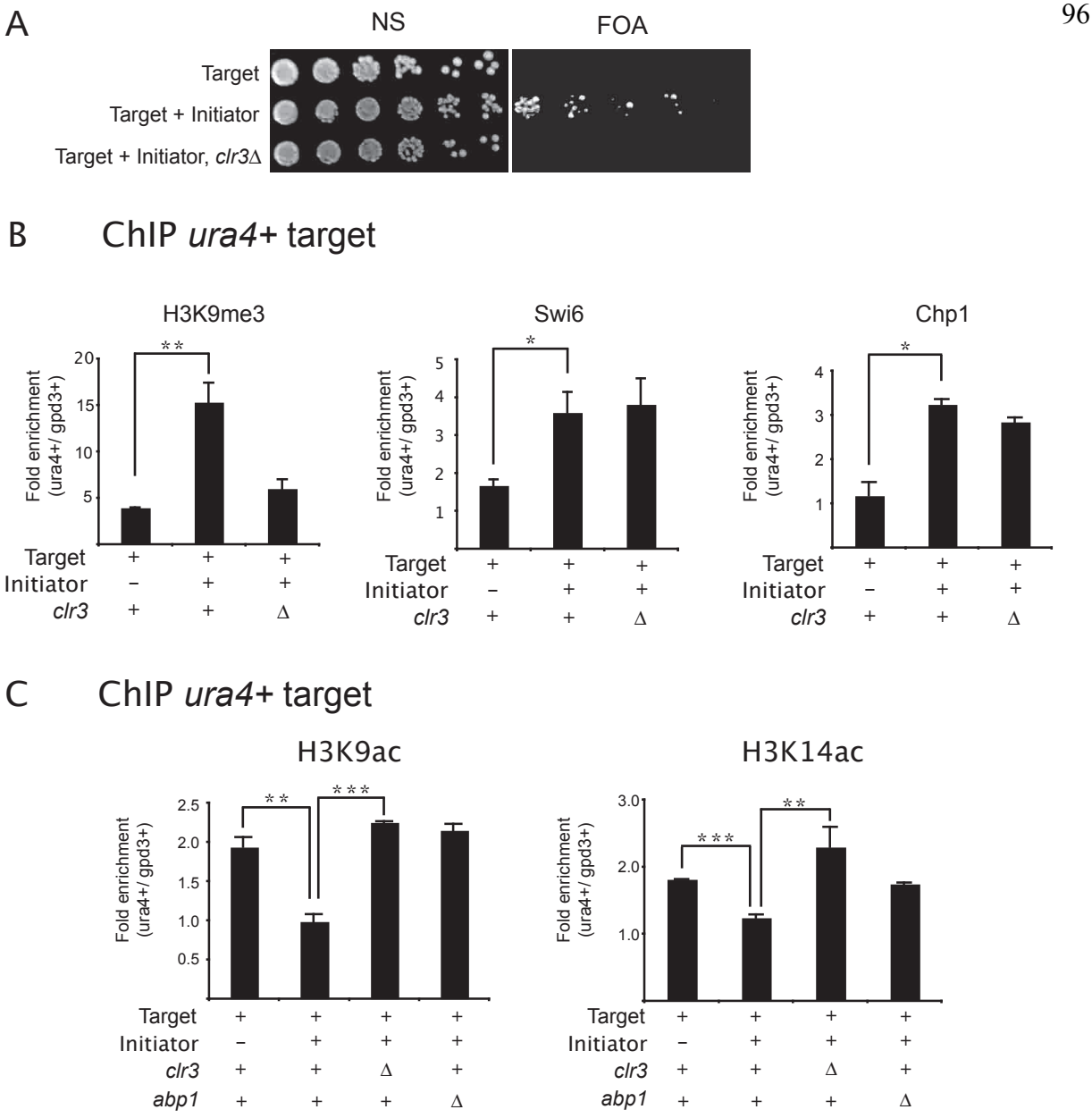


Figure 7. Trans targeting of heterochromatin is dependent on the HDAC, Clr3

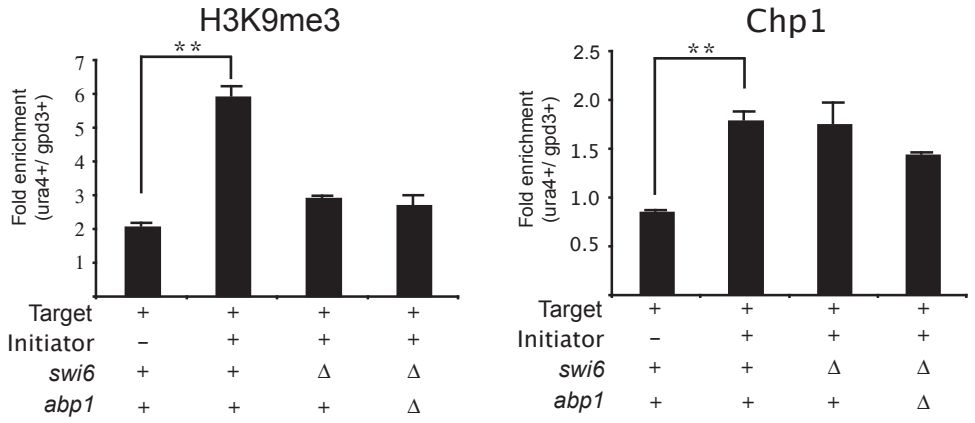
(A) Serial dilution assay with the target *ura4+*, the target plus the initiator, or the target plus the initiator with *clr3*Δ (CK1254), on non-selective (NS) medium or supplemented with 5-FOA (FOA). (B) Quantitative chromatin immunoprecipitation (qChIP) analysis of H3K9me, Swi6, and Chp1 enrichment at the target *ura4+* at *cen1* in the target *ura4+,* target plus initiator with or without *clr3*Δ. (C) Quantitative chromatin immunoprecipitation (qChIP) analysis of H3K9ac and H3K14ac at the target *ura4+* at *cen1* in the target *ura4+*, target plus initiator, or the target plus the initiator with *clr3*Δ or *abp1*Δ. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)

only strain (Fig. 7C). In contrast, we find an increase in the enrichment of H3K9ac and H3K14ac in both *abp1Δ* and *clr3Δ* mutants suggesting that Abp1 is involved in the recruitment of Clr3 activity to the *trans* target in order for Clr3 to deacetylate histone H3 (Fig. 7C). Presumably this deacetylation is required for H3K9me3.

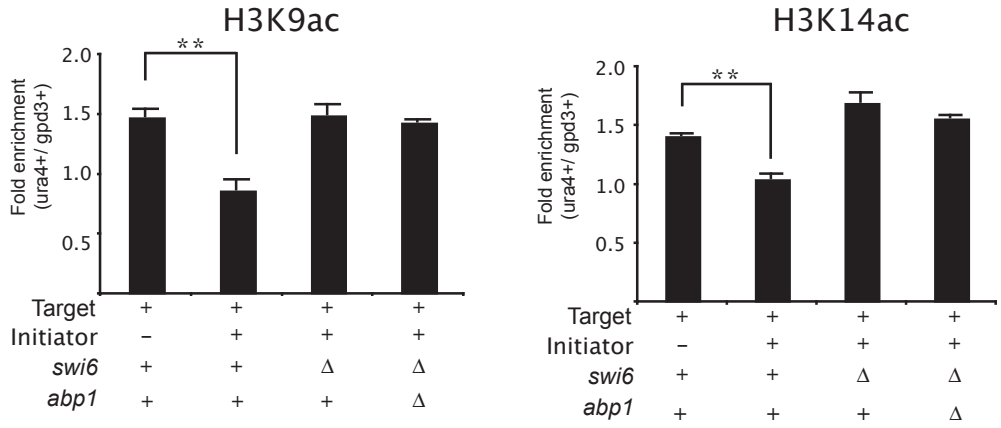
Clr3 recruitment by Swi6 is Dependent on Abp1

The above data suggest a linear heterochromatin assembly pathway during *trans* silencing. We hypothesized that RITS recruits H3K9me3 via a RITS-Kmt1 interaction; a reaction that can only be catalyzed in a domain enriched with CENP-B's, which recruit Swi6. Swi6 can then recruit Clr3, and thus establish a heritable, silent state. In order to test this we assayed for recruitment of H3K9me3 and Chp1 to the *ura4*$^{+}$ target in a *swi6Δ* strain, as well as in *swi6Δ abp1Δ* double mutants. If Swi6 and Abp1 are in the same pathway, then a *swi6* single mutant should mimic the double mutant by ChIP analysis. Indeed, H3K9me3 was decreased at the target *ura4*$^{+}$in both single and double mutants but Chp1 was still recruited suggesting these factors act in the same pathway for RNAi mediated *trans* silencing (Fig. 8A). We also found similar increases in enrichment of

A ChIP *ura4+* target



B ChIP *ura4+* target



C ChIP *ura4+* target

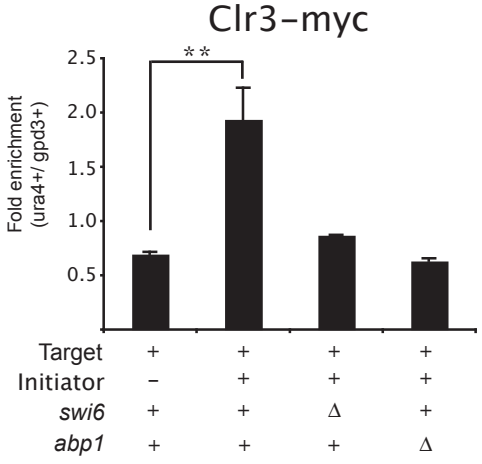


Figure 8

Figure 8. Swi6 and Abp1 are in the same *trans* targeting pathway

(A) Quantitative chromatin immunoprecipitation (qChIP) analysis of H3K9me and Chp1 enrichment at the target *ura4*$^+$ at *cen1* in the indicated strains. (B) Quantitative chromatin immunoprecipitation (qChIP) analysis of H3K9ac and H3K14ac at the target *ura4*$^+$ at *cen1* in the indicated strains. (C) Quantitative chromatin immunoprecipitation (qChIP) analysis of Clr3-myc at the target *ura4*$^+$ at *cen1* in the indicated strains. Error bars reflect SE. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)

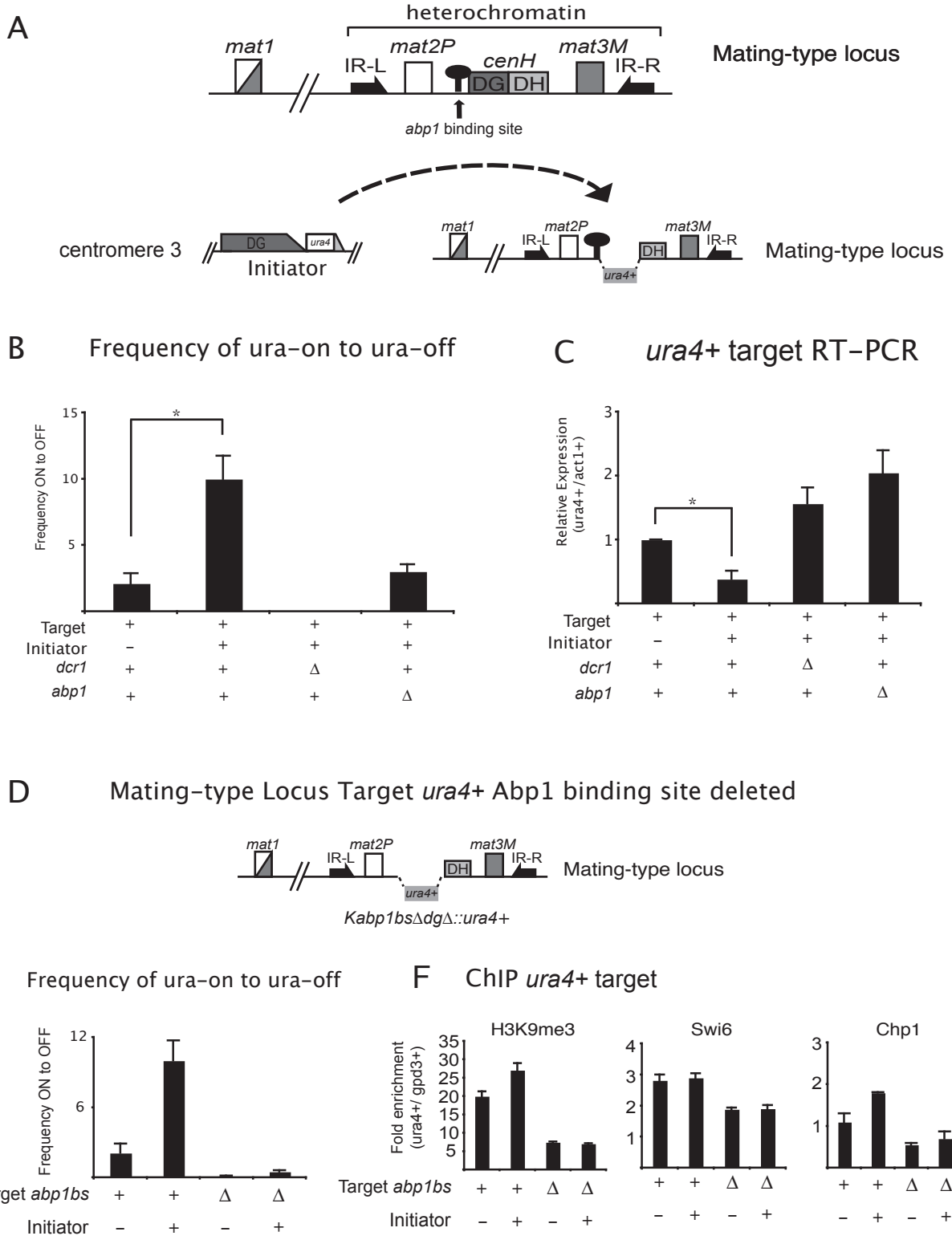
H3K9ac and H3K14ac in both the single and double mutants consistent with a model whereby Abp1 recruits Swi6, which in turn recruits Clr3 (Fig. 8B).

To verify these results, we performed a ChIP assay to detect the fold enrichment of epitope tagged Clr3-myc at the target *ura4*$^+$ in *swi6Δ* and *abp1Δ* strains. Consistent with the proposed model, we found Clr3-myc enriched specifically in the target plus initiator strain, but not in the target only strain. We also saw a loss of enrichment of Clr3 at the target *ura4*$^+$ in *swi6Δ* and *abp1Δ* (Fig. 8C)

RNAi Dependent *Trans* Silencing of the Mating-type Locus is Dependent on Abp1

The mating-type locus contains a region of homology to the *dg* and *dh* repeats at the centromeres, termed *cenH*, that has been shown to be required for heterochromatin establishment (Fig. 9A) (Hall et al. 2002). Recently, an Abp1 binding site was also mapped to a region adjacent to and on the centromere proximal side of *cenH* (Aguilar-Arnal et al. 2008; Cam et al. 2008). We hypothesized that this portion of the mating-type region might therefore be capable of recruiting heterochromatin *in trans* via an Apb1 dependent mechanism.

In order to test this, we inserted a *ura4*$^+$ reporter adjacent to the Abp1 binding site at the mating-type region, replacing the region of homology at the centromere *dg* sequence (*KdgΔ::ura4*$^+$). The *ura4*$^+$ initiator at *cen3* was then introduced into this background in order to assay for *trans* silencing to the mating-type locus. We used an assay that allows measurement of the frequency of the target *ura4*$^+$ (*KdgΔ::ura4*$^+$) at the mating-type locus to switch from the euchromatic state to the heterochromatic state. The


A
heterochromatin
mat1
mat2P
IR-L
cenH
DG
DH
mat3M
IR-R
Mating-type locus
abp1 binding site
centromere 3
DG
ura4
Initiator
mat1
mat2P
IR-L
DH
mat3M
IR-R
ura4+
Mating-type locus
B
Frequency of ura- on to ura- off
Frequency ON to OFF
15
10
5
*
Target + + + +
Initiator − + + +
dcr1 + + Δ +
abp1 + + + Δ
C
ura4+ target RT-PCR
Relative Expression (ura4+/act1+)
3
2
1
*
Target + + + +
Initiator − + + +
dcr1 + + Δ +
abp1 + + + Δ
D
Mating-type Locus Target ura4+ Abp1 binding site
mat1
mat2P
IR-L
mat3M
DH
IR-R
ura4+
Mating-type locus
Kabp1bsΔdgΔ::ura4+
E
Frequency of ura- on to ura- off
Frequency ON to OFF
12
6
Target abp1bs + + Δ Δ
Initiator − + − +
F
ChIP ura4+ target
H3K9me3
Swi6
Chp1
Fold enrichment (ura4+/ gpd3+)
35
30
25
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10
5
4
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2
1
3
2
1
Target abp1bs + + Δ Δ
Initiator − + − +

Figure 9

Figure 9. RNAi-mediated *trans* targeting at the mating-type locus is enhanced by Abp1 (A) Schematic of the mating-type locus of *S. pombe* (top). Below is a schematic of the initiating *ura4*$^+$ at *cen3* targeting *in trans* a *ura4*$^+$ replacing the *dg* homology region at the mating-type locus (*KdgΔ::ura4*$^+$) (CK1023). (B) Single colonies were picked from medium lacking uracil and then grown in non-selective media. The cells were then plated onto non-selective medium and medium supplemented with 5-FOA and the percentage of cells growing on FOA compared to on non-selective medium was determined in the indicated strains. (C) Expression of the target *ura4*$^+$ at the mating-type locus. RNA was extracted from the indicated strains grown in non-selective medium. Quantitive RT-PCR for *ura4*$^+$ was performed and normalized to the target *ura4*$^+$ alone. (D) Schematic of the mating-type locus with the Abp1 binding site deleted. (E) Frequency assays were performed as in (B) in the indicated strains. No significant change was detected in the target *ura4*$^+$ with the Apb1 binding site deleted with or without the initiator present. (F) Quantitative chromatin immunoprecipitation (qChIP) analysis of H3K9me, Swi6, and Chp1 enrichment at the target *ura4*$^+$ at the mating type locus in the indicated strains. No significant changes were detected in these strains. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)

use of a frequency type assay is required for determining silencing at the mating-type locus due to the Atf1/Pcr1 silencing pathway also contributing to low level silencing (Jia et al. 2004). Accordingly, instead of assaying for a loss of silencing, we assay for an increase in silencing. We selected colonies growing on media lacking uracil (Ura-On) re-cultured them in non-selective media to allow the switch to Ura-Off. These cells were then plated on non-selective and 5-FOA and the percentage of colonies growing on FOA relative to non-selective media was determined. This percentage reflects the frequency that individual cells initiate heterochromatin assembly and switch from Ura-On to Ura-Off. The target *ura4*$^+$ switched to Ura-Off at a frequency of 2.1%; this low frequency switching to Ura-Off is likely due to heterochromatin spreading from the RNAi- nucleation element located in the dh sequence (Lawrence, R.J. et. al., submitted)**.** In the presence of the initiating *ura4*$^+$, the *KdgΔ::ura4*$^+$ target cells switched to Ura-Off at a much greater frequency (10%) (Fig. 9B). The dramatic increase in the ability of the target *ura4*$^+$ to switch to a silenced state suggests that the initiating *ura4*$^+$ at *cen3* is able to silence *in trans* to the mating-type locus as well. We also tested if the *trans* targeting to the mating-type locus was also dependent on RNAi. As expected, in a *dcr1Δ* strain we observed an inability of the target *ura4*$^+$ reporter to switch to the off state both with and without the initiator (Fig. 9B). The inability of the target *ura4*$^+$ to be silenced in a *dcr1Δ* background is consistent with a role for RNAi in spreading of heterochromatin from *dh* loci (Lawrence, R.J. et. al., submitted).

Because Abp1 played an integral role in the *trans ta*rgeting of *ura4*$^+$ at *cen1* we also tested if Abp1 was required for *trans* targeting to the mating-type locus. The ability of the *ura4*$^+$ mating-type target to switch to the Ura-Off state in an *abp1Δ* strain was reduced to a similar frequency of the target *ura4*$^+$ alone (Fig. 9B). Abp1 therefore also is required for *trans* silencing

to the mating-type locus. We also tested steady-state transcript levels corresponding to the *ura4*$^+$ mating-type target: qRT-PCR analysis with primers that amplify the mating-type target *ura4*$^+$ demonstrated that the transcript levels of the target *ura4*$^+$ in the presence of the initiator *ura4*$^+$ were relatively less than the target alone (Fig. 9C). In addition, in *dcr1Δ* and *abp1Δ* strains *ura4*$^+$ target transcript levels were also increased (Fig. 9C).

Because there is only one known Apb1 binding site at the mating-type locus compared to Abp1's binding at multiple sites throughout the centromeres we were able to test this further by deleting the known Abp1 binding site located within the mating-type locus (Aguilar-Arnal et al. 2008). We once again replaced the mating-type *dg* element with a *ura4*$^+$ reporter but also deleted the mating-type Abp1 binding site (*Kabp1bsΔdgΔ::ura4*$^+$) (Fig. 9D). Remarkably, deletion of the Abp1 binding site eliminates *trans* silencing, but also influences endogenous mating-type silencing as seen by a lower frequency of ura-on to ura-off in the binding site deletion strain (Fig. 9E). This is the first report of Abp1 regulating recruitment of heterochromatin to the mating-type locus. We next tested whether deletion of the Abp1 binding site resulted in altered heterochromatin marks at the target *ura4*$^+$. Enrichment of H3K9me3, Chp1, and Swi6 as shown by ChIP confirms the presence of additional silencing pathways acting at the mating-type locus. Consistent with results from frequency assays (Fig. 9B), the target *ura4*$^+$recruits additional H3K9me3 and Chp1in the presence of the initiator. Interestingly, Swi6 levels remain unchanged regardless of whether the initiator *ura4*$^+$ is present or not. However, when the Abp1 binding site is deleted, enrichments of H3K9me3, Swi6, and Chp1 are decreased below levels detected at the target *ura4*$^+$ when the Abp1 binding site is intact (Fig. 9F). This suggests a novel role for Abp1 in maintenance of heterochromatin at the mating-type locus.

Overall these results suggest a role for Abp1 in heterochromatin maintenance as well as enhancement of silencing *in trans* to the mating-type locus.

Discussion

This work describes RNAi-mediated targeting of heterochromatin *in trans*, and demonstrates a novel role for CENP-B proteins in the establishment of heterochromatin domains in *S. pombe*. Double stranded transcripts from initiator *ura4*$^+$ reporters at *cen3* are processed into siRNAs, which are then loaded into the RITS complex and subsequently targeted to the target *ura4*$^+$ at *cen1*. Not only do we detect nucleation of heterochromatin, but remarkably we also observe spreading of heterochromatin at least 1kb divergently from the site of nucleation at the target *ura4*$^+$. Thus, heterochromatin can be targeted by the RITS complex and subsequently spread to establish large heterochromatin domains.

