Molecular Mechanisms of Heterochromatin Establishment and Maintenance in Fission Yeast

Molecular mechanisms of heterochromatin establishment and maintenance in fission yeast

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Abstract

In most eukaryotes, H3K9-mediated heterochromatin is found at repetitive DNA elements and serves to facilitate chromosome segregation, maintain genome stability, and silence transposons. While many of its molecular effectors have been identified, it is still unclear how heterochromatin located at pericentromeric repeats is established in mammalian cells. Fission yeast *Schizosaccharomyces pombe* heterochromatin shares molecular features with mammalian heterochromatin and provides a useful model system for studying mechanisms of heterochromatin establishment. In *S. pombe*, the RNAi machinery is first recruited to nascent transcripts emerging from pericentromeric DNA repeats, and in turn, recruits the histone methyltransferase Clr4, which catalyzes histone H3 lysine 9 di- and tri-methylation (H3K9me2 and H3K9me3). H3K9me recruits chromo domain proteins to stabilize the RNAi machinery and to promote transcriptional gene silencing (TGS).

Clr4 recruitment requires pol II transcription as the RNAi machinery is recruited via siRNA base-pairing with nascent transcripts. Given that H3K9me also recruits factors that promote TGS, it has remained unclear how Clr4 recruitment can proceed concomitantly with transcriptional silencing. In Chapter 2, I demonstrate that this process is coordinated by H3K9me2 and H3K9me3. H3K9me2 robustly recruits the RNAi machinery via Chp1, a chromo domain protein required for RNAi, while H3K9me3 recruits HP1 homologs, Swi6 and Chp2, to promote TGS. During heterochromatin establishment, the appearance of H3K9me2 precedes H3K9me3, suggesting a temporal separation of active transcription and TGS.

In RNAi mutants, the level of pericentromeric H3K9me is decreased to around 5 – 20% of wild-type level. In Chapter 3, I exclude the possibility that this residual H3K9me results from

an alternative pathway for Clr4 recruitment, and demonstrate that 1) a functional Clr4 chromo domain and 2) the ability of Clr4 to catalyze H3K9me3 are both required for epigenetically maintaining residual H3K9me. Furthermore, these requirements were confirmed using an inducible system designed to examine epigenetic maintenance at an ectopic locus. Given that the Clr4 chromo domain has higher affinity for H3K9me3 over H3K9me2, these results suggest that in the absence of RNAi-mediated Clr4 recruitment, the interaction between the chromo domain and H3K9me3 becomes critical for Clr4 to recognize pre-existing H3K9me and subsequently catalyze H3K9me on newly deposited histones.

Table of Contents

List of Figures

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Chapter 1

Introduction

I. Introduction to heterochromatin

The words “euchromatin” and “heterochromatin” were coined in the 1920’s by the German botanist Emil Heitz while observing moss nuclei undergo cell division. He noticed that some regions of the nuclei remained densely stained throughout the cell cycle and named them “heterochromatin.” Heterochromatin is associated with transcriptional repression as well as specific histone modifications, such as H3K9me and H3K27me. Today, heterochromatin is broadly separated into two types: constitutive and facultative. Constitutive heterochromatin, the densely stained chromatin Heitz had observed, is invariable across cells types in a multi-cellular organism, and mainly found at pericentromeric regions and telomeres. In large genomes, constitutive heterochromatin is also found on chromosomal arms. In yeast, constitutive heterochromatin is also found at the mating type genes, where it regulates cell type. In contrast, facultative heterochromatin refers to repressive chromatin domains that are cell-type specific in the context of multi-cellular organism, and mostly located within euchromatic chromosome arms.