Chp1 recruitment (and likely RITS) occurs *in trans* even in the absence of Swi6, Clr3, or Abp1 and this recruitment is not dependent on H3K9 methylation. H3K9me3, however, seems to be necessary to maintain a stable silent state. Our results suggest a linear pathway whereby CENP-B proteins recruit Swi6, which in turn recruits Clr3 to deacetylate histones. Because Swi6 enrichment at the target is only seen when the initiator *ura4*$^+$ is present, Swi6 may have a transient interaction with Abp1 that is stabilized by RITS recruitment and/or Kmt1 H3 methylation. Presumably the deacetylation of histones by Clr3 would be necessary for histone H3 lysine 9 to be methylated by the histone methyltransferase Kmt1, and thus establish heterochromatin. Furthermore, H3 is only deacetylated when the initiator *ura4*$^+$ is present, suggesting a role for RITS in activating Clr3 or perhaps stabilizing deacetylation by the recruitment of H3K9 methylation. The dependence on Clr3 for both H3K9ac and H3K14ac is

consistent with recent data, suggesting it may have a promiscuous role in deacetylating histones (Wiren et al. 2005).

As Abp1 has been postulated to recruit Clr6 to TF2 LTRs for silencing, we also tested the requirement for Alp13, a component of the Clr6 histone deacetylase complex, in *trans* heterochromatin assembly (Nakayama et al. 2003; Cam et al. 2008). We did not find losses of H3K9me3 and Swi6 enrichment or increased enrichments of H3K9ac and H3K14ac in an *alp13Δ* strain at the *trans* target, suggesting that Clr6 does not act in *trans* heterochromatin assembly at centromeres (data not shown). Thus, we illustrate that both RNAi as well as the recruitment of Clr3 are necessary for the establishment of a stable heterochromatin domain. This likely leads to transcriptional silencing of the target *ura4*$^{+}$.

Previous results from other labs suggest a *cis*-restricted mechanism of silencing at centromeres and the mating-type locus (Buhler et al. 2006). *Trans* targeting of heterochromatin to the endogenous *ura4*$^{+}$ via siRNAs generated from RITS tethering to *ura4*$^{+}$ mRNA was shown to occur only in an *eri1Δ* strain, suggesting an active restriction of silencing to a *cis* based mechanism (Buhler et al. 2006). Eri1 is a nuclease that has been described as a negative regulator of siRNA biogenesis. We also tested the ability for *trans* targeting in an *eri1Δ* background and found *trans* silencing to the target *ura4*$^{+}$ both at the centromere and mating-type locus was not enhanced, suggesting the mechanism of Eri1 restriction is not as simple as previously proposed (Fig. 10A-C). In *C. elegans*, Eri1 was shown to be necessary for endogenous siRNA biogenesis, but exogenous siRNAs are more abundant in an *eri1Δ* strain suggesting that Eri1 is involved in promoting endogenous silencing mechanisms while inhibiting the effects of foreign double stranded RNA (Duchaine et al. 2006). Eri1 in *S. pombe* may therefore act similarly and have

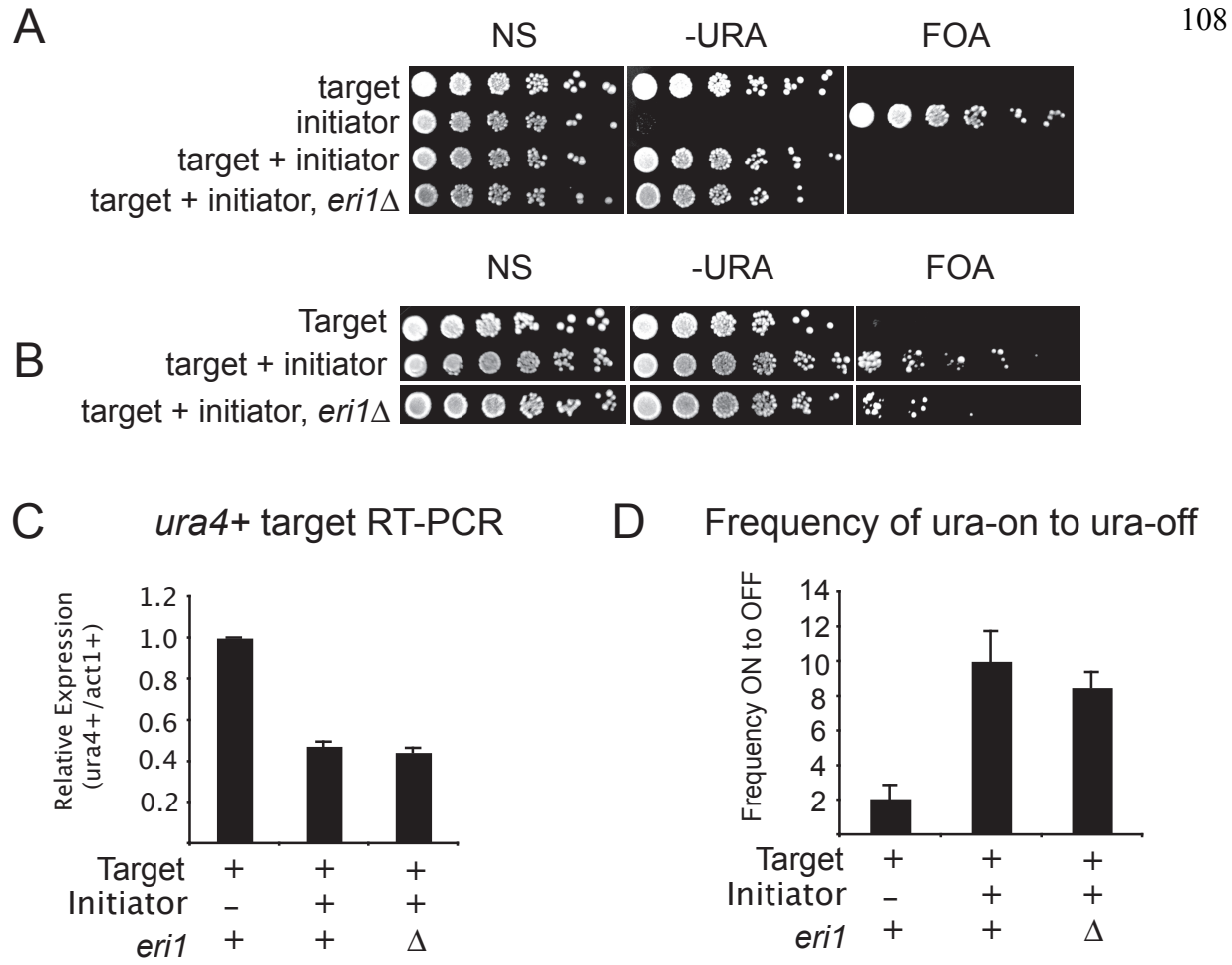


Figure 10. The *eri1*Δ does not effect RNA-mediated *trans* targeting at the centromeres or the mating-type locus.

(A) Serial dilution assay with the target endogenous *ura4*+ (CK306), the initiator *ura4*+ at *cen3* (CK326), the target + initiator (CK327), and target + initiator, *eri1*Δ (CK1317) on non-selective medium (NS), medium lacking uracil (-URA), or medium supplemented with 5-FOA (FOA). (B) Serial dilution assay with the target at *cen1 ura4*+ (CK1180), the target + initiator (CK1101), and target + initiator, *eri1*Δ (CK1316) on non-selective medium (NS), medium lacking uracil (-URA), or medium supplemented with 5-FOA (FOA). (C) Expression of the *cen1* target *ura4*+ (CK1180), the target + initiator (CK1101), and target + initiator, *eri1*Δ (CK1316). Quantitative RT-PCR for *ura4*+ was performed and normalized to the target *ura4+.* (D) Single colonies were picked from medium lacking uracil and then grown in non-selctive media. The cells were then plated onto non-selective medium and medium supplemented with 5-FOA and the percentage of cell growing on FOA compared to on non-selective medium was determined in the indicated strains.

different functions in the endogenous *trans* targeting of heterochromatin versus the tethering of RITS to induce silencing *in trans*.

It is possible that *cis*-directed (or co-transcriptional) and *trans*-directed heterochromatin establishment models are not mutually exclusive. Our results suggest that heterochromatin is targeted *in trans* to *dh* elements and that this *trans* targeting is dependent on RNAi. However, we have also shown recently that RNAi spreads heterochromatin *in cis* (Lawrence, R.J. et. al., submitted). Furthermore, we have recently defined minimal RNAi-dependent heterochromatin nucleation elements at *dg* loci that appear to be *cis*-directed (Lawrence, R.J. et. al., submitted).

We also reveal a specific requirement for Abp1 (and Cbh1 and Cbh2 to a lesser extent) in *trans* silencing. This result is consistent with Abp1 acting upstream of Cbh1 and Cbh2 by recruiting the latter two CENP-Bs. Alternatively, Abp1 may play a specific role in establishment whereas Cbh1 and Cbh2 may play other distinct roles. CENP-B proteins have been shown to dimerize in mammalian systems (Masumoto et al. 2004). The CENP-B proteins are necessary for initiation of heterochromatin assembly *in trans*, but in addition to recruiting Swi6, their dimerization in *S. pombe* may directly contribute to a higher order chromatin structure necessary for stability.

We demonstrate Abp1 is required for *trans* targeting of heterochromatin, to *cen1* and the mating type locus however, it is possible that other factors are also required. RNAi-mediated heterochromatin targeting *in trans* may be restricted to heterochromatin domains, for example regions where alternative nucleation or initiation mechanisms exist. Along these lines, nuclear localization of chromatin domains may also contribute to the ability of a chromatin domain to be targeted *in trans*. For example, heterochromatin domains may be sensitive to trans targeting

because of their localization at the nuclear periphery whereas targeting to euchromatic regions might be inhibited by their interior nuclear localization.

We reveal a role for CENP-B's in the establishment of heterochromatin through cooperativity of Swi6/HP1 binding and the RNAi pathway. Thus, such mechanisms may provide future avenues of research in mammalian systems. Overall, since the data presented herein provide the first insight into the establishment of a stable heterochromatin domain via RNAi *in trans*, this work will inevitably lead to a greater understanding of the mechanisms of RNAi-directed heterochromatin establishment and maintenance.

Materials and Methods

Growth of Yeast Strains

S. pombe strains were grown at 33^{O}C in YEA medium (yeast extract supplemented with adenine), medium lacking uracil, or medium supplemented with 850mg/L of 5-FOA. Strains for siRNA blots were grown in EMM (minimal media) at 33^{O}C. Strains used are listed in Appendix 1.

Construction of Strains

All deletion strains and epitope tags were constructed by a two-step PCR method with a vector template as previously described (Bahler et al. 1998). All deletions or tags were stably integrated by standard transformation protocols for *S. pombe*, and then confirmed using primers outside the integration site. *ura4*$^{+}$ initiators were integrated into the *dh* region of *cen3* between the following sequences: 5'-agcatgggtatagaaagaagacg-3' and 5-gaatgaacgtagcaatagatacaag-3 and transformed into *ago1Δ* strains and selected on media lacking uracil. The target *ura4+* at *cen1* was integrated as previously described (Lawrence, R.J. et. al., submitted). The target *ura4*$^{+}$ at the mating-type locus replacing the *dg* homology region was integrated between the following sequences: 5'-cctcacatcatttgttccgtct -3' and 5'-gttgctttcggacttgaacg-3'. The target *ura4*$^{+}$ at the mating-type locus replacing the *dg* homology region plus the Abp1 binding site was integrated between the following sequences: 5'- tcatccaacgataaccaatca -3' and 5'-gttgctttcggacttgaacg-3'.

RNA extraction and RT-PCR

RNA was extracted from strains according to standard yeast protocols. 50ml *S. pombe* cultures were grown to an $O.D._{600}$ of ~0.5, pelleted, and frozen at -80°C. Frozen pellets were extracted with a solution of 50 mM sodium acetate, 10 mM EDTA, 1% SDS, and three phenol chloroform extractions. RNA was ethanol precipitated. cDNA was made with 1ug of RNA, DNase treated with 2 Units of DNAse RQ1 (Promega) at 37°C for 60 minutes. Reactions were then used in a cDNA reaction with 100 units of SuperScript III (Invitrogen), and primed with oligo-dT for 50 minutes at 42°C. cDNA for strand-specific RT-PCR was performed with similar methods except the RT reaction was done with a gene specific primer and incubated at 55°C**.** All RT-PCR reactions were done in triplicate.

Chromatin Immunoprecipitation

Chromatin immuoprecipitation was performed as previously described(Volpe et al. 2002). The Clr3-myc chromatin immunoprecipitation was performed as previously described (Wiren et al. 2005). Antibodies used were the following: α-H3K9me3 (Upstate Biotechnology #07-442), α-Swi6 (Abcam #14898), α–Chp1 (Abcam #18191), α-Myc (abcam #56), α–Pol II (Covance #MMS-128P). ChIPs were repeated three times.

Quantitative PCR

Quantitative PCR was performed on an MJ Research/BioRad Chromo4 Thermocycler using Opticon 3.0 software. The optimal annealing temperature was determined for each primer set. The thermocycling conditions were as follows: an initial denaturation at 95°C for 3 minutes followed by 40 cycles at 95°C for 10 seconds, 50-60°C for 30 seconds, and 72°C for 1 minute. Fold enrichments and relative expressions were calculated using the Pfaffl method on Gene Ex

software (Biorad). The reactions done for qRT-PCR were normalized to *act1+* and the qPCR for ChIP assays were normalized to *gpd3+*. Primers are listed in Table 1. Reactions were performed in duplicate.

Frequency Assays

Strains were plated onto medium lacking uracil and then single colonies were picked and grown overnight in non-selective YEA medium. Cultures were then diluted and plated onto non-selective YEA medium and medium supplemented with 850mg/L of 5-FOA. Cells were counted and the percentage of cells growing on 5-FOA compared to the number growing on non-selective medium was determined. Each assay was done three times.

Ago1 Purification and siRNA blots

Ago1 was epitope tagged with HA on its N-terminus. HA-Ago1 was immuno-purified from twelve grams of yeast cells grown in minimal media (EMM) as previously described (Verdel and Moazed 2005). Small RNAs were then extracted from protein by phenol chloroform extraction and EtOH precipitation. siRNAs were run a 12% polyacrylamide gel and detected by northern blot analysis using DNA oligos corresponding to the entire ORF of *ura4*$^{+}$. The oligos used were previously described (Buhler et al. 2007). 5% of total siRNAs purified from HA-Ago1 were end labeled with cordycepin as previously described (Verdel and Moazed 2005) and run on a 12% polyacrylamide gel as a control. 2.5% of the beads used in the immunopurifying of HA-Ago1 were subjected to Western Blot analysis to verify the pull-down.

Table 1. Oligonucleotides used in this study

ID#	Sequence	Purpose
B86	AATTCGCAGACATTGGAAATACC	*ura4*+ initiator RT-PCR
B87	TGTGATATGAGCCCAAGAAGC	
B66	AAATAAGCCTTAATGCCCTTGC	*ura4*+ promoter qPCR
B67	CATCTTAATTATACCTCACAGAACTATC	
B68	CTAGGCGTTTTATGTCAGAAGG	*ura4*+ 3'UTR qPCR
B69	TTATTCCCAAGGTGTTTATCTATAATAG	
D29	ATTGCCTTGTTCTTGAGTAC	Cen I (outside target) qPCR
D30	AGGGAGTAACTTCTTCACC	
C48	TCAAGTGGTCTGCCTCTGG	*gpd3*$^+$ qPCR
C49	CACCGACGACGAACATGG	
B1	CCATTGAGCACGGTATTGTC	*act1*$^+$ qPCR
B2	CTTCTCACGGTTGGATTTGG	

CHAPTER IV

RNA-dependent RNA polymerase is a molecular link between heterochromatin stabilization, spreading, and secondary siRNA biogenesis

Abstract

Double-stranded RNA triggers the conserved mechanism RNAi, which processes dsRNA into siRNAs. RNAi plays a key role in post-transcriptional silencing as well as heterochromatin assembly. Whereas the original dsRNA trigger results in primary siRNA biogenesis, in some organisms the activity of RNA-dependent RNA Polymerase results in secondary siRNA biogenesis by synthesizing dsRNA using an RNA template. The relationship between primary and secondary siRNA biogenesis during heterochromatin assembly is currently unclear. Here, we detect previously unidentified primary siRNAs in the absence of RNA-dependent RNA polymerase (Rdp1) in fission yeast. Furthermore, we find similar levels of siRNAs in *rdp1Δ* and mutants of the heterochromatic histone modification pathway, suggesting that heterochromatin promotes siRNA amplification but not primary siRNA biogenesis. Remarkably, we demonstrate that heterochromatin can be nucleated ectopically in the absence of Rdp1, but Rdp1 is required for stabilization of ectopic heterochromatin throughout the cell cycle and spreading of heterochromatin to adjacent loci. Collectively, these data suggest primary siRNAs can direct initial heterochromatin nucleation but the function of Rdp1 is to amplify secondary siRNAs to reinforce and spread the heterochromatic state in a process that requires heterochromatin itself.

Results and Discussion

Double-stranded RNAs (dsRNAs) are processed by the RNAse III enzyme, Dicer (Dcr1 in *S. pombe*), into 21-25 nucleotide RNAs referred to as small interfering RNAs (siRNAs) (Lippman and Martienssen 2004; Meister and Tuschl 2004; Mello and Conte 2004; Sontheimer 2005). Presumably, dsRNA can be generated by convergent, bidirectional transcription by DNA-dependent RNA polymerases (i.e. Pol II) or in some organisms by RNA-dependent RNA polymerase (Rdp1 in *S. pombe*) synthesis of complementary transcripts to amplify dsRNA (Lippman and Martienssen 2004; Motamedi et al. 2004). Two distinct populations of siRNAs have been shown to exist in *C. elegans*: primary siRNAs, which are generated by Dicer processing of an original dsRNA trigger, and secondary siRNAs which require RNA-dependent RNA Polymerase (Pak and Fire 2007; Sijen et al. 2007). siRNAs are loaded into RNAi effector complexes such as RITS in *S. pombe* (Motamedi et al. 2004). RITS is composed of the Argonaute family endonuclease Ago1, which uses siRNAs as guides for "slicing" cognate mRNAs, the adaptor protein Tas3, and the chromodomain protein Chp1 (Martienssen et al. 2005). RITS is thought to target cognate RNAs for heterochromatin assembly at the corresponding chromosomal locus (Buhler et al. 2006).

RNAi has been shown to play a role in heterochromatin assembly (Volpe et al. 2002). Heterochromatin is typically marked by di- or tri-methylation of histone H3 at lysine 9 (H3K9me2/3) (Richards and Elgin 2002). This modification is catalyzed by the *S. pombe* methyltransferase, Clr4 (also referred to as Kmt1) in a complex that includes the DDB1-like protein, Rik1 (Horn et al. 2005). H3K9 methylation serves as a binding site for chromodomain proteins, notably Swi6, a homolog of heterochromatin binding protein 1 (HP1), and the RITS component Chp1 (Bannister et al. 2001; Lachner et al. 2001).

Distinct populations of primary and secondary siRNAs have not been identified in *S. pombe*. The possibility exists that these populations have not been observed because primary siRNAs, which would be presumed to be present in the absence of Rdp1, might be below the limits of detection of previously used methods. Thus, we designed a highly sensitive methodology using RNase protection to analyze siRNA populations in *S. pombe* RNAi and H3K9 methylation machinery mutants. We focused this analysis on regions of heterochromatin where siRNAs have been mapped previously; these include the *dg* and *dh* repeats of pericentric heterochromatin (Figure 1A) (Cam et al. 2005). As expected we detected abundant siRNAs corresponding to *dg* sequences that were dependent on Dcr1 (Figure 1B). Remarkably, we were able to detect *dg* siRNAs in *rdp1Δ*, *clr4Δ*, and *rik1Δ* mutants (Figure 1B), suggesting a distinct population of siRNAs in *S. pombe* that are dependent on the RNA-dependent RNA polymerase and H3K9 methylation machinery. Intriguingly, when the same analysis was performed using *dh* sequences as a probe, we were unable to detect siRNAs in *dcr1Δ*, *rdp1Δ*, *clr4Δ*, and *rik1Δ* mutants (Figure 1B). These results suggest that a low level of primary siRNAs can be generated at *dg* loci independently of Rdp1 and also that siRNAs at *dh* loci are exclusively secondary siRNAs; however, we cannot rule out that siRNAs at *dh* sequences exist in *rdp1Δ*, *clr4Δ*, and *rik1Δ* but are below the limits of detection. Furthermore, the observation that similar levels of siRNAs exist in *rdp1Δ*, *clr4Δ*, and *rik1Δ* suggests that secondary siRNA biogenesis requires heterochromatin itself; consistent with the proposal that siRNA biogenesis is coupled to heterochromatin (Buhler et al. 2006).

We next sought to corroborate the finding that primary siRNAs can be generated in *rdp1Δ*, *clr4Δ*, and *rik1Δ* mutants at *dg* loci using two different methodologies. (1) Analysis of

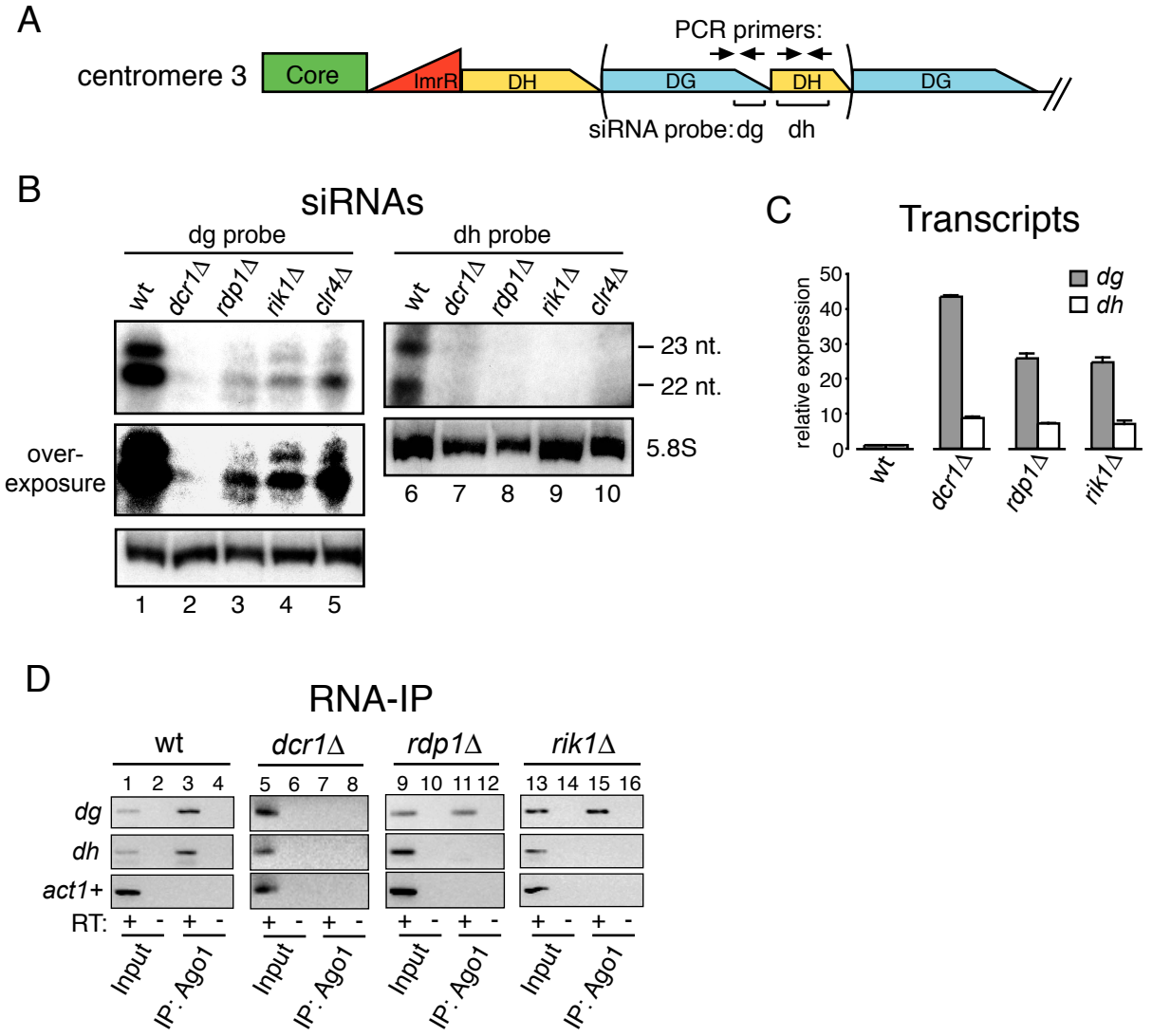


Figure 1: Analysis of siRNA accumulation in rdp1Δ and H3K9 methylation mutants.