The Moazed Lab has been studying RNA interference (RNAi)-mediated establishment of constitutive heterochromatin at the pericentromeric DNA repeats in *S. pombe* for many years. Because it constitutes RNA-mediated regulation of chromatin changes and gene expression, lessons learned from *S. pombe* have contributed to both the chromatin and RNA-mediated silencing fields. *S. pombe* heterochromatin is decorated with histone H3 lysine 9 di- and tri-methylation (H3K9me2 and H3K9me3, respectively; but categorically labeled together as “H3K9me” in this dissertation, unless otherwise stated), which is a hallmark of constitutive heterochromatin in all model organisms (with the exception of budding yeast). For the majority of this chapter, I will survey the function and establishment of H3K9me-mediated heterochromatin (constitutive and facultative) in various model organisms because the work described in Chapter 2 concerns the establishment of H3K9me at *S. pombe* pericentromeric repeats. In the last part of this chapter, I will briefly describe Polycomb and H3K27me-mediated

facultative heterochromatin in the context of epigenetic inheritance, which is related to the results on epigenetic inheritance of heterochromatin in *S. pombe* as described in Chapter 3.

II. Cellular functions of H3K9me-mediated heterochromatin

H3K9me is traditionally considered a hallmark for constitutive heterochromatin found at pericentromeric regions and telomeres. However, research over the past decade has shown that H3K9me is also involved in the establishment of facultative heterochromatin required for silencing transposable elements in the germline and embryonic stem cells. In this section, I will describe the cellular functions of both types of H3K9-decorated heterochromatin.

A. Pericentromeric heterochromatin facilitates proper chromosome segregation

Pericentromeric heterochromatin is formed over large arrays of major and minor DNA repeats. Shortly after the discovery of the H3K9-specific methyltransferase, *Suv39h* (Rea et al., 2000), Peters and colleagues showed that passaged murine embryonic fibroblasts null for both copies of *Suv39h*, *Suv39h1* and *Suv39h2*, had impaired segregation of the entire set of chromosomes, resulting in cells with tetraploid and octaploid DNA contents (Peters et al., 2001). Missegregated chromosomes had normal morphology and no chromosomal fusions. While the molecular basis of this phenotype is not well investigated, it could be explained by the partial requirement for H3K9me in the recruitment of cohesin, which ensures sister chromatid cohesion prior to anaphase. In *S. pombe*, cohesin is recruited by the HP1 homolog Swi6 (Nonaka et al., 2002). In human cells, cohesin is not found to interact with HP1 proteins, but can directly interact with Suv4-20h2, which is a histone H4 lysine 20 methyltransferase recruited to the pericentromeric regions by HP1 proteins (Hahn et al., 2013; Schotta et al., 2004). The chromosome missegregation phenotype observed in *Suv39h* homozygous mutants could be due to defective recruitment of HP1 proteins and Suv4-20h2, resulting in decreased levels of cohesin at pericentromeric regions.

B. Constitutive heterochromatin protects DNA repeats from recombination

The composition of the eukaryotic genome includes a high percentage of repetitive DNA sequences. Structural components of the chromosome, including centromeres, pericentromeric regions, telomeres, and rDNA, are composed of repetitive DNA sequences. Constitutive heterochromatin protects DNA repeats from recombination. In *S. pombe*, the RNAi machinery mediates H3K9me at pericentromeric regions, which are made up of repetitive DNA repeats. In RNAi mutants, these repeats are subjected to recombination during meiosis, resulting in double strand breaks (Ellermeier et al., 2010). In both *S. pombe* and *Drosophila*, heterochromatin loss at rDNA repeats results in increased mitotic recombination at rDNA repeats (Cam et al., 2005).

C. Facultative heterochromatin silences transposable elements

Transposable elements are nucleic acid elements that can insert themselves into other locations in the genome. If left unchecked, transposable elements present a threat to genome integrity as insertion into genes critical for cell function might result in cell death. Repression of transposable elements is first established in the germline of many multicellular organisms via piRNA-mediated recruitment of H3K9 methyltransferase to establish heterochromatic silencing (Czech and Hannon, 2016). In somatic cells, the main mechanism responsible for maintaining the silenced transposons is mediated by DNA methylation. And in a relatively new discovery, transposable elements in mouse embryonic stem cells are found to be repressed by H3K9 methyltransferases recruited by DNA-binding factors during a period in which much of the DNA methylation is erased (Rowe and Trono, 2011).