(A) Schematic representation of centromere 3. PCR primers and RNase protection probes are indicated. (B) RNase protection analysis of siRNAs at dg and dh elements in the indicated strains. Loading control using 5.8S rRNA is indicated. (C) Quantitative RT-PCR analysis of steady-state non-coding dg and dh transcripts in the indicated strains, normalized to wt control + SEM. (D) RNA-IP analysis of dg or dh transcripts enriched with Ago1 immunoprecipitations in the indicated strains. Negative control cDNA reactions lacking reverse transcriptase are indicated (-RT).

transcript levels at loci corresponding to siRNAs and (2) RNA-immunoprecipitation for Ago1 association with transcripts corresponding to siRNAs.

As siRNA biogenesis requires RNAi processing of corresponding transcripts by Dcr1, we expected that *dg* transcripts in *dcr1Δ* should accumulate at higher levels than *rdp1Δ*, *clr4Δ*, or *rik1Δ* mutants. Indeed, we find this is the case, wild-type *dg* transcripts are very low in abundance and dramatically elevated in *dcr1Δ* but in *rdp1Δ* and *rik1Δ* the steady-state levels of *dg* transcripts are significantly lower than in *dcr1Δ* (Figure 1C). Furthermore, the steady-state levels of *dh* transcripts accumulate to similar levels in *dcr1Δ*, *rdp1Δ*, and *rik1Δ* relative to wild-type (Figure 1C) consistent with the above observation that siRNA biogenesis at *dh* loci is dependent on *dcr1Δ*, *rdp1Δ*, and *rik1Δ*.

If siRNAs are generated at *dg* loci independently of Rdp1 and Rik1 then these siRNAs should guide the RNAi-effector Ago1 to corresponding transcripts. Using RNA immunoprecipitation, we specifically detect Ago1 association with *dg* and *dh* transcripts in wild-type cells and the association is abrogated in *dcr1Δ* (Figure 1D). Remarkably, Ago1 associates with *dg* transcripts in *rdp1Δ* and *rik1Δ* but not with *dh* transcripts (Figure 1D). These results are consistent with the interpretation that primary siRNAs can guide Ago1 to *dg* transcripts in *rdp1Δ* and *rik1Δ* mutants.

The *dg* locus where convergent bidirectional transcripts and primary siRNAs are generated corresponds to a *cis* sequence that is sufficient to nucleate heterochromatin at an ectopic locus RNAi-dependently (referred to as *dgNucH*, Lawrence and Volpe, submitted (Partridge et al. 2002; Volpe et al. 2002; Volpe et al. 2003) and is similar to a previously characterized *dg cis* sequence that is sufficient to nucleate heterochromatin, denoted as "L5" (ref). In our initial studies we found that in wild-type cells H3K9me3, Swi6, and Chp1 are

efficiently recruited to *dgNucH* in wild-type cells but not in *rdp1Δ* (data not shown and Lawrence and Volpe, submitted). However, recently it has been shown that RNAi occurs transiently during S phase of the cell cycle and convergent bidirectional genes within euchromatin can transiently form heterochromatin during G1 of the cell cycle (Chen et al. 2008; Gullerova and Proudfoot 2008; Kloc et al. 2008). We thus sought to test whether the primary siRNAs in *rdp1Δ* could initiate heterochromatin assembly at this ectopic *dg* fragment transiently during the cell cycle.

Using previously established methods, we synchronized wild-type, *rdp1Δ,* and *dcr1Δ* cells harboring *dgNucH* (Figure 2A) (Kloc et al. 2008). Cells that accumulated in M, G1/S, S, and G2 were monitored by septation index as before (Figure 2A) and subjected to ChIP analysis for H3K9me3, Swi6, Chp1, and Ago1. Interestingly, we find that the dynamics of RNAi and heterochromatin assembly previously observed at centromeres also occurs at the ectopic *dgNucH*, suggesting *dgNucH* is sufficient to recapitulate the endogenous RNAi-mediated heterochromatin assembly pathway (Kloc et al. 2008). As such, primers specific for the nucleation element detect Chp1 and Ago1 enrichment at *dgNucH* in G1 to S (Figure 2B), when siRNAs are generated (Kloc et al. 2008), and H3K9me3 and Swi6 are disassembled in M to S and peak in G2 (Figure 2B). As expected, there was a lack of enrichment of H3K9me3, Swi6, Chp1, and Ago1 throughout the cell cycle at *dgNucH* in *dcr1Δ* (Figure 2B). Remarkably, in *rdp1Δ*, we found that Ago1, Chp1, and H3K9me3 but not Swi6 were enriched at *dgNucH* specifically in S phase and not at other stages of the cell cycle (Figure 2B) thus

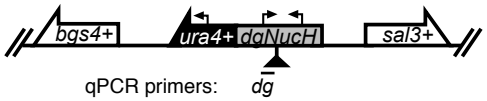
A

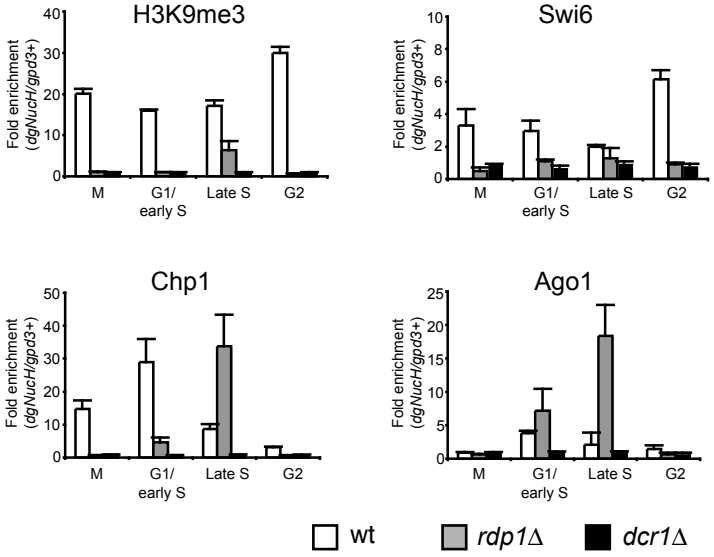
Septation (%)

	M	G1/ early S	Late S	G2
wt	0	11	76	0
*rdp1*Δ	0	9	81	0
*dcr1*Δ	0	7	72	0

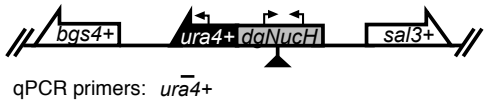
Figure 2

B ChIP





C ChIP



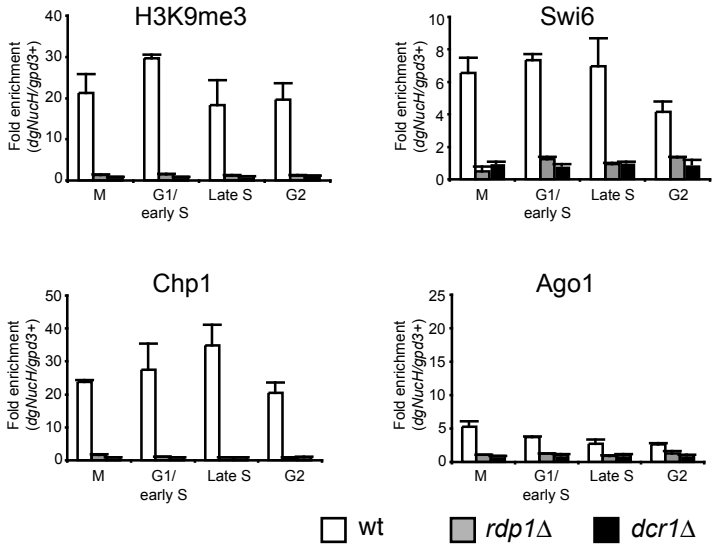


Figure 2. ChIP analysis demonstrates transient recruitment of heterochromatin in *rdp1Δ* to an ectopic heterochromatin nucleation element.

(A) Septation frequency in synchronized cells of the indicated strains. (B-C) ChIP analysis for H3K9me3, Swi6, Chp1 and Ago1 in using primers specific for the *dgNucH* nucleation element (B) or for *ura4*$^{+}$ (C). Primers specific for *dgNucH* in (B) recognize a unique 30-mer (filled triangle) that is integrated into *dgNucH*. Fold enrichment is calculated as the ratio ChIP for the primer set versus the euchromatic locus *gpd3*$^{+}$ + SEM.

suggesting that the primary siRNAs in *rdp1Δ* can transiently establish H3K9 methylation and RITS recruitment in S phase of the cell cycle. However, in the absence of Rdp1 heterochromatin at the ectopic nucleation element is unstable and not maintained throughout the cell cycle suggesting Rdp1 is required to stablize heterochromatin assembly throughout the cell cycle. Furthermore, as H3K9 methylation does not accumulate to wild-type levels in *rdp1Δ* mutants these results suggest a role for Rdp1 in facilitating Kmt1 activity or recruitment (see below).

We next performed the same analysis using primers within the *ura4*$^+$ reporter adjacent to *dgNucH*. Analysis of heterochromatin accumulation at *ura4*$^+$ reflects spreading from the ectopic nucleation element into *ura4*$^+$. Intriguingly, in wild-type cells H3K9me3, Chp1, Swi6, and Ago1 essentially remain stable throughout the cell cycle (Figure 2C). In contrast to heterochromatin assembly at the *dgNucH* nucleator, H3K9 methylation, Swi6, Chp1, and Ago1 enrichment at *ura4*$^+$ requires both Rdp1 and Dcr1 (Figure 2C), suggesting Rdp1 is required for spreading of heterochromatin into the adjacent reporter.

This work suggests that a discrete class of primary siRNAs is present in the absence of Rdp1 at fission yeast centromeres. Interestingly, as the primary siRNAs we have detected are very low in abundance, this suggests that the majority of siRNAs in fission yeast are secondary siRNAs, as is the case for *C. elegans* (Pak and Fire 2007) (Sijen et al. 2007). Furthermore, we also find similar amounts of siRNAs in *clr4Δ*, *rik1Δ*, and *rdp1Δ* mutants relative to wild-type suggesting that H3K9 methylation itself is required for amplification of siRNAs and perhaps coupled to secondary siRNA biogenesis. This finding is consistent with the interpretation that H3K9 methylation and secondary siRNA biogenesis occur in a self-enforcing loop that is coupled *in cis* to chromatin (Sugiyama et al. 2005; Buhler et al. 2006). This model was previously suggested, however we extend these findings to by proposing that in fact secondary

siRNA biogenesis or siRNA amplification is coupled to H3K9 methylation and primary siRNA biogenesis can occur independently of H3K9 methylation (Sugiyama et al. 2005; Buhler et al. 2006).

Remarkably, we find that primary siRNAs in the absence of Rdp1 can target H3K9 methylation to a heterochromatin nucleation element transiently during the cell cycle, which suggests a role for Rdp1 in stabilizing ectopic heterochromatin assembly throughout the cell cycle. Furthermore, Rdp1 is required for spreading from the ectopic heterochromatin to adjacent reporter genes. Several models could explain these results: (1) Rdp1 mediated synthesis of dsRNA could result in siRNA amplification that is required to recruit a threshold amount of H3K9 methylation that specifies a stable heterochromatin state; (2) Rdp1 could antagonize H3K9 demethylases; (3) A RITS – Rdp1 interaction could be necessary to stabilize the recruitment of the H3K9 metyltransferase, Clr4, throughout the cell cycle; (4) or Rdp1 could enhance the activity of Clr4. Overall, this study lays the groundwork for future investigations into mechanisms of the coupling of secondary siRNA biogenesis and heterochromatin assembly.

Materials and Methods

Growth of Yeast Strains

S. pombe was cultured at 33˚ C in non-selective YEA. Strains used are listed in Appendix 1. Cell cycle synchronization was performed as previously described with hydroxyurea to arrest the cells. Cells were analyzed by septation index to verify that they had progressed through the cell cycle similarly to previously described results. Cells were crosslinked with formaldehyde for ChIP at the in M, G1/S, S, and G2 at 70, 90, 120, and 200 minutes, respectively, after washing out the hydroxyurea.

RNase Protection analysis of siRNAs

DNA fragments of centromere 3 to be used as riboprobes were amplified by PCR, cloned into the pCRII-Topo vector according to the manufacturers directions (Invitrogen), and the vector was digested in order to in vitro transcribe off of the vector's T7 promoter. The Maxiscript T7/SP6 kit (Ambion) was used for *in vitro* transcription according to the manufacturers directions using [α−32P]-UTP (Perkin-Elmer). In vitro transcription products were subsequently run on a 5% PAGE - 8 M urea gel and gel isolated by cutting the corresponding fragments from the gel and eluting in 500 µl TE overnight at 37°C. Small RNAs were enriched from total RNA using the miRvana miRNA isolation kit (Ambion) according to the manufacturers directions. 45 µg of small RNA was mixed with 40 µl of gel isolated probe and ethanol precipitated. The pellet was then resuspended in 20 µl hybridization buffer (40 mM Pipes, 1 mM EDTA, 0.4 M NaCl, 80% formamide) and incubated overnight at 42°C. The hybridizations were then digested with

1 µl of each of the following: RNase A (10 mg/ml), RNase I (10 units/µl), and RNase T1 (80 units/µl) in a high salt buffer (0.3 M NaCl, 10 mM Tris, 5 mM EDTA) for one hour and 30 minutes at 37°C. 15 µl of 10% SDS and 5 µl of 10 mg/ml proteinase K were added to the digests and incubated at 37°C for 15 minutes. The reactions were then phenol:: chloroform extracted, ethanol precipitated, and run on a 12% PAGE - 8 M urea gel along with a labeled DECADE marker (Ambion). The gel was dried for 1 hour and exposed to film overnight. Probes used to detect siRNAs are indicated in figures 3 and 4. For the detection of 5.8S loading controls, total small RNA fractions were end labeled with Poly(A) polymerase (USB) and 3'[α−32P]-cordycepin (Perkin Elmer) according to the manufacturers directions for one hour at 37°C. The reactions were then phenol:: chloroform extracted, ethanol precipitated, and run on a 8% PAGE – 8 M urea gel. The gel was dried for 1 hour and exposed to film overnight.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation was performed as previously described. Three microliters of the following antibodies were used per 500ul of chromatin: α-H3K9me3 (Upstate Biotechnology #07-442), α-Swi6 (Abcam #14898), α -Chp1 (Abcam #18191), α –Ago1 (Abcam #18190).

RNA-IP

RNA-IP was performed essentially as previously described. Briefly, RNA-IPs were performed as chromatin immunoprecipitations with the additon of 100 units of RNase inhibitor (Promega) to cell lysis, sonication, immunoprecipitation, wash, and RNA extraction steps. Chromatin was

digested with DNase I in the presence of 100 units of RNase inhibitor as previously described prior to immunoprecipitation.

Quantitative PCR

Quantitative PCR was performed on an MJ Research / BioRad Chromo4 Thermocycler using Opticon 3.0 software. The optimum annealing temperature and efficiency of the qPCR reaction for each primer set was determined empirically. The thermocycling conditions were as follows: an initial denaturation at 95˚ C for 3 minutes followed by 40 cycles of 95˚ C for 10 seconds, 50-60˚ C for 30 seconds, and 72˚ C for 1 minute. Quantitative PCR reactions were performed in duplicate. Fold enrichments and relative expressions were calculated using the Pfaffl method on GeneEx software (BioRad). Raw Ct values for each primer were input using either *act1*$^+$ (for expression) or *gpd3*$^+$ (for ChIP) as reference genes and normalized to wild-type controls. Essentially this methodology normalizes the background enrichment from immunoprecipitations, represented by the Ct value obtained for either *gpd3*$^+$, relative to the Ct value obtained by immunoprecipitation using a primer set of interest. This value is then normalized to the ratio of Ct values for the DNA isolated from input samples for the primer set of interest to input samples of the *gpd3*$^+$ control. This yields fold enrichment that is simplified as follows and analogous to standard semi-quantitative methods:

(immunoprecipitated *primer set* / immunoprecipitated *gpd3*$^+$) / (input *primer set* / input *gpd3*$^+$)

Primers are listed in Table 1.

GAAAACACATCGTTGTCTTCAGAGTAGTGATAGTTCTTATCGTTGTAGTTATAGTTGTA 129
GTTATAGTTATAGTTGTAGTTGTAATAACTCTTTCAACAAGTCCTGAATCTTGGCAAAC
AGACCCTCATACAGTGCTGTCAGCTCACTCAAGTCCAATCGCACCATGTTAATTAAACG
CATCTCCAATGCACGGGTACATAGAATTACTTCGAGACTGAAACAATACGGTGCTTGGG
CTTAGTCCTTGTAGTGTATTTTGCATACATACCCTTCTGTTCGAATGATATCCC

Figure 3: *dg* sequence used as a probe for RNase protection

ATTAACTGTCAGGATGTGTTGTCGTTCTTGAAGTAAGTCTTGTGAAAGAAACCACCGAG 130
TTAAAAATAGAGATCTTGATGGTTTTATATATAAAAGTTTAATGCTTGCCTATTTATAC
ATTTCCCAAGGACTGCTGAGGTAGATGCGTTGTTCAGTTGACTAGGATTTGTTTGAATT
CAAAGATTATCTATTCAAGTCTTCTTTATACGTTTGCATGTGTTTGATAAGAATTCCAT
TGATTCAGAAAAAAAGTGCCAACAGTTTTTCCAACTTGACTGACACTACAACCTTCCAA
TCATAGCCATACTACTATGGTATAAAAATAAAAGTTTACAATTTTACTACTGTTATATA
TATTTCGATGGTAGTGAAGCTTTAAAAAAAAGAAGGAATAGCATACCGTCAAGTCGTTA
GTTGATGGGTAGGGTTGTTAGCCTAATTATGAGTTTTATTTGTAAAATAGGTTTAGGTT
TTAGTTTTAGTTTTAAATTATTAGTCCAATCATGAGTTGAGCTGCTAGTTTAGGCGTGC
GTTGACCTTTTAGCT

Figure 4: *dh* sequence used as probe for RNase protection

Table 1. Oligonucleotides used in this study

ID#	Sequence	Purpose
C9	CCGCAGTTGGGAGTACATCATTC	*dg* primers
C10	ACAGCACTCAACAACAGTCTTGG	
C15	ATTAACTGTCAGGATGTGTTGTCGTTCTTG	*dh* primers
C16	CGCATCTACCTCAGCAGTCCTTGG	
B86	AATTCGCAGACATTGGAAATACC	*ura4*$^{+}$ primers
B87	TGTGATATGAGCCCAAGAAGC	
C48	TCAAGTGGTCTGCCTCTGG	*gpd3*$^{+}$ primers
C49	CACCGACGACGAACATGG	
B74	TGCGGTTCACCCTTAACATC	*dgNucH* primers (unique 30-mer primers)
B75	CTTCTCATCACTGAAATCTTTAACG	

CHAPTER V

Retrotransposon LTRs repress adjacent antisense and sense transcripts by RNA turnover

Abstract

Barbara McClintock postulated that transposons could act as "controlling elements" for gene regulation. Indeed, transposable elements have recently been shown to play a role in gene regulation through epigenetic regulation and/or inhibition of Pol II transcription. Here, we define a new role for retrotransposon Tf2 LTRs from the fission yeast, *S. pombe*, in controlling both sense and antisense transcripts in adjacent non-coding RNAs. Importantly, this work suggests Tf2 LTRs repress RNA expression via a novel mechanism that involves RNA turnover rather than Pol II transcriptional inhibition. Consistent with this proposal, genetic analyses suggests that the nuclear exosome component, Rrp6, suppresses the accumulation of both sense and antisense transcripts at loci adjacent to LTRs. Overall, this work defines a novel link between nuclear RNA surveillance and transposon-mediated gene regulation.

Introduction

Transposable elements are discrete DNA segments that can translocate between non-homologous insertion sites within the genome. Barbara McClintock first described transposons in maize (Jones 2005). A large fraction of the genome is composed of transposons and they compromise two separate classes based on their mechanism of transposition. The first class is DNA mediated and the second class is RNA mediated. RNA mediated transposons are called retrotransposons and are a close relative to retroviruses both in genome structure and replication mechanisms (Slotkin and Martienssen 2007). *S. pombe* has two classes of LTR (long terminal repeat) retrotransposons, Tf1 and Tf2 (Kelly and Levin 2005). LTR retrotransposons consist of a coding sequence surrounded by two LTRs on either side. The sequenced laboratory strain of *S. pombe* does not contain copies of the Tf1-type retrotransposons, but has thirteen full-length Tf2-type retrotransposons (Kelly and Levin 2005). Interestingly, remnants of Tf2 and Tf1 transpositions compromising full-length LTRs as well as fragments of LTRs are dispersed throughout the genome (Kelly and Levin 2005). A possible role for LTRs in the regulation of gene expression has not been investigated.

Results and Discussion

We sought to investigate the role of transposons in gene regulation in the fission yeast *S. pombe*. Initially, we investigated the regulation of the *meu3*$^+$ non-coding RNA adjacent to a Tf2 retroelement long terminal repeat (LTR) on chromosome 3 (Fig. 1A). This locus was chosen for analysis because microarray data indicate that expression of *meu3*$^+$ is upregulated in histone deacetylase mutants (Hansen et al. 2005). As histone deacetylases play a role in maintaining a chromatin structure that is repressive to Pol II transcription and transposons have been implicated in repressing Pol II transcription we investigated this locus for the effect of the LTR on *meu3*$^+$transcriptional regulation (Slotkin and Martienssen 2007).

The Tf2 LTR sequences adjacent to *meu3*$^+$ were either completely deleted or replaced with vector sequence of identical length (*Tf2LTRΔ* or *Tf2LTRΔ::vector*, respectively, Fig. 1B). We found a dramatic increase in *meu3*$^+$ steady-state transcripts by qRT-PCR upon deletion or replacement of the LTR with vector sequence (Fig. 1B). Upon further analysis using strand-specific RT-PCR we found that in the absence of the LTR both sense and remarkably antisense *meu3*$^+$ transcripts accumulated to much greater levels as compared to a control strain with an intact LTR (Fig. 1C).

We sought to determine if suppression of the antisense and sense *meu3*$^+$ transcripts was dependent on the *meu3*$^+$ sequence itself. 5' RACE analysis demonstrated that the *meu3*$^+$ antisense transcript initiated downstream from the previously mapped *meu3*$^+$ locus and putative TATA sequences can be found in this region (data not shown). We replaced the previously defined *meu3*$^+$ sequence with a *ura4*$^+$ coding sequence (lacking the endogenous *ura4*$^+$ promoter and terminator) such that the putative TATA

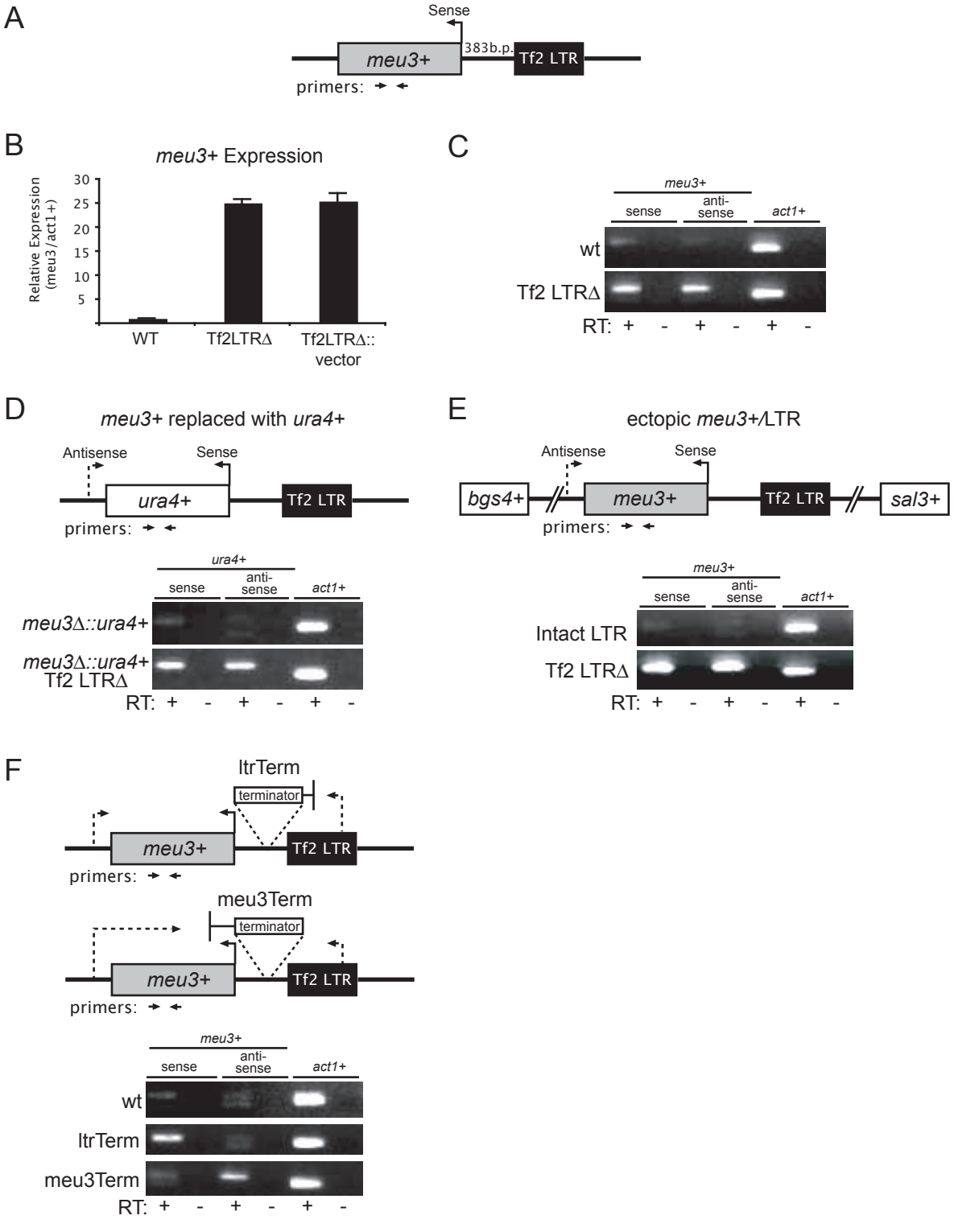

A
Sense
meu3+
383b.p.
Tf2 LTR
primers:
B
meu3+ Expression
Relative Expression (meu3/act1+)
30
25
20
15
10
5
WT
Tf2LTRΔ
Tf2LTRΔ:: vector
C
meu3+
sense
anti-sense
act1+
wt
Tf2 LTRΔ
RT: + - + - + -
D
meu3+ replaced with ura4+
Antisense
Sense
ura4+
Tf2 LTR
primers:
ura4+
sense
anti-sense
act1+
meu3Δ::ura4+
meu3Δ::ura4+ Tf2 LTRΔ
RT: + - + - + -
E
ectopic meu3+/LTR
Antisense
Sense
bgs4+
meu3+
Tf2 LTR
sal3+
primers:
meu3+
sense
anti-sense
act1+
Intact LTR
Tf2 LTRΔ
RT: + - + - + -
F
ltrTerm
terminator
meu3+
Tf2 LTR
primers:
meu3Term
terminator
meu3+
Tf2 LTR
primers:
meu3+
sense
anti-sense
act1+
wt
ltrTerm
meu3Term
RT: + - + - + -

Figure 1

Figure 1. *meu3*$^+$ sense and antisense transcripts are suppressed by an adjacent Tf2 LTR.

(A) Schematic of the *meu3*$^+$ locus with the adjacent Tf2 LTR. (B) qRT-PCR analysis for *meu3*$^+$ transcripts in wild-type or strains in which the Tf2 LTR has been deleted (*Tf2LTRΔ*) or replaced with vector sequence (*Tf2LTRΔ::vector*). Error bars indicate s.e.m. (C) Strand-specific RT-PCR (ssRT-PCR) analysis of wild-type or a *Tf2LTRΔ* strain, using primers indicated in part A to specifically amplify *meu3*$^+$ sense or antisense transcripts. A control reaction to amplify the sense *act1*$^+$ transcript is indicated. Control reactions lacking reverse transcriptase (-RT) are indicated. (D) ssRT-PCR analysis of a strain in which the *ura4*$^+$ coding sequence replaces *meu3*$^+$ (*meu3Δ::ura4*$^+$) or a strain in which the *ura4*$^+$ coding sequence replaces *meu3*$^+$ and the Tf2 LTR is deleted (*meu3Δ::ura4*$^+$*Tf2LTRΔ*), using primers to specifically amplify *ura4*$^+$ sense or antisense transcripts. Control sense *act1*$^+$ transcripts and reactions lacking reverse transcriptase (-RT) are indicated. (E) ssRT-PCR analysis of strains in which the *meu3*$^+$ locus (including downstream TATA sequences) is integrated at the *bgs4*$^+$*/sal3*$^+$ intergenic locus with or without the adjacent Tf2 LTR. Control sense *act1*$^+$ transcripts and reactions lacking reverse transcriptase (-RT) are indicated. (F) ssRT-PCR analysis of strains in which a transcriptional terminator derived from the *ura4*$^+$ 3' UTR is integrated in between *meu3*$^+$ and the adjacent Tf2 LTR in one of two orientations: such that the terminator stops transcription initiated at the Tf2 LTR (ltrTerm) or such that the terminator stops the antisense transcription initiated downstream of *meu3*$^+$ (meu3Term). Control sense *act1*$^+$ transcripts and reactions lacking reverse transcriptase (-RT) are indicated.

sequences for the antisense transcript were not perturbed (Fig. 1D). This experiment demonstrated that the *meu3*$^+$ sequence itself does not play a role in LTR-mediated regulation of neighboring transcripts, as *ura4*$^+$ sense and antisense transcripts were suppressed by the adjacent LTR and accumulated to much greater levels in the absence of the LTR (Fig. 1D).

We next tested whether the LTR mediated suppression of *meu3*$^+$ transcripts was subject to chromosomal position-effects by integrating *meu3*$^+$ (including the putative TATA sequence that specifies the antisense RNA) and the adjacent LTR at an intergenic locus on chromosome 3. Indeed, LTR-mediated suppression of *meu3*$^+$ sense and antisense transcripts was not subject to this change in chromosomal position as *meu3*$^+$ sense and antisense transcripts were suppressed by the presence of the LTR at this locus as well (Fig. 1E).

Mapping of putative transcripts at the *meu3*$^+$ locus in wild-type cells by 5' RACE revealed the presence of a transcript that initiates in the LTR and reads through into *meu3*$^+$ in addition to the previously mapped *meu3*$^+$ transcript (data not shown). Furthermore, the *meu3*$^+$ antisense transcript reads through the LTR (data not shown). This led us to investigate whether read-through transcription either into or from the LTR played a role in the suppression of *meu3*$^+$ sense and antisense transcripts. Thus, we inserted a transcriptional terminator in between the LTR and *meu3*$^+$ in two orientations: one that terminates transcription initiated at the LTR (ltrTerm, Fig. 1F) and one that terminates antisense *meu3*$^+$ transcription reading through into the LTR (meu3Term, Fig. 1F). Remarkably these results revealed a role for read-through transcription into or from the LTR in suppression of *meu3*$^+$ sense and antisense transcripts. As such, in the ltrTerm strain *meu3*$^+$ sense transcripts accumulated to greater levels than the wild-type strain lacking the terminator (Fig. 1F). Furthermore, in the ltrTerm strain *meu3*$^+$ antisense transcripts accumulated to levels similar to that of the wild-type strain (Fig. 1F). In addition, in

the meu3Term strain, *meu3*$^+$ antisense transcripts accumulated to levels much greater than the wild-type control strain lacking the terminator, whereas *meu3*$^+$ sense transcripts accumulated to levels similar to wild-type (Fig. 1F). Overall, these results suggest that transcription of the TF2 LTR sequences themselves is required for LTR-mediated suppression of adjacent transcripts.

In plants and mammals transposable elements are often associated with histone modifications typical of silenced genes and/or heterochromatin (Slotkin and Martienssen 2007). The canonical heterochromatic histone modification is H3 lysine 9 di- or tri-methylation (H3K9me2/3), which serves as a binding site for HP1/Swi6 (Bannister et al. 2001; Lachner et al. 2001). Chromatin immunoprecipitation (ChIP) analysis using primers specific for the Tf2 LTR adjacent to *meu3*$^+$ demonstrated a lack of enrichment of H3K9me1/me2/me3 and also a lack of the heterochromatin binding protein Swi6 at this locus (Fig. 2), suggesting that the Tf2 exerts repression of *meu3*$^+$ transcripts by a mechanism distinct from heterochromatic silencing.

Tf2 LTRs have recently been shown to recruit the CENP-B DNA binding protein, Abp1, which itself recruits the CENP-Bs Cbh1 and Cbh2 (Cam et al. 2008). These DNA binding proteins in turn recruit histone deacetylases (HDACs) to deacetylate histones (Cam et al. 2008). Deacetylation mediated by HDACs has also been shown to reduce Pol II occupancy, thus we tested whether deletion of the LTR adjacent to *meu3*$^+$ results in increased histone acetylation and Pol II occupancy at *meu3*$^+$. ChIP analysis demonstrated a modest increase in histone H3K9 and H3K14 acetylation at *meu3*$^+$ in the Tf2 LTR deleted strain (Fig. 3). Surprisingly, the deletion of the LTR did not result in increased Pol II occupancy (Fig. 3).

These results led us to investigate other mechanisms that would be distinct from Pol II transcriptional repression to could account for the increase in *meu3*$^+$ sense and antisense transcripts in the absence of the adjacent LTR. The lack of an increase in Pol II occupancy at

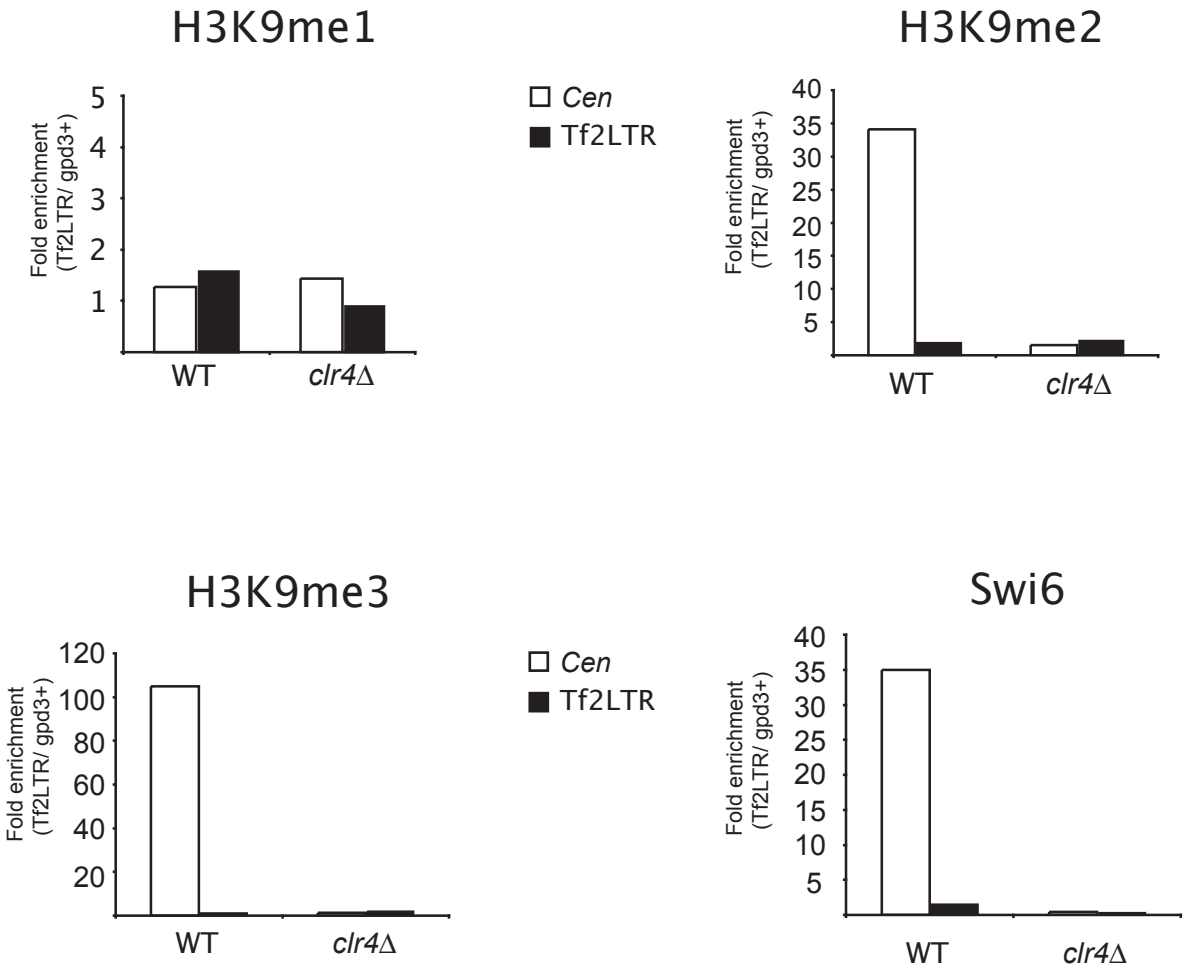


Figure 2: Lack of heterochromatin at the LTR adjacent to *meu3+.* ChIP analysis for H3K9me1/2/3 or Swi6 was performed in wild-type or *clr4*Δ strains using primers specific for the centromere or the Tf2 LTR.

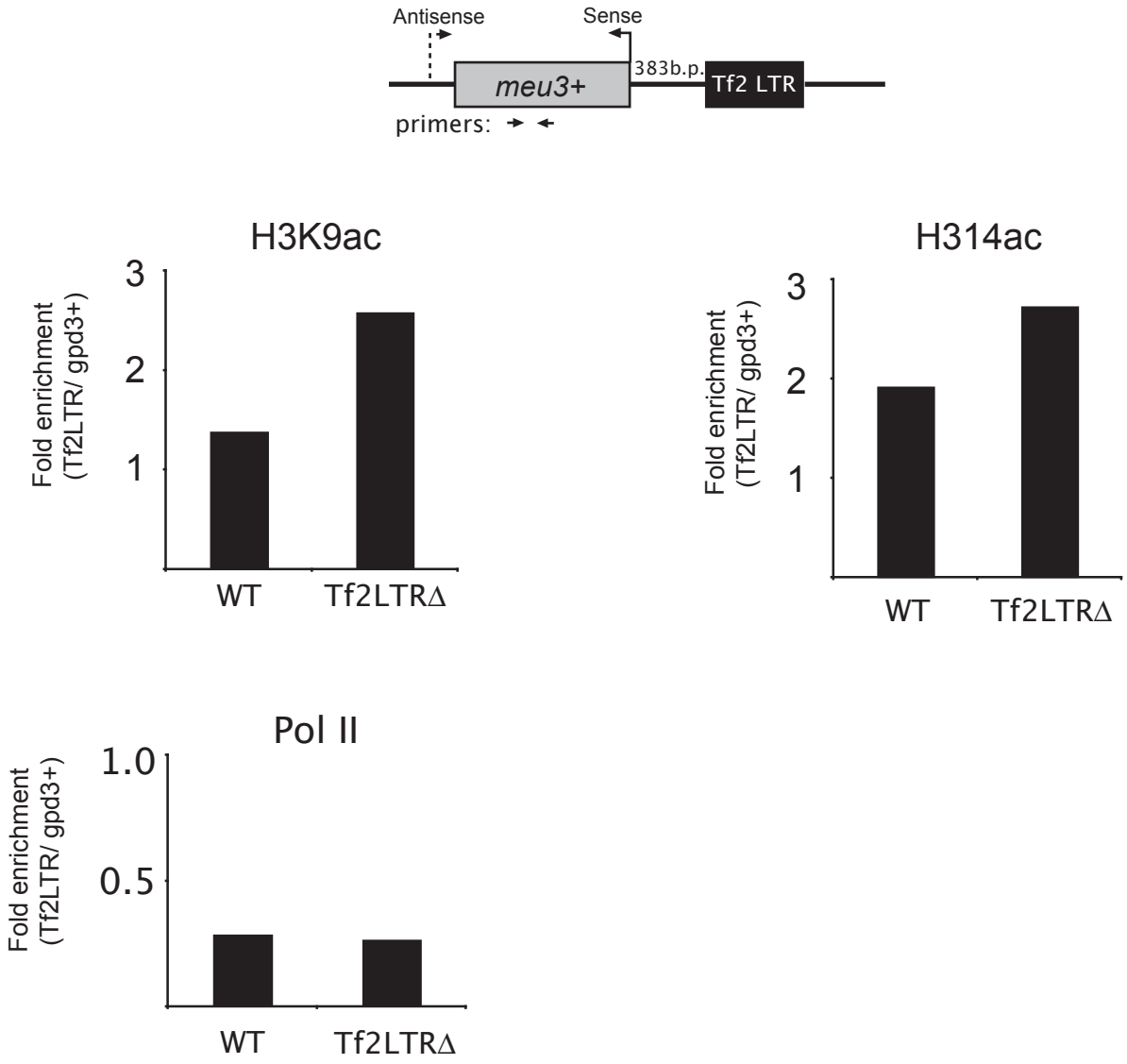


Figure 3. The Tf2 LTR does not suppress Pol II occupancy at meu3+.

Chromatin immunoprecipitation (ChIP) analysis for H3K9ac, H3K14ac, and Pol II in wild-type and a Tf2LTRΔ strain using primers specific for *meu3+*. Fold enrichment is calculated as the ratio of immunoprecipitated DNA at *meu3+* to immunoprecipitated DNA at the control *gpd3+* locus + s.e.m.

meu3$^+$ in the absence of the LTR (Fig. 3), suggested that the TF2 LTR exerts its suppression of *meu3*$^+$ transcripts via RNA turnover rather than Pol II repression. Therefore, we tested whether genomic deletions of components of the nuclear (Rrp6) and cytoplasmic (Cid14) exosome or the RNAi nuclease, Dcr1, had any affect on the accumulation of *meu3*$^+$ transcripts (Bernstein et al. 2001; Buhler et al. 2007). We also tested an Abp1 deletion strain and genomic deletions for the HDAC Clr3, and Alp13, a component of the Clr6 HDAC complex; these two HDACs have been proposed to be recruited by Abp1. Remarkably, these results demonstrated that in an *rrp6Δ* mutant both *meu3*$^+$ sense and antisense transcripts accumulated to essentially identical levels as the LTR deletion strain (Fig. 4). Interestingly, this effect was specific for the nuclear exosome as *cid14Δ* had little effect on *meu3*$^+$ transcripts (Fig. 4). Furthermore, RNAi did not play a role in suppression of *meu3*$^+$ transcripts as *dcr1Δ* also had little effect on *meu3*$^+$ transcripts. Interestingly, *clr3Δ*, *alp13Δ*, and *abp1Δ* only had modest effects on the accumulation of *meu3*$^+$ sense transcripts (Fig. 4). Although these proteins have recently been proposed to silence genes adjacent to LTRs, *clr3Δ*, *alp13Δ*, and *abp1Δ* mutants did not pheno-copy the LTR deletion and also had very little effect on the accumulation of antisense *meu3*$^+$ transcripts (Fig. 4 and 5). Overall, these results suggest a role for the nuclear exosome in targeting transcripts adjacent to a TF2 LTR for degradation.

We next tested whether Tf2 LTR mediated suppression of adjacent transcripts was a conserved feature of other Tf2 LTRs. We deleted the Tf2 LTRs at loci adjacent to *meu19*$^+$ and *meu4*$^+$ by replacing the LTRs with *ura4*$^+$. In both cases we find upregulation of the adjacent *meu19*$^+$ and *meu4*$^+$ transcripts (Fig. 6) suggesting that LTR mediated suppression of adjacent transcripts is conserved at other loci in the fission yeast genome.