III. Mechanisms of H3K9-mediated establishment of constitutive heterochromatin

The central theme of H3K9me-mediated heterochromatin establishment is the recruitment of methyltransferase(s), which deposit H3K9me2/3 to recruit effector proteins of heterochromatic silencing. In this section, I will first use the model organism *Drosophila* to

illustrate the discovery of the main molecular components in establishing constitutive heterochromatin. The remainder of this section will be devoted to highlighting the roles of homologs in other model organisms in conjunction with what is currently known about the establishment mechanisms of pericentromeric heterochromatin. In the following section (Section IV), I will describe the mechanisms for establishing H3K9me-mediated facultative heterochromatin in germline and embryonic stem cells.

A. *Drosophila melanogaster*

The study of constitutive heterochromatin has laid the foundation for the field of epigenetics and has shed light on the properties of heterochromatin. A decade after the cytological observation of heterochromatin, Hermann Muller and colleagues uncovered its silencing properties through studying a phenomenon called position effect variegation (PEV) in *Drosophila*. Muller observed red-white mosaic patterns in the eyes of progenies of flies exposed to radiation (Muller, 1930). This is due to a chromosome translocation, resulting in the white gene relocating to the proximity of the pericentromeric heterochromatin, identified by observation of stained polythene chromosomes (Cooper, 1959). Progenies of flies with this translocation had varying degrees of red-white variegation in the eyes, providing clues as to the properties of the pericentromeric heterochromatin – it can spread, exert silencing on euchromatic gene, and be stably inherited.

To identify molecular factors that contribute to the formation of pericentromeric heterochromatin, genetic screens were used to uncover genes that enhanced or repressed the variegation. *Su(var)3-9* and *Su(var)2-5* were two genes identified to lose variegation upon mutagenesis due to loss of heterochromatin. *Su(var)3-9* encodes a protein with a chromo domain and a SET domain (Tschiersch et al., 1994). The human homolog of Su(var)3-9 was later found to di- and tri-methylate H3K9 via the SET domain in a landmark study (Rea et al., 2000). *Su(var)2-5* encodes the heterochromatin protein 1 (HP1a), which has a chromo domain

and a chromo-shadow domain. This protein was also independently identified in biochemical and immune-staining experiments as a structural component of heterochromatin (James and Elgin, 1986). During spreading of heterochromatin, HP1a is thought to bind to existing H3K9me via its chromo domain and to recruit Su(var)3-9 via its chromo-shadow domain (Jacobs et al., 2001; Schotta et al., 2002). While this model does not entirely hold true in other model organisms, most of which have homologs of these proteins, the discovery Su(var)3-9 and HP1a laid the foundation for studying molecular mechanisms of H3K9-mediated heterochromatin.

B. *Schizosaccharomyces pombe*

The fission yeast *S. pombe* has three chromosomes, ranging from 3.5 to 5.7 Mb (Wood et al., 2002). Domains of di- and tri-methylated H3K9 are found at DNA repeats flanking the centromeres, the mating type region, telomeres, and rDNA repeats (Cam et al., 2005). *S. pombe* does not have DNA methylation (5-methylcytosine) (Antequera et al., 1984), a classical modification associated with gene silencing, nor does it have H3K27 methylation, a hallmark of facultative heterochromatin in metazoans. Because *S. pombe* presents a pared-down version of mammalian pericentromeric heterochromatin, which also consists of H3K9me-decorated DNA repeats, numerous biochemical and genetic studies have been carried out using *S. pombe* to understand the molecular mechanisms of heterochromatin establishment.