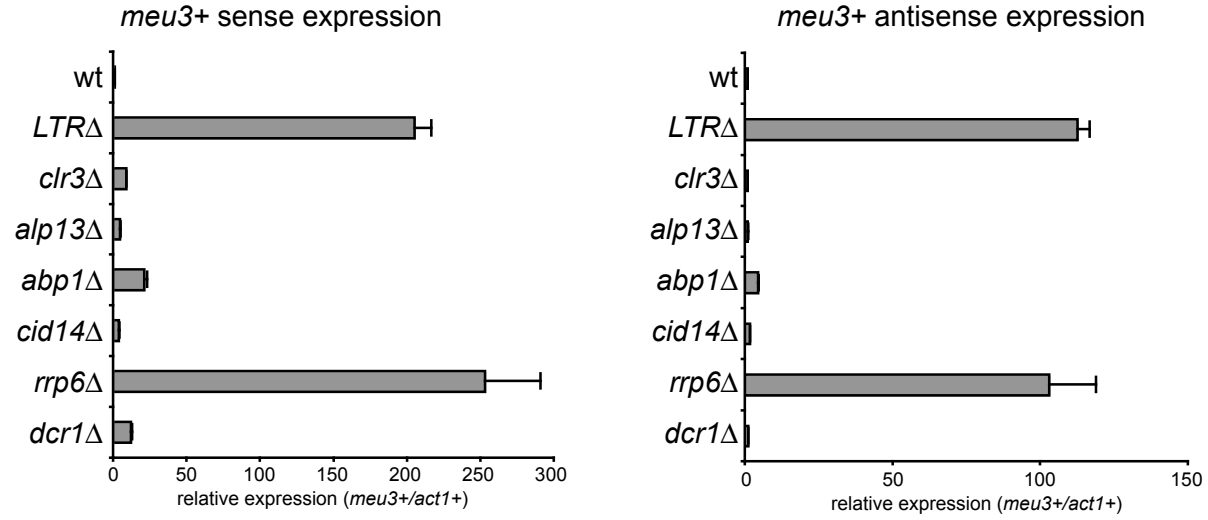
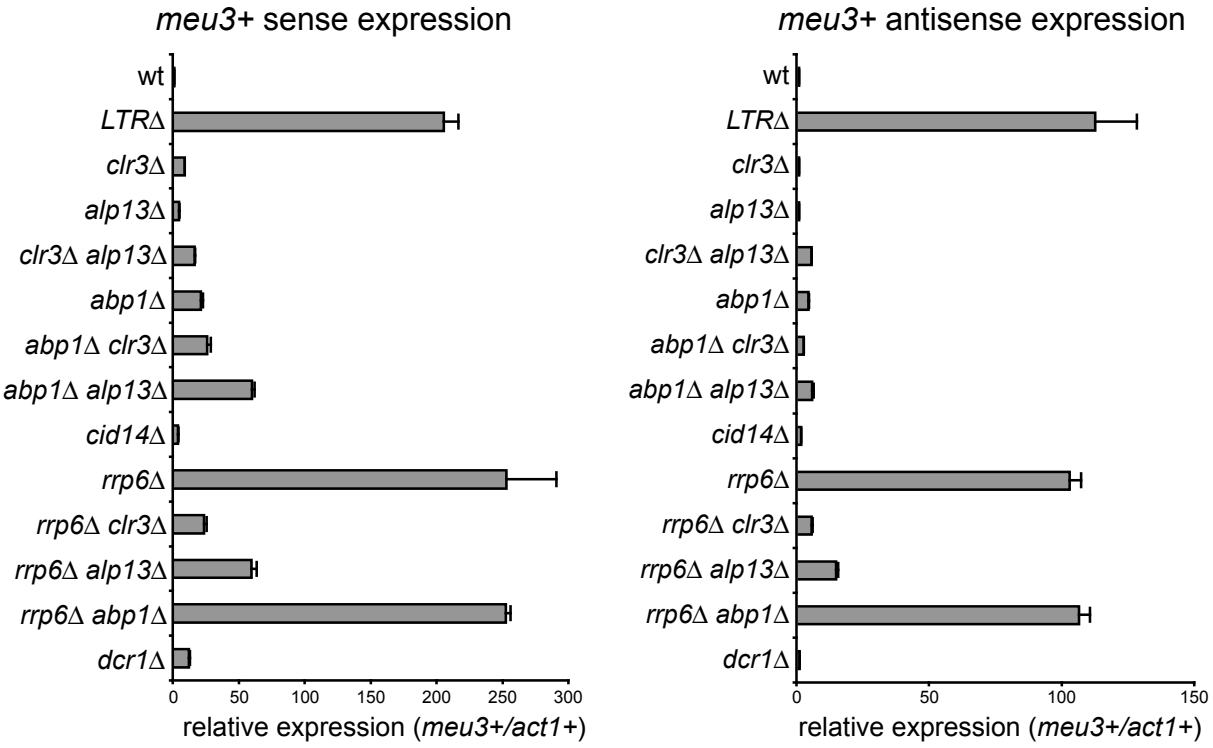


Figure 4. *meu3+* sense and antisense transcripts are regulated by the nuclear exosome component Rrp6.

qRT-PCR analysis for sense and antisense *meu3+* transcripts in the indicated strains. Relative expression is calculated as the fold change in expression of *meu3+* transcripts relative to wild-type (set as 1.0), using *act1+* sense expression as an internal control.



Fiigure 5: Quantitative RT-PCR analysis of *meu3+* sense and antisense transcripts in the indicated strains. Data from wt, LTRΔ, *clr3*Δ, *alp13*Δ, *abp1*Δ, *cid14*Δ, *rrp6*Δ, and *dcr1*Δ are from Fig. 3, as the experiments in Figs. 3 and S2 were performed at the same time.

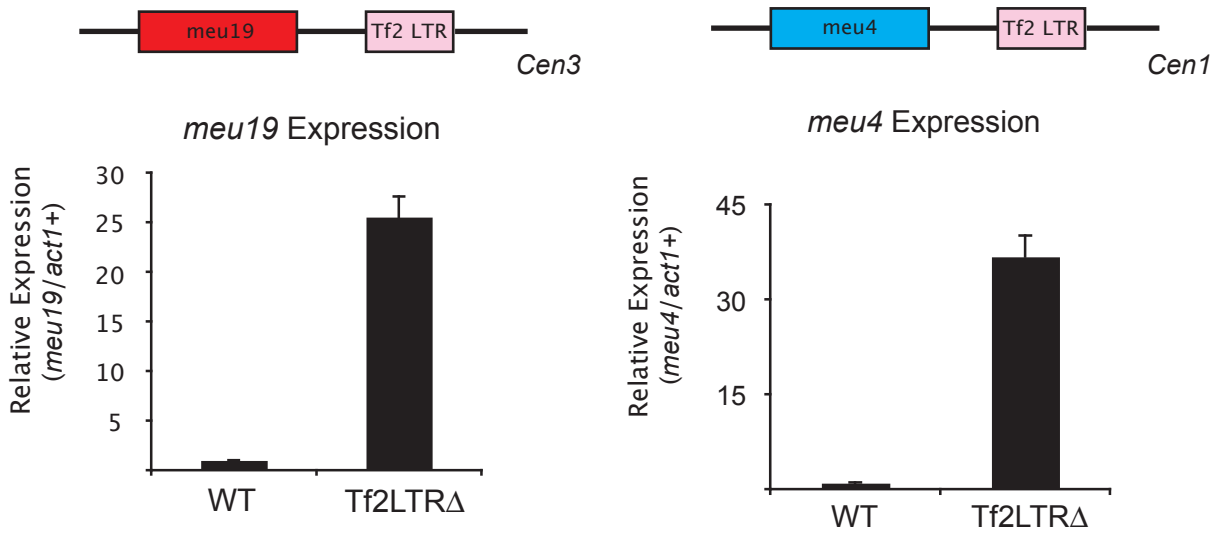


Figure 6. Tf2 LTRs control the accumulation of *meu4+* and *meu19+* transcripts.

qRT-PCR analysis for sense and antisense *meu4+* or *meu19+* transcripts in wild-type or strains in which the adjacent LTR has been deleted (Tf2LTRΔ). Relative expression is calculated as the fold change in expression of *meu4+* or *meu19+* transcripts relative to wild-type (set as 1.0), using *act1+* sense expression as an internal control.

The data presented here define a new mechanism whereby LTRs contribute to silencing of nearby genes by RNA turnover via the exosome. As LTRs are widespread throughout the genome, we hypothesize that retroelements that have transposed into intergenic regions could lead to the quelling of spurious intergenic transcripts—a mechanism that would presumably be evolutionarily favorable; whereas transposition of retroelements near genes can also lead to regulation of these genes via the exosome as shown here.

Materials and Methods

Growth of Yeast Strains

S. pombe was cultured at 33˚ C in non-selective YEA. Strains used are listed in Appendix 1.

Construction of Strains

Deletion mutants were generated using a two-step PCR method with previously described vector templates. Long ~300 bp. "adaptamers" were generated by PCR and then used in a second PCR reaction with vectors that either delete the gene of interest with a kanMX marker. Each deletion PCR product was then stably integrated by standard *S. pombe* transformation and each strain was confirmed using PCR with primers outside the genes.

RNA extraction and RT-PCR

RNA was extracted according to standard fission yeast protocols. Briefly, 50 ml *S. pombe* cultures were grown to an $O.D._{600}$ of ~0.5, pelleted, and frozen at – 80˚ C. Frozen pellets were extracted with a solution of 50 mM sodium acetate, 10 mM EDTA, 1% SDS, and three extractions of phenol::chloroform (1:1) equilibrated in DEPC treated water. RNA was precipitated with sodium acetate and ethanol. To make cDNA, 1 μg of RNA was DNAse treated with 2 Units of DNAse RQ1 (Promega), with the buffer supplied by the manufacturer at 37˚ C for 60 minutes. Purified RNA was then used in a cDNA reaction with 100 units of SuperScript III (Invitrogen), using buffer supplied by the manufacturer and primed with random 9-mers or strand-specific primers. cDNA reactions were incubated at 42˚ C and then the enzyme was inactivated at 85˚ C for 5 minutes.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation was performed as previously described. Three microliters of the following antibodies were used per 500ul of chromatin: α-H3K9me3 (Upstate Biotechnology #07-442), α-Swi6 (Abcam #14898).

Quantitative PCR

Quantitative PCR was performed on an MJ Research / BioRad Chromo4 Thermocycler using Opticon 3.0 software. The optimum annealing temperature and efficiency of the qPCR reaction for each primer set was determined empirically. The thermocycling conditions were as follows: an initial denaturation at 95˚ C for 3 minutes followed by 40 cycles of 95˚ C for 10 seconds, 50-60˚ C for 30 seconds, and 72˚ C for 1 minute. Quantitative PCR reactions were performed in duplicate. Fold enrichments and relative expressions were calculated using the Pfaffl method on GeneEx software (BioRad). Raw Ct values for each primer were input using either *act1*$^+$ (for expression) or *gpd3*$^+$ (for ChIP) as reference genes and normalized to wild-type controls. Essentially this methodology normalizes the background enrichment from immunoprecipitations, represented by the Ct value obtained for either *gpd3*$^+$, relative to the Ct value obtained by immunoprecipitation using a primer set of interest. This value is then normalized to the ratio of Ct values for the DNA isolated from input samples for the primer set of interest to input samples of the *gpd3*$^+$ control. This yields fold enrichment that is simplified as follows and analogous to standard semi-quantitative methods: (immunoprecipitated *primer set* / immunoprecipitated *gpd3*$^+$) / (input *primer set* / input *gpd3*$^+$) Primers are listed in Table 1.

Table 1. Oligonucleotides used in this study

ID#	Sequence	Purpose
C7	TGTGCCTCGTCAAATTATCATCCATCC	*cen3* primers
C8	ACTTGGAATCGAATTGAGAACTTGTTATGC	
Meu3rt5'	GGCTACCCTCTTTTGGTTACTTCC	*meu3*$^+$ primers
Meu3rt3"	GCCAGATATTGCCATTGTTTTGACC	
B86	AATTCGCAGACATTGGAAATACC	*ura4*$^+$ primers
B87	TGTGATATGAGCCCAAGAAGC	
Meu19rt5'	GGCTACCCTCTTTTGGTTACTTCC	*meu19*$^+$ primers
Meu19rt3'	TTACAATGGAGACGAGTTACC	
Meu4rt5'	CAAATGCTGTGAGAAGAAGAAACC	*Meu4*$^+$ primers
Meu4rt3'	AAATATGTGATGGGACCAGTAACG	

CHAPTER VI

Conclusions and Future Perspectives

The work described in this thesis offers new insights into the mechanisms of RNA silencing in the organism *S. pombe*. First, we demonstrated obvious differences in the regulation of the forward and reverse transcripts at centromere repeats. The regulation of the reverse *dh* transcript seems to be under the control of a novel translational silencing mechanism. Next we described a RNAi-mediated *trans* silencing pathway in *S. pombe* that induces transcriptional silencing of a target mRNA via the recruitment of heterochromatin. In addition, we describe a class of Rdp1 dependent secondary siRNAs required for the amplication of the RNAi pathway. Finally, we uncover a new silencing mechanism linking Tf2 LTRs to the transcriptional control of non-coding RNAs.

The first comprehensive analysis of centromere transcript regulation by RNAi, RNA-turnover, and histone modification pathways is described in chapter two. The regulation of the centromere transcripts synthesized from both DNA strands of outer *dg* and *dh* sequences has not been fully investigated. We therefore used *ura4*$^+$ reporter genes under the regulation of endogenous centromere promoters to analyze centromere transcript regulation in *S. pombe*. Our results reveal both strand-specific and locus dependent differential regulation of heterochromatic repeats and reveal for the first time that homologous centromere repeat sequences are subject to distinct repression pathways. Interestingly, we also detected a novel silencing mechanism contributing to the silencing of the *ura4*$^+$integrated at the *dh* repeat at centromere three in the reverse orientation. A novel translational inhibition mechanism controlled by the exosome component Cid14 may be required for *ura4*$^+$ silencing. Other possible mechanisms to explain these observations include nuclear retention of mRNA, RNA deadenylation, or decapping of RNA, which could all be mediated by the exosome. Further investigation into these possible

mechanisms may uncover additional components necessary for silencing at the centromeres. In addition, the various *ura4*$^+$ reporters created for use in this study will provide a valuable tool for future studies involving differential regulation of the *dg* and *dh* outer repeat transcripts of the *S. pombe* centromeres, including possible variations in cell cycle regulation of the forward and reverse transcripts.

Chapter three describes a novel mechanism of RNAi-mediated targeting of heterochromatin *in trans*, and demonstrates a novel role for CENP-B proteins in the establishment of heterochromatin domains in *S. pombe*. Double stranded transcripts from initiator *ura4*$^+$ reporters at *cen3* are processed into siRNAs, which are then loaded into the RITS complex and subsequently targeted to the target *ura4*$^+$ at *cen1*. Not only do we detect nucleation of heterochromatin, but remarkably we also observe spreading of heterochromatin from the site of nucleation at the target *ura4*$^+$. Thus, heterochromatin can be targeted by the RITS complex and subsequently spread to establish large heterochromatin domains. Chp1 recruitment (and likely RITS) occurs *in trans* even in the absence of Swi6, Clr3, or Abp1 and this recruitment is not dependent on H3K9 methylation. H3K9me3, however, seems to be necessary to maintain a stable silent state. Our results suggest a linear pathway whereby CENP-B proteins recruit Swi6, which in turn recruits Clr3 to deacetylate histones in order for H3K9me3 to occur.

Interestingly, only the forward *ura4*$^+$ initiator was able to target *in trans*. It is possible that sense *ura4*$^+$ siRNAs loaded into RITS are unable to target the nascent sense mRNA at the target locus whereas antisense *ura4*$^+$ siRNAs from the "forward" initiator can. This data also suggests that siRNAs loaded into RITS target the homologous mRNA and not the DNA. Targeting of RITS to

double stranded DNA should work with either sense or antisense siRNAs. Furthermore, a deletion of the target *ura4*$^+$ promoter to eliminate a *ura4*$^+$ transcript, would also directly test whether RITS is targeting the RNA or DNA. Presumably, Chp1 and H3K9me3 would still be targeted in the absence of a transcript if RITS was targeted to the DNA. Studies have also suggested that RNAi-directed transcriptional gene silencing does not affect changes in RNA polymerase II occupancy at the target locus (Buhler et al. 2006). However, we see a significant decrease in the enrichment of Pol II at the target *ura4*$^+$ when heterochromatin is targeted *in trans* suggesting an inhibition of transcription leading to silencing. The data presented herein provide the first insight into the establishment of a stable heterochromatin domain via RNAi *in trans*, and will provide a system for future studies involving RNAi-directed heterochromatin establishment.

Contrary to recent work suggesting that Rdp1 (RNA-dependent RNA polymerase) is required for the production of dicer dependent siRNAs in the intiation of the RNAi pathway, this work suggests that a discrete class of primary siRNAs is present in the absence of Rdp1 at fission yeast centromeres. Interestingly, as the primary siRNAs we have detected are very low in abundance, this suggests that the majority of siRNAs in fission yeast are secondary siRNAs, as is the case for *C. elegans* (Pak and Fire 2007; Sijen et al. 2007). Mapping of primary versus secondary siRNAs by sequencing populations of siRNAs from *rdp1Δ* strains compared to wild-type strains across the genome would distinguish areas of initiation and amplification in the RNAi pathway. Overall, this study lays the groundwork for future investigations into mechanisms of the coupling of secondary siRNA biogenesis and heterochromatin assembly.

The data presented in chapter five defines a new mechanism whereby LTRs contribute to silencing of nearby genes by RNA turnover via the exosome. A recent study has implicated Abp1 in recruiting the HDACs Clr3 and Clr6 to LTRs to silence adjacent genes (Cam et al. 2008). Our analysis of *clr3Δ*, *alp13Δ* (a component of the Clr6 HDAC complex), and *abp1Δ* mutants showed little effect of these mutants on the accumulation of *meu3*$^{+}$ transcripts. Thus, it is possible that mechanisms distinct from those previously characterized are acting at the *meu3*$^{+}$ locus. Furthermore, double mutants for *clr3Δ abp1Δ* and *alp13Δ abp1Δ* resulted in additive *meu3*$^{+}$ transcript accumulation relative to the respective single mutants, suggesting that the HDACs and Abp1 act in distinct pathways at this locus; however our results are consistent with genomewide mapping of Clr3 and Abp1 which shows that Clr3 and Abp1 occupancy throughout the *S. pombe* genome is only rarely coincident (Cam et al. 2005). Additionally, *rrp6Δ clr3Δ* and *rrp6Δ alp13Δ* double mutants actually resulted in less *meu3*$^{+}$ transcript accumulation than the *rrp6Δ* single mutant suggesting that Clr3 and Alp13 act in a distinct pathway from Rrp6. This observation also suggests Clr3 and Alp13 antagonize Rrp6. In contrast, an *rrp6Δ abp1Δ* double mutant resulted in non-additive *meu3*$^{+}$ transcript accumulation relative to the respective single mutants suggesting that if Abp1 has a function at this locus it is in the same pathway as Rrp6. This result further suggests that Abp1 acts in a pathway distinct from Clr3 and Alp13 at the *meu3*$^{+}$ locus. The investigation into the silencing of the *meu3*$^{+}$ non-coding RNA has helped to shed light on the separable functions of proteins previously described in *S. pombe* retrotransposon silencing pathways.

Collectively, the work presented in this thesis contributes to the understanding of RNA silencing mechanisms in *S. pombe*. The architecture of the centromeres as well as heterochromatin

structures in *S. pombe* are similar to mammalian systems, therefore the studies described herein are applicable to higher eukaryotes. A developing class of diseases has begun to emerge involving defects in the molecular components of the RNAi and heterochromatin assembly pathways. Consequently, ongoing studies of the molecular mechanisms of both pathways will be essential to the understanding and effective treatment of these diseases. In addition, many pharmaceutical and biotechnology companies have declared an interest in RNAi-based therapeutics to silence disease associated genes. However, the effective use of RNAi as a therapeutic requires a comprehensive knowledge of the basic pathways activating silencing. Continuing studies similar to those presented in this thesis, utilizing basic model systems, will undoubtedly be necessary for the understanding of silencing pathways in higher eukaryotes.

References

Aguilar-Arnal, L., Marsellach, F.X., and Azorin, F. 2008. The fission yeast homologue of CENP-B, Abp1, regulates directionality of mating-type switching. *EMBO J* **27**(7): 1029-1038.

Ahmad, K. and Henikoff, S. 2002. The histone variant H3.3 marks active chromatin by replication-independent nucleosome assembly. *Mol Cell* **9**(6): 1191-1200.

Allfrey, V.G., Faulkner, R., and Mirsky, A.E. 1964. Acetylation and methylation of histones and their possible role in the regulation of RNA synthesis. *Proc Natl Acad Sci U S A* **51**: 786-794.

Allis, C.D., Richman, R., Gorovsky, M.A., Ziegler, Y.S., Touchstone, B., Bradley, W.A., and Cook, R.G. 1986. hv1 is an evolutionarily conserved H2A variant that is preferentially associated with active genes. *J Biol Chem* **261**(4): 1941-1948.

Allshire, R.C., Javerzat, J.P., Redhead, N.J., and Cranston, G. 1994. Position effect variegation at fission yeast centromeres. *Cell* **76**(1): 157-169.

Allshire, R.C., Nimmo, E.R., Ekwall, K., Javerzat, J.P., and Cranston, G. 1995. Mutations derepressing silent centromeric domains in fission yeast disrupt chromosome segregation. *Genes Dev* **9**(2): 218-233.

Andrulis, E.D., Neiman, A.M., Zappulla, D.C., and Sternglanz, R. 1998. Perinuclear localization of chromatin facilitates transcriptional silencing. *Nature* **394**(6693): 592-595.

Andrulis, E.D., Zappulla, D.C., Ansari, A., Perrod, S., Laiosa, C.V., Gartenberg, M.R., and Sternglanz, R. 2002. Esc1, a nuclear periphery protein required for Sir4-based plasmid anchoring and partitioning. *Mol Cell Biol* **22**(23): 8292-8301.

Aravin, A.A. and Bourc'his, D. 2008. Small RNA guides for de novo DNA methylation in mammalian germ cells. *Genes Dev* **22**(8): 970-975.

Aravin, A.A., Lagos-Quintana, M., Yalcin, A., Zavolan, M., Marks, D., Snyder, B., Gaasterland, T., Meyer, J., and Tuschl, T. 2003. The small RNA profile during Drosophila melanogaster development. *Dev Cell* **5**(2): 337-350.

Aravin, A.A., Sachidanandam, R., Bourc'his, D., Schaefer, C., Pezic, D., Toth, K.F., Bestor, T., and Hannon, G.J. 2008. A piRNA pathway primed by individual transposons is linked to de novo DNA methylation in mice. *Mol Cell* **31**(6): 785-799.

Aufsatz, W., Mette, M.F., van der Winden, J., Matzke, M., and Matzke, A.J. 2002. HDA6, a putative histone deacetylase needed to enhance DNA methylation induced by double-stranded RNA. *EMBO J* **21**(24): 6832-6841.

Bahler, J., Wu, J.Q., Longtine, M.S., Shah, N.G., McKenzie, A., 3rd, Steever, A.B., Wach, A., Philippsen, P., and Pringle, J.R. 1998. Heterologous modules for efficient and versatile PCR-based gene targeting in Schizosaccharomyces pombe. *Yeast* **14**(10): 943-951.

Bannister, A.J. and Kouzarides, T. 1996. The CBP co-activator is a histone acetyltransferase. *Nature* **384**(6610): 641-643.

Bannister, A.J., Zegerman, P., Partridge, J.F., Miska, E.A., Thomas, J.O., Allshire, R.C., and Kouzarides, T. 2001. Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. *Nature* **410**(6824): 120-124.

Barlev, N.A., Candau, R., Wang, L., Darpino, P., Silverman, N., and Berger, S.L. 1995. Characterization of physical interactions of the putative transcriptional adaptor, ADA2, with acidic activation domains and TATA-binding protein. *J Biol Chem* **270**(33): 19337-19344.

Bartee, L., Malagnac, F., and Bender, J. 2001. Arabidopsis cmt3 chromomethylase mutations block non-CG methylation and silencing of an endogenous gene. *Genes Dev* **15**(14): 1753-1758.

Bernard, P., Maure, J.F., Partridge, J.F., Genier, S., Javerzat, J.P., and Allshire, R.C. 2001. Requirement of heterochromatin for cohesion at centromeres. *Science* **294**(5551): 2539-2542.

Bernstein, E., Caudy, A.A., Hammond, S.M., and Hannon, G.J. 2001. Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* **409**(6818): 363-366.

Bird, A. 2002. DNA methylation patterns and epigenetic memory. *Genes Dev* **16**(1): 6-21.

Bohmert, K., Camus, I., Bellini, C., Bouchez, D., Caboche, M., and Benning, C. 1998. AGO1 defines a novel locus of Arabidopsis controlling leaf development. *EMBO J* **17**(1): 170-180.

Braunstein, M., Rose, A.B., Holmes, S.G., Allis, C.D., and Broach, J.R. 1993. Transcriptional silencing in yeast is associated with reduced nucleosome acetylation. *Genes Dev* **7**(4): 592-604.