S. pombe has the machinery for RNA interference (RNAi): Argonaute, Dicer and RNA-dependent RNA polymerase (encoded by *ago1*$^+$, *dcr1*$^+$, and *rdp1*$^+$, respectively). RNAi was first observed in *C. elegans*, where dsRNA is cleaved by Dicer into siRNAs, loaded onto Argonaute, and targeted to cognate mRNAs, which are silenced (Hannon, 2002). Because silencing occurs at the level of mRNA, this process is called post-transcriptional gene silencing (PTGS). Deletion of RNAi genes in *S. pombe* results in the loss of heterochromatin, demonstrating that RNAi could also mediate transcriptional gene silencing (TGS) (Volpe et al., 2002). How the RNAi

machinery can mediate changes at the chromatin level became a subject of intense investigation.

Our lab's foray into RNAi-mediate heterochromatin establishment in *S. pombe* started with the purification of Chp1, a chromo domain protein that binds to H3K9me. Chp1 was found in a complex with Argonaute (Ago1) and Tas3, a GW domain protein that serves as an adapter between Chp1 and Ago1 (Verdel et al., 2004). The complex also contains pericentromeric siRNAs, and localizes to the pericentromeric region in a siRNA-dependent manner. For its role in mediating silencing at the chromatin, the complex was named RNA-induced transcriptional silencing (RITS). Two observations suggest that RITS is targeted to the chromatin via siRNA base-pairing with nascent transcripts: First, RITS could be crosslinked to pericentromeric transcripts in a Clr4-dependent manner. Second, tethering of the complex to nascent transcript at a euchromatic locus induced RNAi-mediated silencing (Buhler et al., 2006; Motamedi et al., 2004)(Figure 1.1).

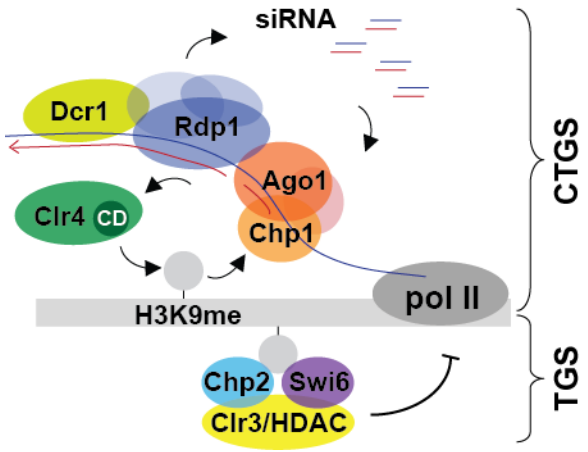

Figure 1.1 Model of RNAi-mediated heterochromatin establishment.
CTGS: Co-transcriptional gene silencing; TGS: Transcriptional gene silencing

In the presence of functional RNAi, RITS associates with the RNA-directed RNA polymerase complex (RDRC), which contains Rdp1, an RNA-dependent polymerase, Hrr1, a helicase, and Cid12, a poly(A) polymerase (Motamedi et al., 2004). Rdp1 has RNA-directed RNA polymerase activity in vitro, and converts the single stranded pericentromeric transcript

into double stranded RNA, which can then be processed by the ribonuclease Dcr1 (Motamedi et al., 2004). RDRC physically associates with RITS and localizes to pericentromeric DNA region in a Clr4-, RITS- and siRNA-dependent manner, suggesting that siRNA-loaded RITS directs RDRC to specific chromatin-bound RNA templates to initiate dsRNA synthesis and siRNA amplification (Gerace et al., 2010a; Motamedi et al., 2004).

Ultimately, the establishment of pericentromeric heterochromatin requires the recruitment of an H3K9 methyltransferase to the chromatin. In *S. pombe*, Clr4 is responsible for H3K9 di- and tri-methylation, and resides in a multiprotein complex called CLRC (Hong et al., 2005; Horn et al., 2005; Nakayama et al., 2001). Subunits of RITS and RDRC associate with CLRC in a siRNA-dependent manner, demonstrating how RNAi machinery can recruit Clr4 to the chromatin (Gerace et al., 2010a). In addition, Stc1 serves as an adaptor protein between CLRC and Ago1 (Bayne et al., 2010).