Brennecke, J., Aravin, A.A., Stark, A., Dus, M., Kellis, M., Sachidanandam, R., and Hannon, G.J. 2007. Discrete small RNA-generating loci as master regulators of transposon activity in Drosophila. *Cell* **128**(6): 1089-1103.

Briggs, S.D., Bryk, M., Strahl, B.D., Cheung, W.L., Davie, J.K., Dent, S.Y., Winston, F., and Allis, C.D. 2001. Histone H3 lysine 4 methylation is mediated by Set1 and required for cell growth and rDNA silencing in Saccharomyces cerevisiae. *Genes Dev* **15**(24): 3286-3295.

Brower-Toland, B., Findley, S.D., Jiang, L., Liu, L., Yin, H., Dus, M., Zhou, P., Elgin, S.C., and Lin, H. 2007. Drosophila PIWI associates with chromatin and interacts directly with HP1a. *Genes Dev* **21**(18): 2300-2311.

Brownell, J.E., Zhou, J., Ranalli, T., Kobayashi, R., Edmondson, D.G., Roth, S.Y., and Allis, C.D. 1996. Tetrahymena histone acetyltransferase A: a homolog to yeast Gcn5p linking histone acetylation to gene activation. *Cell* **84**(6): 843-851.

Bryk, M., Briggs, S.D., Strahl, B.D., Curcio, M.J., Allis, C.D., and Winston, F. 2002. Evidence that Set1, a factor required for methylation of histone H3, regulates rDNA silencing in S. cerevisiae by a Sir2-independent mechanism. *Curr Biol* **12**(2): 165-170.

Buhler, M., Haas, W., Gygi, S.P., and Moazed, D. 2007. RNAi-dependent and -independent RNA turnover mechanisms contribute to heterochromatic gene silencing. *Cell* **129**(4): 707-721.

Buhler, M., Spies, N., Bartel, D.P., and Moazed, D. 2008. TRAMP-mediated RNA surveillance prevents spurious entry of RNAs into the Schizosaccharomyces pombe siRNA pathway. *Nat Struct Mol Biol* **15**(10): 1015-1023.

Buhler, M., Verdel, A., and Moazed, D. 2006. Tethering RITS to a nascent transcript initiates RNAi- and heterochromatin-dependent gene silencing. *Cell* **125**(5): 873-886.

Cam, H. and Grewal, S.I. 2004. RNA interference and epigenetic control of heterochromatin assembly in fission yeast. *Cold Spring Harb Symp Quant Biol* **69**: 419-427.

Cam, H.P., Noma, K., Ebina, H., Levin, H.L., and Grewal, S.I. 2008. Host genome surveillance for retrotransposons by transposon-derived proteins. *Nature* **451**(7177): 431-436.

Cam, H.P., Sugiyama, T., Chen, E.S., Chen, X., FitzGerald, P.C., and Grewal, S.I. 2005. Comprehensive analysis of heterochromatin- and RNAi-mediated epigenetic control of the fission yeast genome. *Nat Genet* **37**(8): 809-819.

Cao, X., Aufsatz, W., Zilberman, D., Mette, M.F., Huang, M.S., Matzke, M., and Jacobsen, S.E. 2003. Role of the DRM and CMT3 methyltransferases in RNA-directed DNA methylation. *Curr Biol* **13**(24): 2212-2217.

Chalker, D.L. and Yao, M.C. 2001. Nongenic, bidirectional transcription precedes and may promote developmental DNA deletion in Tetrahymena thermophila. *Genes Dev* **15**(10): 1287-1298.

Chan, S.W., Zilberman, D., Xie, Z., Johansen, L.K., Carrington, J.C., and Jacobsen, S.E. 2004. RNA silencing genes control de novo DNA methylation. *Science* **303**(5662): 1336.

Chang, B., Chen, Y., Zhao, Y., and Bruick, R.K. 2007. JMJD6 is a histone arginine demethylase. *Science* **318**(5849): 444-447.

Chen, E.S., Zhang, K., Nicolas, E., Cam, H.P., Zofall, M., and Grewal, S.I. 2008. Cell cycle control of centromeric repeat transcription and heterochromatin assembly. *Nature* **451**(7179): 734-737.

Chen, H., Lin, R.J., Schiltz, R.L., Chakravarti, D., Nash, A., Nagy, L., Privalsky, M.L., Nakatani, Y., and Evans, R.M. 1997. Nuclear receptor coactivator ACTR is a novel histone acetyltransferase and forms a multimeric activation complex with P/CAF and CBP/p300. *Cell* **90**(3): 569-580.

Chiang, Y.C., Komarnitsky, P., Chase, D., and Denis, C.L. 1996. ADR1 activation domains contact the histone acetyltransferase GCN5 and the core transcriptional factor TFIIB. *J Biol Chem* **271**(50): 32359-32365.

Cogoni, C. and Macino, G. 1997. Isolation of quelling-defective (qde) mutants impaired in posttranscriptional transgene-induced gene silencing in Neurospora crassa. *Proc Natl Acad Sci U S A* **94**(19): 10233-10238.

-. 1999. Gene silencing in Neurospora crassa requires a protein homologous to RNA-dependent RNA polymerase. *Nature* **399**(6732): 166-169.

Djupedal, I., Portoso, M., Spahr, H., Bonilla, C., Gustafsson, C.M., Allshire, R.C., and Ekwall, K. 2005. RNA Pol II subunit Rpb7 promotes centromeric transcription and RNAi-directed chromatin silencing. *Genes Dev* **19**(19): 2301-2306.

Downes, M., Ordentlich, P., Kao, H.Y., Alvarez, J.G., and Evans, R.M. 2000. Identification of a nuclear domain with deacetylase activity. *Proc Natl Acad Sci U S A* **97**(19): 10330-10335.

Drysdale, C.M., Jackson, B.M., McVeigh, R., Klebanow, E.R., Bai, Y., Kokubo, T., Swanson, M., Nakatani, Y., Weil, P.A., and Hinnebusch, A.G. 1998. The Gcn4p activation domain interacts specifically in vitro with RNA polymerase II holoenzyme, TFIID, and the Adap-Gcn5p coactivator complex. *Mol Cell Biol* **18**(3): 1711-1724.

Duchaine, T.F., Wohlschlegel, J.A., Kennedy, S., Bei, Y., Conte, D., Jr., Pang, K., Brownell, D.R., Harding, S., Mitani, S., Ruvkun, G., Yates, J.R., 3rd, and Mello, C.C. 2006. Functional proteomics reveals the biochemical niche of C. elegans DCR-1 in multiple small-RNA-mediated pathways. *Cell* **124**(2): 343-354.

Eissenberg, J.C., James, T.C., Foster-Hartnett, D.M., Hartnett, T., Ngan, V., and Elgin, S.C. 1990. Mutation in a heterochromatin-specific chromosomal protein is associated with suppression of position-effect variegation in Drosophila melanogaster. *Proc Natl Acad Sci U S A* **87**(24): 9923-9927.

Ekwall, K., Javerzat, J.P., Lorentz, A., Schmidt, H., Cranston, G., and Allshire, R. 1995. The chromodomain protein Swi6: a key component at fission yeast centromeres. *Science* **269**(5229): 1429-1431.

Ekwall, K., Nimmo, E.R., Javerzat, J.P., Borgstrom, B., Egel, R., Cranston, G., and Allshire, R. 1996. Mutations in the fission yeast silencing factors clr4+ and rik1+ disrupt the localisation of the chromo domain protein Swi6p and impair centromere function. *J Cell Sci* **109 (Pt 11)**: 2637-2648.

Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. 2001a. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411**(6836): 494-498.

Elbashir, S.M., Lendeckel, W., and Tuschl, T. 2001b. RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev* **15**(2): 188-200.

Elgin, S.C. and Grewal, S.I. 2003. Heterochromatin: silence is golden. *Curr Biol* **13**(23): R895-898.

Fagard, M., Boutet, S., Morel, J.B., Bellini, C., and Vaucheret, H. 2000. AGO1, QDE-2, and RDE-1 are related proteins required for post-transcriptional gene silencing in plants, quelling in fungi, and RNA interference in animals. *Proc Natl Acad Sci U S A* **97**(21): 11650-11654.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., and Mello, C.C. 1998. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* **391**(6669): 806-811.

Fischle, W., Wang, Y., Jacobs, S.A., Kim, Y., Allis, C.D., and Khorasanizadeh, S. 2003. Molecular basis for the discrimination of repressive methyl-lysine marks in histone H3 by Polycomb and HP1 chromodomains. *Genes Dev* **17**(15): 1870-1881.

Fodor, B.D., Kubicek, S., Yonezawa, M., O'Sullivan, R.J., Sengupta, R., Perez-Burgos, L., Opravil, S., Mechtler, K., Schotta, G., and Jenuwein, T. 2006. Jmjd2b antagonizes H3K9 trimethylation at pericentric heterochromatin in mammalian cells. *Genes Dev* **20**(12): 1557-1562.

Gartenberg, M.R., Neumann, F.R., Laroche, T., Blaszczyk, M., and Gasser, S.M. 2004. Sir-mediated repression can occur independently of chromosomal and subnuclear contexts. *Cell* **119**(7): 955-967.

Gasciolli, V., Mallory, A.C., Bartel, D.P., and Vaucheret, H. 2005. Partially redundant functions of Arabidopsis DICER-like enzymes and a role for DCL4 in producing trans-acting siRNAs. *Curr Biol* **15**(16): 1494-1500.

Gorovsky, M.A., Pleger, G.L., Keevert, J.B., and Johmann, C.A. 1973. Studies on histone fraction F2A1 in macro- and micronuclei of Tetrahymena pyriformis. *J Cell Biol* **57**(3): 773-781.

Grewal, S.I. and Elgin, S.C. 2007. Transcription and RNA interference in the formation of heterochromatin. *Nature* **447**(7143): 399-406.

Grewal, S.I. and Jia, S. 2007. Heterochromatin revisited. *Nat Rev Genet* **8**(1): 35-46.

Gullerova, M. and Proudfoot, N.J. 2008. Cohesin complex promotes transcriptional termination between convergent genes in S. pombe. *Cell* **132**(6): 983-995.

Guo, S. and Kemphues, K.J. 1995. par-1, a gene required for establishing polarity in C. elegans embryos, encodes a putative Ser/Thr kinase that is asymmetrically distributed. *Cell* **81**(4): 611-620.

Hall, I.M., Shankaranarayana, G.D., Noma, K., Ayoub, N., Cohen, A., and Grewal, S.I. 2002. Establishment and maintenance of a heterochromatin domain. *Science* **297**(5590): 2232-2237.

Hamilton, A.J. and Baulcombe, D.C. 1999. A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* **286**(5441): 950-952.

Hammond, S.M., Bernstein, E., Beach, D., and Hannon, G.J. 2000a. An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. *Nature* **404**(6775): 293-296.

-. 2000b. An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. *Nature* **404**(6775): 293-296.

Hansen, K.R., Burns, G., Mata, J., Volpe, T.A., Martienssen, R.A., Bahler, J., and Thon, G. 2005. Global effects on gene expression in fission yeast by silencing and RNA interference machineries. *Mol Cell Biol* **25**(2): 590-601.

Hansen, K.R., Ibarra, P.T., and Thon, G. 2006. Evolutionary-conserved telomere-linked helicase genes of fission yeast are repressed by silencing factors, RNAi components and the telomere-binding protein Taz1. *Nucleic Acids Res* **34**(1): 78-88.

Hassig, C.A., Tong, J.K., Fleischer, T.C., Owa, T., Grable, P.G., Ayer, D.E., and Schreiber, S.L. 1998. A role for histone deacetylase activity in HDAC1-mediated transcriptional repression. *Proc Natl Acad Sci U S A* **95**(7): 3519-3524.

Hebbes, T.R., Thorne, A.W., and Crane-Robinson, C. 1988. A direct link between core histone acetylation and transcriptionally active chromatin. *Embo J* **7**(5): 1395-1402.

Heitz, E. 1928. Das heterochromatin der Moose. *Jahrbuecher Wiss Botanik* **69**: 762-818.

Herr, A.J., Jensen, M.B., Dalmay, T., and Baulcombe, D.C. 2005. RNA polymerase IV directs silencing of endogenous DNA. *Science* **308**(5718): 118-120.

Horn, P.J., Bastie, J.N., and Peterson, C.L. 2005. A Rik1-associated, cullin-dependent E3 ubiquitin ligase is essential for heterochromatin formation. *Genes Dev* **19**(14): 1705-1714.

Howman, E.V., Fowler, K.J., Newson, A.J., Redward, S., MacDonald, A.C., Kalitsis, P., and Choo, K.H. 2000. Early disruption of centromeric chromatin organization in centromere protein A (Cenpa) null mice. *Proc Natl Acad Sci U S A* **97**(3): 1148-1153.

Huarte, M., Lan, F., Kim, T., Vaughn, M.W., Zaratiegui, M., Martienssen, R.A., Buratowski, S., and Shi, Y. 2007. The fission yeast Jmj2 reverses histone H3 Lysine 4 trimethylation. *J Biol Chem* **282**(30): 21662-21670.

Imai, S., Armstrong, C.M., Kaeberlein, M., and Guarente, L. 2000. Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. *Nature* **403**(6771): 795-800.

Irvine, D.V., Zaratiegui, M., Tolia, N.H., Goto, D.B., Chitwood, D.H., Vaughn, M.W., Joshua-Tor, L., and Martienssen, R.A. 2006. Argonaute slicing is required for heterochromatic silencing and spreading. *Science* **313**(5790): 1134-1137.

Jackson, J.P., Lindroth, A.M., Cao, X., and Jacobsen, S.E. 2002. Control of CpNpG DNA methylation by the KRYPTONITE histone H3 methyltransferase. *Nature* **416**(6880): 556-560.

Jacobs, S.A., Taverna, S.D., Zhang, Y., Briggs, S.D., Li, J., Eissenberg, J.C., Allis, C.D., and Khorasanizadeh, S. 2001. Specificity of the HP1 chromo domain for the methylated N-terminus of histone H3. *Embo J* **20**(18): 5232-5241.

Jacobson, R.H., Ladurner, A.G., King, D.S., and Tjian, R. 2000. Structure and function of a human TAFII250 double bromodomain module. *Science* **288**(5470): 1422-1425.

Jenuwein, T. and Allis, C.D. 2001. Translating the histone code. *Science* **293**(5532): 1074-1080.

Jia, S., Noma, K., and Grewal, S.I. 2004. RNAi-independent heterochromatin nucleation by the stress-activated ATF/CREB family proteins. *Science* **304**(5679): 1971-1976.

Jones, R.N. 2005. McClintock's controlling elements: the full story. *Cytogenet Genome Res* **109**(1-3): 90-103.

Kadosh, D. and Struhl, K. 1997. Repression by Ume6 involves recruitment of a complex containing Sin3 corepressor and Rpd3 histone deacetylase to target promoters. *Cell* **89**(3): 365-371.

-. 1998. Histone deacetylase activity of Rpd3 is important for transcriptional repression in vivo. *Genes Dev* **12**(6): 797-805.

Kanno, T., Huettel, B., Mette, M.F., Aufsatz, W., Jaligot, E., Daxinger, L., Kreil, D.P., Matzke, M., and Matzke, A.J. 2005. Atypical RNA polymerase subunits required for RNA-directed DNA methylation. *Nat Genet* **37**(7): 761-765.

Kanoh, J., Sadaie, M., Urano, T., and Ishikawa, F. 2005. Telomere binding protein Taz1 establishes Swi6 heterochromatin independently of RNAi at telomeres. *Curr Biol* **15**(20): 1808-1819.

Kato, H., Goto, D.B., Martienssen, R.A., Urano, T., Furukawa, K., and Murakami, Y. 2005. RNA polymerase II is required for RNAi-dependent heterochromatin assembly. *Science* **309**(5733): 467-469.

Kelly, F.D. and Levin, H.L. 2005. The evolution of transposons in Schizosaccharomyces pombe. *Cytogenet Genome Res* **110**(1-4): 566-574.

Kim, D.H., Villeneuve, L.M., Morris, K.V., and Rossi, J.J. 2006. Argonaute-1 directs siRNA-mediated transcriptional gene silencing in human cells. *Nat Struct Mol Biol* **13**(9): 793-797.

Kinoshita, T., Miura, A., Choi, Y., Kinoshita, Y., Cao, X., Jacobsen, S.E., Fischer, R.L., and Kakutani, T. 2004. One-way control of FWA imprinting in Arabidopsis endosperm by DNA methylation. *Science* **303**(5657): 521-523.

Klenov, M.S., Lavrov, S.A., Stolyarenko, A.D., Ryazansky, S.S., Aravin, A.A., Tuschl, T., and Gvozdev, V.A. 2007. Repeat-associated siRNAs cause chromatin silencing of retrotransposons in the Drosophila melanogaster germline. *Nucleic Acids Res* **35**(16): 5430-5438.

Kloc, A., Zaratiegui, M., Nora, E., and Martienssen, R. 2008. RNA interference guides histone modification during the S phase of chromosomal replication. *Curr Biol* **18**(7): 490-495.

Klose, R.J., Yamane, K., Bae, Y., Zhang, D., Erdjument-Bromage, H., Tempst, P., Wong, J., and Zhang, Y. 2006. The transcriptional repressor JHDM3A demethylates trimethyl histone H3 lysine 9 and lysine 36. *Nature* **442**(7100): 312-316.

Kornberg, R.D. 1974. Chromatin structure: a repeating unit of histones and DNA. *Science* **184**(139): 868-871.

Kouzarides, T. 2007. Chromatin modifications and their function. *Cell* **128**(4): 693-705.

Kuo, M.H., Brownell, J.E., Sobel, R.E., Ranalli, T.A., Cook, R.G., Edmondson, D.G., Roth, S.Y., and Allis, C.D. 1996. Transcription-linked acetylation by Gcn5p of histones H3 and H4 at specific lysines. *Nature* **383**(6597): 269-272.

Kuo, M.H., Zhou, J., Jambeck, P., Churchill, M.E., and Allis, C.D. 1998. Histone acetyltransferase activity of yeast Gcn5p is required for the activation of target genes in vivo. *Genes Dev* **12**(5): 627-639.

Kuramochi-Miyagawa, S., Watanabe, T., Gotoh, K., Totoki, Y., Toyoda, A., Ikawa, M., Asada, N., Kojima, K., Yamaguchi, Y., Ijiri, T.W., Hata, K., Li, E., Matsuda, Y., Kimura, T., Okabe, M., Sakaki, Y., Sasaki, H., and Nakano, T. 2008. DNA methylation of retrotransposon genes is regulated by Piwi family members MILI and MIWI2 in murine fetal testes. *Genes Dev* **22**(7): 908-917.

LaCava, J., Houseley, J., Saveanu, C., Petfalski, E., Thompson, E., Jacquier, A., and Tollervey, D. 2005. RNA degradation by the exosome is promoted by a nuclear polyadenylation complex. *Cell* **121**(5): 713-724.

Lachner, M., O'Carroll, D., Rea, S., Mechtler, K., and Jenuwein, T. 2001. Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. *Nature* **410**(6824): 116-120.

Lan, F., Zaratiegui, M., Villen, J., Vaughn, M.W., Verdel, A., Huarte, M., Shi, Y., Gygi, S.P., Moazed, D., and Martienssen, R.A. 2007. S. pombe LSD1 homologs regulate heterochromatin propagation and euchromatic gene transcription. *Mol Cell* **26**(1): 89-101.

Laroche, T., Martin, S.G., Gotta, M., Gorham, H.C., Pryde, F.E., Louis, E.J., and Gasser, S.M. 1998. Mutation of yeast Ku genes disrupts the subnuclear organization of telomeres. *Curr Biol* **8**(11): 653-656.

Lee, M.G., Wynder, C., Cooch, N., and Shiekhattar, R. 2005. An essential role for CoREST in nucleosomal histone 3 lysine 4 demethylation. *Nature* **437**(7057): 432-435.

Lee, R.C. and Ambros, V. 2001. An extensive class of small RNAs in Caenorhabditis elegans. *Science* **294**(5543): 862-864.

Li, C.F., Pontes, O., El-Shami, M., Henderson, I.R., Bernatavichute, Y.V., Chan, S.W., Lagrange, T., Pikaard, C.S., and Jacobsen, S.E. 2006. An ARGONAUTE4-containing nuclear processing center colocalized with Cajal bodies in Arabidopsis thaliana. *Cell* **126**(1): 93-106.

Lindroth, A.M., Cao, X., Jackson, J.P., Zilberman, D., McCallum, C.M., Henikoff, S., and Jacobsen, S.E. 2001. Requirement of CHROMOMETHYLASE3 for maintenance of CpXpG methylation. *Science* **292**(5524): 2077-2080.

Lippman, Z., Gendrel, A.V., Black, M., Vaughn, M.W., Dedhia, N., McCombie, W.R., Lavine, K., Mittal, V., May, B., Kasschau, K.D., Carrington, J.C., Doerge, R.W., Colot, V., and Martienssen, R. 2004. Role of transposable elements in heterochromatin and epigenetic control. *Nature* **430**(6998): 471-476.

Lippman, Z. and Martienssen, R. 2004. The role of RNA interference in heterochromatic silencing. *Nature* **431**(7006): 364-370.

Lippman, Z., May, B., Yordan, C., Singer, T., and Martienssen, R. 2003. Distinct mechanisms determine transposon inheritance and methylation via small interfering RNA and histone modification. *PLoS Biol* **1**(3): E67.

Litt, M.D., Simpson, M., Recillas-Targa, F., Prioleau, M.N., and Felsenfeld, G. 2001. Transitions in histone acetylation reveal boundaries of three separately regulated neighboring loci. *Embo J* **20**(9): 2224-2235.

Liu, J., Carmell, M.A., Rivas, F.V., Marsden, C.G., Thomson, J.M., Song, J.J., Hammond, S.M., Joshua-Tor, L., and Hannon, G.J. 2004a. Argonaute2 is the catalytic engine of mammalian RNAi. *Science* **305**(5689): 1437-1441.

Liu, Y., Mochizuki, K., and Gorovsky, M.A. 2004b. Histone H3 lysine 9 methylation is required for DNA elimination in developing macronuclei in Tetrahymena. *Proc Natl Acad Sci U S A* **101**(6): 1679-1684.

Lorentz, A., Ostermann, K., Fleck, O., and Schmidt, H. 1994. Switching gene swi6, involved in repression of silent mating-type loci in fission yeast, encodes a homologue of chromatin-associated proteins from Drosophila and mammals. *Gene* **143**(1): 139-143.

Luger, K., Mèader, A.W., Richmond, R.K., Sargent, D.F., and Richmond, T.J. 1997. Crystal structure of the nucleosome core particle at 2.8 A resolution. *Nature* **389**(6648): 251-260.

Ma, J., Hwang, K.K., Worman, H.J., Courvalin, J.C., and Eissenberg, J.C. 2001. Expression and functional analysis of three isoforms of human heterochromatin-associated protein HP1 in Drosophila. *Chromosoma* **109**(8): 536-544.

Malagnac, F., Bartee, L., and Bender, J. 2002. An Arabidopsis SET domain protein required for maintenance but not establishment of DNA methylation. *Embo J* **21**(24): 6842-6852.

Malone, C.D., Anderson, A.M., Motl, J.A., Rexer, C.H., and Chalker, D.L. 2005. Germ line transcripts are processed by a Dicer-like protein that is essential for developmentally programmed genome rearrangements of Tetrahymena thermophila. *Mol Cell Biol* **25**(20): 9151-9164.

Mandell, J.G., Bahler, J., Volpe, T.A., Martienssen, R.A., and Cech, T.R. 2005. Global expression changes resulting from loss of telomeric DNA in fission yeast. *Genome Biol* **6**(1): R1.

Martienssen, R.A., Zaratiegui, M., and Goto, D.B. 2005. RNA interference and heterochromatin in the fission yeast Schizosaccharomyces pombe. *Trends Genet* **21**(8): 450-456.

Martinez, J., Patkaniowska, A., Urlaub, H., Luhrmann, R., and Tuschl, T. 2002. Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. *Cell* **110**(5): 563-574.

Masumoto, H., Nakano, M., and Ohzeki, J. 2004. The role of CENP-B and alpha-satellite DNA: de novo assembly and epigenetic maintenance of human centromeres. *Chromosome Res* **12**(6): 543-556.

Mathieu, O. and Bender, J. 2004. RNA-directed DNA methylation. *J Cell Sci* **117**(Pt 21): 4881-4888.

Matranga, C., Tomari, Y., Shin, C., Bartel, D.P., and Zamore, P.D. 2005. Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes. *Cell* **123**(4): 607-620.

Matzke, M., Aufsatz, W., Kanno, T., Daxinger, L., Papp, I., Mette, M.F., and Matzke, A.J. 2004. Genetic analysis of RNA-mediated transcriptional gene silencing. *Biochim Biophys Acta* **1677**(1-3): 129-141.

Matzke, M., Kanno, T., Huettel, B., Daxinger, L., and Matzke, A.J. 2007. Targets of RNA-directed DNA methylation. *Curr Opin Plant Biol* **10**(5): 512-519.

May, B.P., Lippman, Z.B., Fang, Y., Spector, D.L., and Martienssen, R.A. 2005. Differential regulation of strand-specific transcripts from Arabidopsis centromeric satellite repeats. *PLoS Genet* **1**(6): e79.

Mc, C.B. 1950. The origin and behavior of mutable loci in maize. *Proc Natl Acad Sci U S A* **36**(6): 344-355.

Meister, G. and Tuschl, T. 2004. Mechanisms of gene silencing by double-stranded RNA. *Nature* **431**(7006): 343-349.

Mello, C.C. and Conte, D., Jr. 2004. Revealing the world of RNA interference. *Nature* **431**(7006): 338-342.

Meneghini, M.D., Wu, M., and Madhani, H.D. 2003. Conserved histone variant H2A.Z protects euchromatin from the ectopic spread of silent heterochromatin. *Cell* **112**(5): 725-736.

Mette, M.F., van der Winden, J., Matzke, M.A., and Matzke, A.J. 1999. Production of aberrant promoter transcripts contributes to methylation and silencing of unlinked homologous promoters in trans. *EMBO J* **18**(1): 241-248.

Metzger, E., Wissmann, M., Yin, N., Muller, J.M., Schneider, R., Peters, A.H., Gunther, T., Buettner, R., and Schule, R. 2005. LSD1 demethylates repressive histone marks to promote androgen-receptor-dependent transcription. *Nature* **437**(7057): 436-439.

Milne, T.A., Briggs, S.D., Brock, H.W., Martin, M.E., Gibbs, D., Allis, C.D., and Hess, J.L. 2002. MLL targets SET domain methyltransferase activity to Hox gene promoters. *Mol Cell* **10**(5): 1107-1117.

Mirkovitch, J., Gasser, S.M., and Laemmli, U.K. 1987. Relation of chromosome structure and gene expression. *Philos Trans R Soc Lond B Biol Sci* **317**(1187): 563-574.

Mizzen, C.A., Yang, X.J., Kokubo, T., Brownell, J.E., Bannister, A.J., Owen-Hughes, T., Workman, J., Wang, L., Berger, S.L., Kouzarides, T., Nakatani, Y., and Allis, C.D. 1996. The TAF(II)250 subunit of TFIID has histone acetyltransferase activity. *Cell* **87**(7): 1261-1270.

Mochizuki, K., Fine, N.A., Fujisawa, T., and Gorovsky, M.A. 2002. Analysis of a piwi-related gene implicates small RNAs in genome rearrangement in tetrahymena. *Cell* **110**(6): 689-699.

Morris, K.V., Chan, S.W., Jacobsen, S.E., and Looney, D.J. 2004. Small interfering RNA-induced transcriptional gene silencing in human cells. *Science* **305**(5688): 1289-1292.

Motamedi, M.R., Verdel, A., Colmenares, S.U., Gerber, S.A., Gygi, S.P., and Moazed, D. 2004. Two RNAi complexes, RITS and RDRC, physically interact and localize to noncoding centromeric RNAs. *Cell* **119**(6): 789-802.

Muller, H.J. 1930. Types of viable variations induced by X-rays in Drosophila. *J Genetics* **22**: 299-334

.

Nakagawa, H., Lee, J.K., Hurwitz, J., Allshire, R.C., Nakayama, J., Grewal, S.I., Tanaka, K., and Murakami, Y. 2002. Fission yeast CENP-B homologs nucleate centromeric heterochromatin by promoting heterochromatin-specific histone tail modifications. *Genes Dev* **16**(14): 1766-1778.

Nakayama, J., Rice, J.C., Strahl, B.D., Allis, C.D., and Grewal, S.I. 2001. Role of histone H3 lysine 9 methylation in epigenetic control of heterochromatin assembly. *Science* **292**(5514): 110-113.

Nakayama, J., Xiao, G., Noma, K., Malikzay, A., Bjerling, P., Ekwall, K., Kobayashi, R., and Grewal, S.I. 2003. Alp13, an MRG family protein, is a component of fission yeast Clr6 histone deacetylase required for genomic integrity. *EMBO J* **22**(11): 2776-2787.

Napoli, C., Lemieux, C., and Jorgensen, R. 1990. Introduction of a Chimeric Chalcone Synthase Gene into Petunia Results in Reversible Co-Suppression of Homologous Genes in trans. *Plant Cell* **2**(4): 279-289.

Ng, H.H., Robert, F., Young, R.A., and Struhl, K. 2003. Targeted recruitment of Set1 histone methylase by elongating Pol II provides a localized mark and memory of recent transcriptional activity. *Mol Cell* **11**(3): 709-719.

Noma, K., Allis, C.D., and Grewal, S.I. 2001. Transitions in distinct histone H3 methylation patterns at the heterochromatin domain boundaries. *Science* **293**(5532): 1150-1155.

Noma, K. and Grewal, S.I. 2002. Histone H3 lysine 4 methylation is mediated by Set1 and promotes maintenance of active chromatin states in fission yeast. *Proc Natl Acad Sci U S A* **99**: 16438-16445.

Noma, K., Sugiyama, T., Cam, H., Verdel, A., Zofall, M., Jia, S., Moazed, D., and Grewal, S.I. 2004. RITS acts in cis to promote RNA interference-mediated transcriptional and post-transcriptional silencing. *Nat Genet* **36**(11): 1174-1180.

Nonaka, N., Kitajima, T., Yokobayashi, S., Xiao, G., Yamamoto, M., Grewal, S.I., and Watanabe, Y. 2002. Recruitment of cohesin to heterochromatic regions by Swi6/HP1 in fission yeast. *Nat Cell Biol* **4**(1): 89-93.

Ogryzko, V.V., Schiltz, R.L., Russanova, V., Howard, B.H., and Nakatani, Y. 1996. The transcriptional coactivators p300 and CBP are histone acetyltransferases. *Cell* **87**(5): 953-959.

Okada, T., Ohzeki, J., Nakano, M., Yoda, K., Brinkley, W.R., Larionov, V., and Masumoto, H. 2007. CENP-B controls centromere formation depending on the chromatin context. *Cell* **131**(7): 1287-1300.

Onodera, Y., Haag, J.R., Ream, T., Nunes, P.C., Pontes, O., and Pikaard, C.S. 2005. Plant nuclear RNA polymerase IV mediates siRNA and DNA methylation-dependent heterochromatin formation. *Cell* **120**(5): 613-622.

Opel, M., Lando, D., Bonilla, C., Trewick, S.C., Boukaba, A., Walfridsson, J., Cauwood, J., Werler, P.J., Carr, A.M., Kouzarides, T., Murzina, N.V., Allshire, R.C., Ekwall, K., and Laue, E.D. 2007. Genome-wide studies of histone demethylation catalysed by the fission yeast homologues of mammalian LSD1. *PLoS ONE* **2**(4): e386.

Owen, D.J., Ornaghi, P., Yang, J.C., Lowe, N., Evans, P.R., Ballario, P., Neuhaus, D., Filetici, P., and Travers, A.A. 2000. The structural basis for the recognition of acetylated histone H4 by the bromodomain of histone acetyltransferase gcn5p. *Embo J* **19**(22): 6141-6149.

Pak, J. and Fire, A. 2007. Distinct populations of primary and secondary effectors during RNAi in C. elegans. *Science* **315**(5809): 241-244.

Pal-Bhadra, M., Leibovitch, B.A., Gandhi, S.G., Rao, M., Bhadra, U., Birchler, J.A., and Elgin, S.C. 2004. Heterochromatic silencing and HP1 localization in Drosophila are dependent on the RNAi machinery. *Science* **303**(5658): 669-672.

Palmer, D.K., O'Day, K., Wener, M.H., Andrews, B.S., and Margolis, R.L. 1987. A 17-kD centromere protein (CENP-A) copurifies with nucleosome core particles and with histones. *J Cell Biol* **104**(4): 805-815.

Partridge, J.F., Scott, K.S., Bannister, A.J., Kouzarides, T., and Allshire, R.C. 2002. cis-acting DNA from fission yeast centromeres mediates histone H3 methylation and recruitment of silencing factors and cohesin to an ectopic site. *Curr Biol* **12**(19): 1652-1660.

Peters, A.H., Kubicek, S., Mechtler, K., O'Sullivan, R.J., Derijck, A.A., Perez-Burgos, L., Kohlmaier, A., Opravil, S., Tachibana, M., Shinkai, Y., Martens, J.H., and Jenuwein, T.

2003. Partitioning and plasticity of repressive histone methylation states in mammalian chromatin. *Mol Cell* **12**(6): 1577-1589.

Peters, A.H., O'Carroll, D., Scherthan, H., Mechtler, K., Sauer, S., Schèofer, C., Weipoltshammer, K., Pagani, M., Lachner, M., Kohlmaier, A., Opravil, S., Doyle, M., Sibilia, M., and Jenuwein, T. 2001. Loss of the Suv39h histone methyltransferases impairs mammalian heterochromatin and genome stability. *Cell* **107**(3): 323-337.

Pidoux, A.L. and Allshire, R.C. 2004. Kinetochore and heterochromatin domains of the fission yeast centromere. *Chromosome Res* **12**(6): 521-534.

Pokholok, D.K., Harbison, C.T., Levine, S., Cole, M., Hannett, N.M., Lee, T.I., Bell, G.W., Walker, K., Rolfe, P.A., Herbolsheimer, E., Zeitlinger, J., Lewitter, F., Gifford, D.K., and Young, R.A. 2005. Genome-wide map of nucleosome acetylation and methylation in yeast. *Cell* **122**(4): 517-527.

Pontier, D., Yahubyan, G., Vega, D., Bulski, A., Saez-Vasquez, J., Hakimi, M.A., Lerbs-Mache, S., Colot, V., and Lagrange, T. 2005. Reinforcement of silencing at transposons and highly repeated sequences requires the concerted action of two distinct RNA polymerases IV in Arabidopsis. *Genes Dev* **19**(17): 2030-2040.

Qi, Y., He, X., Wang, X.J., Kohany, O., Jurka, J., and Hannon, G.J. 2006. Distinct catalytic and non-catalytic roles of ARGONAUTE4 in RNA-directed DNA methylation. *Nature* **443**(7114): 1008-1012.

Rand, T.A., Petersen, S., Du, F., and Wang, X. 2005. Argonaute2 cleaves the anti-guide strand of siRNA during RISC activation. *Cell* **123**(4): 621-629.

Rangasamy, D., Berven, L., Ridgway, P., and Tremethick, D.J. 2003. Pericentric heterochromatin becomes enriched with H2A.Z during early mammalian development. *EMBO J* **22**(7): 1599-1607.

Rangasamy, D., Greaves, I., and Tremethick, D.J. 2004. RNA interference demonstrates a novel role for H2A.Z in chromosome segregation. *Nat Struct Mol Biol* **11**(7): 650-655.

Ratcliff, F., Harrison, B.D., and Baulcombe, D.C. 1997. A similarity between viral defense and gene silencing in plants. *Science* **276**(5318): 1558-1560.

Rea, S., Eisenhaber, F., O'Carroll, D., Strahl, B.D., Sun, Z.W., Schmid, M., Opravil, S., Mechtler, K., Ponting, C.P., Allis, C.D., and Jenuwein, T. 2000. Regulation of chromatin structure by site-specific histone H3 methyltransferases. *Nature* **406**(6796): 593-599.

Reinhart, B.J. and Bartel, D.P. 2002. Small RNAs correspond to centromere heterochromatic repeats. *Science* **297**(5588): 1831.

Rice, J.C., Briggs, S.D., Ueberheide, B., Barber, C.M., Shabanowitz, J., Hunt, D.F., Shinkai, Y., and Allis, C.D. 2003. Histone methyltransferases direct different degrees of methylation to define distinct chromatin domains. *Mol Cell* **12**(6): 1591-1598.

Richards, E.J. and Elgin, S.C. 2002. Epigenetic codes for heterochromatin formation and silencing: rounding up the usual suspects. *Cell* **108**(4): 489-500.

Richmond, T.J. and Davey, C.A. 2003. The structure of DNA in the nucleosome core. *Nature* **423**(6936): 145-150.

Richmond, T.J., Finch, J.T., Rushton, B., Rhodes, D., and Klug, A. 1984. Structure of the nucleosome core particle at 7 A resolution. *Nature* **311**(5986): 532-537.

Romano, N. and Macino, G. 1992. Quelling: transient inactivation of gene expression in Neurospora crassa by transformation with homologous sequences. *Mol Microbiol* **6**(22): 3343-3353.

Rundlett, S.E., Carmen, A.A., Kobayashi, R., Bavykin, S., Turner, B.M., and Grunstein, M. 1996. HDA1 and RPD3 are members of distinct yeast histone deacetylase complexes that regulate silencing and transcription. *Proc Natl Acad Sci U S A* **93**(25): 14503-14508.

Rundlett, S.E., Carmen, A.A., Suka, N., Turner, B.M., and Grunstein, M. 1998. Transcriptional repression by UME6 involves deacetylation of lysine 5 of histone H4 by RPD3. *Nature* **392**(6678): 831-835.

Sadaie, M., Iida, T., Urano, T., and Nakayama, J. 2004. A chromodomain protein, Chp1, is required for the establishment of heterochromatin in fission yeast. *Embo J* **23**(19): 3825-3835.

Santos-Rosa, H., Schneider, R., Bannister, A.J., Sherriff, J., Bernstein, B.E., Emre, N.C., Schreiber, S.L., Mellor, J., and Kouzarides, T. 2002. Active genes are tri-methylated at K4 of histone H3. *Nature* **419**(6905): 407-411.

Schiebel, W., Haas, B., Marinkovic, S., Klanner, A., and Sanger, H.L. 1993. RNA-directed RNA polymerase from tomato leaves. I. Purification and physical properties. *J Biol Chem* **268**(16): 11851-11857.

Shi, Y., Lan, F., Matson, C., Mulligan, P., Whetstine, J.R., Cole, P.A., and Casero, R.A. 2004. Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. *Cell* **119**(7): 941-953.

Shi, Y.J., Matson, C., Lan, F., Iwase, S., Baba, T., and Shi, Y. 2005. Regulation of LSD1 histone demethylase activity by its associated factors. *Mol Cell* **19**(6): 857-864.

Sijen, T., Fleenor, J., Simmer, F., Thijssen, K.L., Parrish, S., Timmons, L., Plasterk, R.H., and Fire, A. 2001. On the role of RNA amplification in dsRNA-triggered gene silencing. *Cell* **107**(4): 465-476.

Sijen, T., Steiner, F.A., Thijssen, K.L., and Plasterk, R.H. 2007. Secondary siRNAs result from unprimed RNA synthesis and form a distinct class. *Science* **315**(5809): 244-247.

Silverman, N., Agapite, J., and Guarente, L. 1994. Yeast ADA2 protein binds to the VP16 protein activation domain and activates transcription. *Proc Natl Acad Sci U S A* **91**(24): 11665-11668.

Slotkin, R.K. and Martienssen, R. 2007. Transposable elements and the epigenetic regulation of the genome. *Nat Rev Genet* **8**(4): 272-285.

Smardon, A., Spoerke, J.M., Stacey, S.C., Klein, M.E., Mackin, N., and Maine, E.M. 2000. EGO-1 is related to RNA-directed RNA polymerase and functions in germ-line development and RNA interference in C. elegans. *Curr Biol* **10**(4): 169-178.

Smith, L.M., Pontes, O., Searle, I., Yelina, N., Yousafzai, F.K., Herr, A.J., Pikaard, C.S., and Baulcombe, D.C. 2007. An SNF2 protein associated with nuclear RNA silencing and the spread of a silencing signal between cells in Arabidopsis. *Plant Cell* **19**(5): 1507-1521.

Song, J.J., Smith, S.K., Hannon, G.J., and Joshua-Tor, L. 2004. Crystal structure of Argonaute and its implications for RISC slicer activity. *Science* **305**(5689): 1434-1437.

Sontheimer, E.J. 2005. Assembly and function of RNA silencing complexes. *Nat Rev Mol Cell Biol* **6**(2): 127-138.

Spencer, T.E., Jenster, G., Burcin, M.M., Allis, C.D., Zhou, J., Mizzen, C.A., McKenna, N.J., Onate, S.A., Tsai, S.Y., Tsai, M.J., and O'Malley, B.W. 1997. Steroid receptor coactivator-1 is a histone acetyltransferase. *Nature* **389**(6647): 194-198.

Stedman, E. 1950. Cell specificity of histones. *Nature* **166**(4227): 780-781.

Stoica, C., Carmichael, J.B., Parker, H., Pare, J., and Hobman, T.C. 2006. Interactions between the RNA interference effector protein Ago1 and 14-3-3 proteins: consequences for cell cycle progression. *J Biol Chem* **281**(49): 37646-37651.

Sugiyama, T., Cam, H., Verdel, A., Moazed, D., and Grewal, S.I. 2005. RNA-dependent RNA polymerase is an essential component of a self-enforcing loop coupling heterochromatin assembly to siRNA production. *Proc Natl Acad Sci U S A* **102**(1): 152-157.

Sugiyama, T., Cam, H.P., Sugiyama, R., Noma, K., Zofall, M., Kobayashi, R., and Grewal, S.I. 2007. SHREC, an effector complex for heterochromatic transcriptional silencing. *Cell* **128**(3): 491-504.

Tabara, H., Sarkissian, M., Kelly, W.G., Fleenor, J., Grishok, A., Timmons, L., Fire, A., and Mello, C.C. 1999. The rde-1 gene, RNA interference, and transposon silencing in C. elegans. *Cell* **99**(2): 123-132.

Taunton, J., Hassig, C.A., and Schreiber, S.L. 1996. A mammalian histone deacetylase related to the yeast transcriptional regulator Rpd3p. *Science* **272**(5260): 408-411.

Taverna, S.D., Coyne, R.S., and Allis, C.D. 2002. Methylation of histone h3 at lysine 9 targets programmed DNA elimination in tetrahymena. *Cell* **110**(6): 701-711.

Thoma, F., Koller, T., and Klug, A. 1979. Involvement of histone H1 in the organization of the nucleosome and of the salt-dependent superstructures of chromatin. *J Cell Biol* **83**(2 Pt 1): 403-427.

Ting, A.H., Schuebel, K.E., Herman, J.G., and Baylin, S.B. 2005. Short double-stranded RNA induces transcriptional gene silencing in human cancer cells in the absence of DNA methylation. *Nat Genet* **37**(8): 906-910.

Tsukada, Y., Fang, J., Erdjument-Bromage, H., Warren, M.E., Borchers, C.H., Tempst, P., and Zhang, Y. 2006. Histone demethylation by a family of JmjC domain-containing proteins. *Nature* **439**(7078): 811-816.

Utley, R.T., Ikeda, K., Grant, P.A., Cãotâe, J., Steger, D.J., Eberharter, A., John, S., and Workman, J.L. 1998. Transcriptional activators direct histone acetyltransferase complexes to nucleosomes. *Nature* **394**(6692): 498-502.

Vaquero, A., Loyola, A., and Reinberg, D. 2003. The constantly changing face of chromatin. *Sci Aging Knowledge Environ* **2003**(14): RE4.

Verdel, A., Jia, S., Gerber, S., Sugiyama, T., Gygi, S., Grewal, S.I., and Moazed, D. 2004. RNAi-mediated targeting of heterochromatin by the RITS complex. *Science* **303**(5658): 672-676.

Verdel, A. and Moazed, D. 2005. Labeling and characterization of small RNAs associated with the RNA interference effector complex RITS. *Methods Enzymol* **392**: 297-307.

Volpe, T., Schramke, V., Hamilton, G.L., White, S.A., Teng, G., Martienssen, R.A., and Allshire, R.C. 2003. RNA interference is required for normal centromere function in fission yeast. *Chromosome Res* **11**(2): 137-146.

Volpe, T.A., Kidner, C., Hall, I.M., Teng, G., Grewal, S.I., and Martienssen, R.A. 2002. Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. *Science* **297**(5588): 1833-1837.

Waddington, C.H. 1942a. Canalization of development and the inheritance of acquired characters. *Nature* **150**(3811): 563-565.

-. 1942b. The Epigenotype. *Endeavor* **1**: 18-20.

Wang, H., Cao, R., Xia, L., Erdjument-Bromage, H., Borchers, C., Tempst, P., and Zhang, Y. 2001. Purification and functional characterization of a histone H3-lysine 4-specific methyltransferase. *Mol Cell* **8**(6): 1207-1217.

Wang, S.W., Stevenson, A.L., Kearsey, S.E., Watt, S., and Bahler, J. 2008. Global role for polyadenylation-assisted nuclear RNA degradation in posttranscriptional gene silencing. *Mol Cell Biol* **28**(2): 656-665.

Wassenegger, M., Heimes, S., Riedel, L., and Sanger, H.L. 1994. RNA-directed de novo methylation of genomic sequences in plants. *Cell* **76**(3): 567-576.

Whetstine, J.R., Nottke, A., Lan, F., Huarte, M., Smolikov, S., Chen, Z., Spooner, E., Li, E., Zhang, G., Colaiacovo, M., and Shi, Y. 2006. Reversal of histone lysine trimethylation by the JMJD2 family of histone demethylases. *Cell* **125**(3): 467-481.

Wiren, M., Silverstein, R.A., Sinha, I., Walfridsson, J., Lee, H.M., Laurenson, P., Pillus, L., Robyr, D., Grunstein, M., and Ekwall, K. 2005. Genomewide analysis of nucleosome density histone acetylation and HDAC function in fission yeast. *EMBO J* **24**(16): 2906-2918.

Wood, V. Gwilliam, R. Rajandream, M.A. Lyne, M. Lyne, R. Stewart, A. Sgouros, J. Peat, N. Hayles, J. Baker, S. Basham, D. Bowman, S. Brooks, K. Brown, D. Brown, S. Chillingworth, T. Churcher, C. Collins, M. Connor, R. Cronin, A. Davis, P. Feltwell, T. Fraser, A. Gentles, S. Goble, A. Hamlin, N. Harris, D. Hidalgo, J. Hodgson, G. Holroyd, S. Hornsby, T. Howarth, S. Huckle, E.J. Hunt, S. Jagels, K. James, K. Jones, L. Jones, M. Leather, S. McDonald, S. McLean, J. Mooney, P. Moule, S. Mungall, K. Murphy, L. Niblett, D. Odell, C. Oliver, K. O'Neil, S. Pearson, D. Quail, M.A. Rabbinowitsch, E. Rutherford, K. Rutter, S. Saunders, D. Seeger, K. Sharp, S. Skelton, J. Simmonds, M.

Squares, R. Squares, S. Stevens, K. Taylor, K. Taylor, R.G. Tivey, A. Walsh, S. Warren, T. Whitehead, S. Woodward, J. Volckaert, G. Aert, R. Robben, J. Grymonprez, B. Weltjens, I. Vanstreels, E. Rieger, M. Schafer, M. Muller-Auer, S. Gabel, C. Fuchs, M. Dusterhoft, A. Fritzc, C. Holzer, E. Moestl, D. Hilbert, H. Borzym, K. Langer, I. Beck, A. Lehrach, H. Reinhardt, R. Pohl, T.M. Eger, P. Zimmermann, W. Wedler, H. Wambutt, R. Purnelle, B. Goffeau, A. Cadieu, E. Dreano, S. Gloux, S. Lelaure, V. Mottier, S. Galibert, F. Aves, S.J. Xiang, Z. Hunt, C. Moore, K. Hurst, S.M. Lucas, M. Rochet, M. Gaillardin, C. Tallada, V.A. Garzon, A. Thode, G. Daga, R.R. Cruzado, L. Jimenez, J. Sanchez, M. del Rey, F. Benito, J. Dominguez, A. Revuelta, J.L. Moreno, S. Armstrong, J. Forsburg, S.L. Cerutti, L. Lowe, T. McCombie, W.R. Paulsen, I. Potashkin, J. Shpakovski, G.V. Ussery, D. Barrell, B.G. and Nurse, P. 2002. The genome sequence of Schizosaccharomyces pombe. *Nature* **415**(6874): 871-880.

Xie, Z., Johansen, L.K., Gustafson, A.M., Kasschau, K.D., Lellis, A.D., Zilberman, D., Jacobsen, S.E., and Carrington, J.C. 2004. Genetic and functional diversification of small RNA pathways in plants. *PLoS Biol* **2**(5): E104.

Yamada, T., Fischle, W., Sugiyama, T., Allis, C.D., and Grewal, S.I. 2005. The nucleation and maintenance of heterochromatin by a histone deacetylase in fission yeast. *Mol Cell* **20**(2): 173-185.

Yang, W.M., Inouye, C., Zeng, Y., Bearss, D., and Seto, E. 1996. Transcriptional repression by YY1 is mediated by interaction with a mammalian homolog of the yeast global regulator RPD3. *Proc Natl Acad Sci U S A* **93**(23): 12845-12850.

Zamore, P.D., Tuschl, T., Sharp, P.A., and Bartel, D.P. 2000. RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* **101**(1): 25-33.

Zhang, K., Mosch, K., Fischle, W., and Grewal, S.I. 2008. Roles of the Clr4 methyltransferase complex in nucleation, spreading and maintenance of heterochromatin. *Nat Struct Mol Biol* **15**(4): 381-388.

Zhimulev, I.F., Belyaeva, E.S., Bgatov, A.V., Baricheva, E.M., and Vlassova, I.E. 1988. Cytogenetic and molecular aspects of position effect variegation in Drosophila melanogaster. II. Peculiarities of morphology and genetic activity of the 2B region in the T(1;2)dorvar7 chromosome in males. *Chromosoma* **96**(3): 255-261.

Zilberman, D., Cao, X., and Jacobsen, S.E. 2003. ARGONAUTE4 control of locus-specific siRNA accumulation and DNA and histone methylation. *Science* **299**(5607): 716-719.

APPENDIX I

Strains Used in Each Study

Chapter Two

Strain	Relevant Genotype	Source
CK833	*cen3dg(3)::ura4^{+} ade6Δ ura4Δ h^{+}*	This Study
CK190	*cen3dg(3)::ura4^{+} ago1Δ::kanMX6 ade6Δ ura4Δ h^{-}*	This Study
CK889	*cen3dg::ura4^{+} dcr1Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK890	*cen3dg::ura4^{+} rdp1Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK845	*cen3dg::ura4^{+} chp1Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK843	*cen3dg::ura4^{+} tas3Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK849	*cen3dg::ura4^{+} rik1Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK847	*cen3dg::ura4^{+} clr4Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK841	*cen3dg::ura4^{+} swi6Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK836	*cen3dg(1)::ura4$^{+}_{Inv}$ ade6Δ ura4Δ h^{-}*	This Study
CK423	*cen3dg(1)::ura4$^{+}_{Inv}$ ago1Δ::kanMX6 ade6Δ ura4Δ h^{-}*	This Study
CK337	*cen3dg::ura4$^{+}_{Inv}$ dcr1Δ::kanMX6 ade6Δ ura4Δ h^{+}*	This Study
CK883	*cen3dg::ura4$^{+}_{Inv}$ rdp1Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK349	*cen3dg::ura4$^{+}_{Inv}$ chp1Δ::kanMX6 ade6Δ ura4Δ h^{-}*	This Study
CK507	*cen3dg::ura4$^{+}_{Inv}$ tas3Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK527	*cen3dg::ura4$^{+}_{Inv}$ rik1Δ::kanMX6 ade6Δ ura4Δ h^{+}*	This Study
CK388	*cen3dg::ura4$^{+}_{Inv}$ clr4Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK509	*cen3dg::ura4$^{+}_{Inv}$ swi6Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK326	*cen3dh(4)::ura4^{+} ade6Δ ura4Δ h^{-}*	This Study
CK302	*cen3dh(4)::ura4^{+} ago1Δ::kanMX6 ade6Δ ura4Δ h^{-}*	This Study
CK715	*cen3dh::ura4^{+} dcr1Δ::kanMX6 ade6Δ ura4Δ h^{-}*	This Study
CK894	*cen3dh::ura4^{+} rdp1Δ::kanMX6 ade6Δ ura4Δ h^{+}*	This Study
CK885	*cen3dh::ura4^{+} chp1Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK1037	*cen3dh::ura4^{+} tas3Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK722	*cen3dh::ura4^{+} rik1Δ::kanMX6 ade6Δ ura4Δ*	This Study

CK887	*cen3dh::ura4*$^{+}$ *clr4Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK892	*cen3dh::ura4*$^{+}$ *swi6Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK918	*cen3dh(4)::ura4*$^{+}_{Inv}$ *ade6Δ ura4Δ h*$^{-}$	This Study
CK480	*cen3dh(3)::ura4*$^{+}_{Inv}$ *ago1Δ::kanMX6 ade6Δ ura4Δ h*$^{-}$	This Study
CK714	*cen3dh::ura4*$^{+}_{Inv}$ *dcr1Δ::kanMX6 ade6Δ ura4Δ h*$^{-}$	This Study
CK973	*cen3dh::ura4*$^{+}_{Inv}$ *rdp1Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK976	*cen3dh::ura4*$^{+}_{Inv}$ *chp1Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK1033	*cen3dh::ura4*$^{+}_{Inv}$ *tas3Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK975	*cen3dh::ura4*$^{+}_{Inv}$ *rik1Δ::kanMX6 ade6Δ ura4Δ h*$^{-}$	This Study
CK1070	*cen3dh::ura4*$^{+}_{Inv}$ *clr4Δ::kanMX6 ade6Δ ura4Δ h*$^{-}$	This Study
CK1029	*cen3dh::ura4*$^{+}_{Inv}$ *swi6Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK900	*cen1dg::ura4*$^{+}_{Inv}$ *ade6Δ ura4Δ h*$^{+}$	This Study
CK859	*cen1dg::ura4*$^{+}_{Inv}$ *ago1Δ::kanMX6 ade6Δ ura4Δ h*$^{-}$	This Study
CK934	*cen1dg::ura4*$^{+}_{Inv}$ *dcr1Δ::kanMX6 ade6Δ ura4Δ h*$^{+}$	This Study
CK932	*cen1dg::ura4*$^{+}_{Inv}$ *rdp1Δ::kanMX6 ade6Δ ura4Δ h*$^{-}$	This Study
CK924	*cen1dg::ura4*$^{+}_{Inv}$ *chp1Δ::kanMX6 ade6Δ ura4Δ*$^{-}$	This Study
CK928	*cen1dg::ura4*$^{+}_{Inv}$ *tas3Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK953	*cen1dg::ura4*$^{+}_{Inv}$ *rik1Δ::kanMX6 ade6Δ ura4Δ h*$^{-}$	This Study
CK926	*cen1dg::ura4*$^{+}_{Inv}$ *clr4Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK930	*cen1dg::ura4*$^{+}_{Inv}$ *swi6Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK896	*cen1dh::ura4*$^{+}$ *ade6Δ ura4Δ h*$^{-}$	This Study
CK855	*cen1dh::ura4*$^{+}$ *ago1Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK948	*cen1dh::ura4*$^{+}$ *dcr1Δ::kanMX6 ade6Δ ura4Δ h*$^{+}$	This Study
CK946	*cen1dh::ura4*$^{+}$ *rdp1Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK955	*cen1dh::ura4*$^{+}$ *chp1Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK1031	*cen1dh::ura4*$^{+}$ *tas3Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK1069	*cen1dh::ura4*$^{+}$ *rik1Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK949	*cen1dh::ura4*$^{+}$ *clr4Δ::kanMX6 ade6Δ ura4Δ h*$^{-}$	This Study

CK951	*cen1dh::ura4*$^{+}$ *swi6Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK936	*cen1dh::ura4*$^{+}_{Inv}$ *ade6Δ ura4Δ h*$^{+}$	This Study
CK921	*cen1dh::ura4*$^{+}_{Inv}$ *ago1Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK958	*cen1dh::ura4*$^{+}_{Inv}$ *dcr1Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK961	*cen1dh::ura4*$^{+}_{Inv}$ *rdp1Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK965	*cen1dh::ura4*$^{+}_{Inv}$ *chp1Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK960	*cen1dh::ura4*$^{+}_{Inv}$ *tas3Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK1072	*cen1dh::ura4*$^{+}_{Inv}$ *rik1Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK967	*cen1dh::ura4*$^{+}_{Inv}$ *clr4Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK963	*cen1dh::ura4*$^{+}_{Inv}$ *swi6Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK1195	*cen3dg::ura4*$^{+}$ *rrp6Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK1107	*cen3dg::ura4*$^{+}$ *cid14Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK1191	*cen3dg::ura4*$^{+}_{Inv}$ *rrp6Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK1201	*cen3dg::ura4*$^{+}_{Inv}$ *cid14Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK1196	*cen3dh::ura4*$^{+}$ *rrp6Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK1102	*cen3dh::ura4*$^{+}$ *cid14Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK1177	*cen3dh::ura4*$^{+}_{Inv}$ *rrp6Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK1118	*cen3dh::ura4*$^{+}_{Inv}$ *cid14Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK1175	*cen3dh::ura4*$^{+}_{Inv}$ *dcr1Δ::kanMX6 rrp6Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK1173	*cen3dh::ura4*$^{+}_{Inv}$ *dcr1Δ::kanMX6 cid14Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK1176	*cen3dh::ura4*$^{+}_{Inv}$ *clr4Δ::kanMX6 rrp6Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK1174	*cen3dh::ura4*$^{+}_{Inv}$ *clr4Δ::kanMX6 cid14Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK1204	*cen3dg(1)::ura4*$^{+}$ *ade6Δ ura4Δ*	This Study
CK401	*cen3dg(1)::ura4*$^{+}$ *ago1Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK1220	*cen3dg(2)::ura4*$^{+}$ *ade6Δ ura4Δ*	This Study
CK1216	*cen3dg(2)::ura4*$^{+}$ *ago1Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK1203	*cen3dg(3)::ura4*$^{+}$ *ade6Δ ura4Δ*	This Study

CK1217	*cen3dg(3)::ura4^{+} ago1Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK396	*cen3dg(4)::ura4^{+} ade6Δ ura4Δ*	This Study
CK1197	*cen3dg(4)::ura4^{+} ago1Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK1312	*cen3dg(2)::ura4$^{+}_{Inv}$ ade6Δ ura4Δ*	This Study
CK1311	*cen3dg(2)::ura4$^{+}_{Inv}$ ago1Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK1011	*cen3dg(3)::ura4$^{+}_{Inv}$ ade6Δ ura4Δ*	This Study
CK436	*cen3dg(3)::ura4$^{+}_{Inv}$ ago1Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK1199	*cen3dg(4)::ura4$^{+}_{Inv}$ ade6Δ ura4Δ h^{-}*	This Study
CK442	*cen3dg(4)::ura4$^{+}_{Inv}$ ago1Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK1200	*cen3dh(2)::ura4^{+} ade6Δ ura4Δ*	This Study
CK1212	*cen3dh(2)::ura4^{+} ago1Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK1313	*cen3dh(3)::ura4^{+} ade6Δ ura4Δ*	This Study
CK1314	*cen3dh(3)::ura4^{+} ago1Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK1206	*cen3dh(1)::ura4$^{+}_{Inv}$ ade6Δ ura4Δ*	This Study
CK1207	*cen3dh(1)::ura4$^{+}_{Inv}$ ago1Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK1255	*cen3dh(2)::ura4$^{+}_{Inv}$ ade6Δ ura4Δ*	This Study
CK1210	*cen3dh(2)::ura4$^{+}_{Inv}$ ago1Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK1205	*cen3dh(3)::ura4$^{+}_{Inv}$ ade6Δ ura4Δ*	This Study
CK875	*cen3dh(4)::ura4$^{+}_{Inv}$ ago1Δ::kanMX6 ade6Δ ura4Δ*	This Study

Chapter Three

Strain	Relevant Genotype	Source
CK326	*cen3dh::ura4^{+} ade6Δ ura4Δ h^{-}*	This Study
CK715	*cen3dh::ura4^{+} dcr1Δ::kanMX6 ade6Δ ura4Δ h^{-}*	This Study
CK918	*cen3dh::ura4$^{+}_{inv}$ ura4Δ h-*	This Study
CK714	*cen3dh::ura4$^{+}_{inv}$ dcr1Δ::kanMX6 ade6Δ ura4Δ h^{-}*	This Study
CK327	*cen3dh::ura4^{+} ade6Δ h^{-}*	This Study
CK1046	*cen3dh::ura4$^{+}_{inv}$*	This Study
CK1101	*dh1RindHNEΔ::ura4^{+} dh1::kanMX6 cen3dh::ura4^{+} ura4Δ h^{-}*	This Study
CK1097	*dh1RindHNEΔ::ura4^{+} dh1::kanMX6 cen3dh::ura4$^{+}_{inv}$ ura4Δ*	This Study
RL699	*dh1RindHNEΔ::ura4^{+} dh1::kanMX6 ura4Δ h^{+}*	This Study
CK1186	*dh1RindHNEΔ::ura4^{+} dh1::kanMX6 cen3dh::ura4^{+} dcr1Δ::kanMX6 ura4Δ h-*	This Study
CK1265	*dh1RindHNEΔ::ura4^{+} dh1::kanMX6 cen3dh::ura4^{+} ago1Δ::kanMX6 ura4Δ*	This Study
CK1188	*dh1RindHNEΔ::ura4^{+} dh1::kanMX6 cen3dh::ura4^{+} rdp1Δ::kanMX6 ura4Δ h+*	This Study
CK1263	*dh1RindHNEΔ::ura4^{+} dh1::kanMX6 cen3dh::ura4^{+} chp1Δ::kanMX6 ura4Δ*	This Study
CK1266	*dh1RindHNEΔ::ura4^{+} dh1::kanMX6 cen3dh::ura4^{+} swi6Δ::kanMX6 ura4Δ h^{-}*	This Study
CK1254	*dh1RindHNEΔ::ura4^{+} dh1::kanMX6 cen3dh::ura4^{+} clr3Δ::kanMX6 ura4Δ*	This Study
CK1222	*dh1RindHNEΔ::ura4^{+} dh1::kanMX6 cen3dh::ura4^{+} abp1Δ::leu2^{+} ura4Δ h+*	This Study
CK1259	*dh1RindHNEΔ::ura4^{+} dh1::kanMX6 cen3dh::ura4^{+} cbh1Δ::kanMX6 ura4Δ*	This Study
CK1258	*dh1RindHNEΔ::ura4^{+} dh1::kanMX6 cen3dh::ura4^{+} cbh2Δ::kanMX6 ura4Δ*	This Study
CK1288	*dh1RindHNEΔ::ura4^{+} dh1::kanMX6 cen3dh::ura4^{+} swi6Δ::kanMX6 abp1Δ::leu2^{+} ura4Δ*	This Study
CK1237	*cen3dh::ura4^{+} kanMX6::P3nmt1-3HA-ago1 ade6Δ ura4Δ*	This Study
CK1249	*cen3dh::ura4^{+} dcr1Δ::KanMX kanMX6::P3nmt1-3HA-ago1 ade6Δ ura4Δ*	This Study
CK1262	*dh1RindHNEΔ::ura4^{+} dh1::KanMX cen3dh::ura4 abp1Δ::leu2^{+} kanMX6::P3nmt1-3HA-ago1 ura4Δ*	This Study
CK1145	*cen3dh::ura4^{+} abp1Δ::leu2^{+} ura4Δ*	This Study
CK1146	*cen3dh::ura4^{+} cbh1Δ::kanMX6 ura4Δ*	This Study
CK1149	*cen3dh::ura4^{+} cbh2Δ::kanMX6 ura4Δ*	This Study
CK1296	*dh1RindHNEΔ::ura4^{+} dh1::kanMX6 clr3-myc::kanMX6 ura4Δ*	This Study
CK1297	*dh1RindHNEΔ::ura4^{+} dh1::KanMX6 cen3dh::ura4^{+} clr3-*	This Study

	myc::kanMX6 ura4Δ	
CK1298	*dh1RindHNEΔ::ura4$^+$ dh1::kanMX6 cen3dh::ura4$^+$ clr3-myc::kanMX6 swi6Δ::kanMX6 ura4Δ*	This Study
CK1299	*dh1RindHNEΔ::ura4$^+$ dh1::kanMX6 cen3dh::ura4$^+$ clr3-myc::kanMX6 abp1Δ::leu2$^+$ ura4Δ*	This Study
CK1023	*KdgΔ::ura4$^+$ ura4Δ*	This Study
CK1090	*KdgΔ::ura4$^+$ cen3dh::ura4$^+$ ura4Δ*	This Study
CK1228	*KdgΔ::ura4$^+$ cen3dh::ura4$^+$ dcr1Δ::kanMX6 ura4Δ h$^+$*	This Study
CK1226	*KdgΔ::ura4$^+$ cen3dh::ura4$^+$ abp1Δ::leu2$^+$ ura4Δ*	This Study
CK1283	*Kabp1bsΔdgΔ::ura4$^+$ ura4Δ*	This Study
CK1282	*Kabp1bsΔdgΔ::ura4$^+$ cen3dh::ura4$^+$ ura4Δ*	This Study
CK1223	*cen3dh::ura4$^+_{inv}$ kanMX6::P3nmt1-3HA-ago1 ade6Δ ura4Δ*	This Study
CK306	wild-type *h$^+$*	975
CK1315	*dh1RindHNEΔ::ura4$^+$ dh1::kanMX6 dcr1Δ::kanMX6 ura4Δ*	This Study
CK1316	*dh1RindHNEΔ::ura4$^+$ dh1::kanMX6 cen3dh::ura4$^+$ eri1Δ::kanMX6 ura4Δ*	This Study
CK1317	*cen3dh::ura4$^+$ eri1Δ::kanMX6*	This Study

Chapter Four

Strain	Relevant Genotype	Source
RL323	*bgs4^{+}/sal3^{+}(IGR)::ura4^{+} dgNucH ura4Δ h^{-}*	This study
RL346	*dcr1Δ::KanMX bgs4^{+}/sal3^{+}(IGR)::ura4^{+}-dgNucH ura4Δ h^{+}*	This study
RL349	*rdp1Δ::KanMX bgs4^{+}/sal3^{+}(IGR)::ura4^{+}-dgNucH ura4Δ*	This study
RL275	*otr1(sph1)::ura4^{+} ura4Δ ade6-210 h^{+}*	This study
RL286	*dcr1Δ::KanMX otr1(sph1)::ura4^{+} ura4Δ ade6-210 h^{+}*	This study
RL303	*clr4Δ::KanMX otr1(sph1)::ura4^{+} ura4Δ ade6-210 h^{+}*	This study
RL107	*rik1Δ::KanMX otr1(sph1)::ura4^{+} ura4ds/e ade6-210 h^{+}*	This study
RL174	*rdp1Δ::KanMX otr1(sph1)::ura4^{+} ura4Δ ade6-210 h^{-}*	This study

NOTE *(IGR)* denotes intergenic region

Chapter Five

Strain	Relevant Genotype	Source
CK100	*Wild-type*	TV281
CK573	*Tf2LTR Δ, ura4Δ*	This Study
CK615	*Tf2LTR D::vector, ura4Δ*	This Study
CK711	*meu3⁺::ura4⁺, ura4Δ*	This Study
CK745	*meu3⁺::ura4⁺, Tf2LTR Δ, ura4Δ*	This Study
CK852	*bgs4⁺/sal3⁺(IGR):: meu3⁺/Tf2LTR Δ, meu3⁺/tf2LTR::ura4⁺, ura4Δ*	This Study
CK780	*bgs4⁺/sal3⁺(IGR):: meu3⁺/Tf2LTR, meu3⁺/tf2LTR::ura4⁺, ura4Δ*	This Study
CK755	*meu3⁺/tf2LTR::ltrTerm, ura4Δ*	This Study
CK782	*meu3⁺/tf2LTR::meu3Term, ura4Δ*	This Study
CK613	*Tf2LTR Δ (meu19⁺), ura4Δ*	This Study
CK614	*Tf2LTR Δ (meu4⁺), ura4Δ*	This Study
RL34	*clr3Δ::kanMX6, ura4Δ*	This Study
CK621	*alp13Δ::kanMX6, ura4Δ*	This Study
CK1226	*KdgΔ::ura4⁺ cen3dh::ura4⁺ abp1Δ::leu2⁺ ura4Δ*	This Study
CK1118	*cen3dh::ura4⁺$_{Inv}$ cid14Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK1177	*cen3dh::ura4⁺$_{Inv}$ rrp6Δ::kanMX6 ade6Δ ura4Δ*	This Study
CK1126	*dcr1Δ::kanMX6, ura4Δ*	This Study
CK674	*clr3Δ::kanMX6, alp13Δ::kanMX6, ura4Δ*	This Study
RL807	*clr3Δ::kanMX6, abp1Δ::leu2⁺, ura4Δ*	This Study
RL808	*alp13Δ::kanMX6, abp1Δ::leu2⁺, ura4Δ*	This Study
RL809	*rrp6Δ::kanMX6, clr3Δ::kanMX6, ura4Δ*	This Study
RL810	*rrp6Δ::kanMX6, alp13Δ::kanMX6, ura4Δ*	This Study
	rrp6Δ::kanMX6, abp1Δ::leu2⁺ ura4Δ	This Study