H3K9me2/3 deposited by Clr4 recruits chromo domain proteins, which are effectors of heterochromatin silencing (Motamedi et al., 2008; Sugiyama et al., 2007). In addition to Chp1, *S. pombe* has two other chromo domain proteins that are HP1 homologs, Swi6 and Chp2. Swi6 serves multiple functions, including facilitating the recruitment of RDRC to chromatin via accessory factor Ers1(Hayashi et al., 2012; Rougemaille et al., 2012). In the absence of Swi6, pericentromeric siRNA levels are dramatically decreased, but H3K9me is not affected, suggesting that Swi6 enhances, but is not critical for RNAi. Swi6 also acts in parallel pathways with Chp2 to recruit Clr3, an H3K14 histone deacetylase required for TGS (Motamedi et al., 2008; Sadaie et al., 2008; Sugiyama et al., 2007).

The machineries described above mediate two types of silencing at the pericentromeric heterochromatin, TGS and RNAi-dependent silencing (Figure 1.1). RNAi-dependent silencing is also called co-transcriptional gene silencing (CTGS), as it requires nascent transcript provided by RNA Polymerase II activity. Given that TGS serves to shut off transcription, how is this

silencing mechanism reconciled with the need for the nascent transcript in CTGS-mediated recruitment of Clr4? This paradox will be addressed in Chapter 2.

C. *Mus musculus*

Because most of the critical players in *S. pombe* heterochromatin establishment have mammalian homologs, the idea that RNAi is involved in establishing pericentromeric heterochromatin in mammalian cells is very tempting. However, no mammalian pericentromeric siRNA has been detected, and the mammalian homolog of Rdp1 has not been identified. Initial observations in dicer null embryonic stem cells suggested that RNAi might play a role in establishing pericentromeric heterochromatin. Dicer is important for embryonic stem cell differentiation and genome stability, and its absence results in defective pericentromeric heterochromatin, indicated by upregulation of pericentromeric transcripts and decreased H3K9me3 (Kanellopoulou et al., 2005). However, heterochromatin defects were later attributed to microRNA-mediated misregulation of DNA methyltransferase expression, and mutant phenotypes were rescued by overexpression of DNA methyltransferase (Benetti et al., 2008; Sinkkonen et al., 2008).

While RNAi is unlikely to play a role in establishing mammalian heterochromatin, there remains the possibility that pericentromeric RNA is required for de novo establishment of pericentromeric heterochromatin in early embryogenesis. In the mouse zygote, maternal and paternal chromosomes carry different histone H3 and histone modifications that are reflective of different chromatin reprogramming histories during gametogenesis (Gill et al., 2012; Puschendorf et al., 2008). Paternal chromosomes have histone H3.3 (non-K9 methylated); maternal chromosomes have H3 decorated with methylated K9 and localized HP1β. Methylation on lysine 27 residue of H3.3 recruits PRC1 via a chromo domain-containing protein Cbx2 to establish pericentromeric heterochromatin on paternal chromosomes (Tardat et al., 2015). In H3.3K27R mutant zygote (but not in H3.3K9R zygote), paternal pericentromeric

heterochromatin cannot be established and HP1β is mislocalized, resulting in dysfunctional chromosome segregation, and developmental arrest (Santenard et al., 2010). Mutant zygotes were rescued by the injection of double-stranded RNA derived from pericentromeric transcripts, but not single-stranded RNA (both sense and antisense were tested). Interestingly, the hinge domain, not chromo domain, of HP1β was critical for its localization after double-stranded RNA injection. Though H3K9 methylation is not involved, RNA clearly plays an important role in pericentromeric heterochromatin establishment.

Maintenance of pericentromeric heterochromatin requires H3K9 di- and tri-methyltransferase Suv39h1 (or Suv39h2 in male germline) as well as Prdm3 and Prdm16, H3K9 mono-methyltransferases that act redundantly on cytoplasmic histones (Peters et al., 2001; Pinheiro et al., 2012). Simultaneous loss of these enzymes prevents the formation of pericentromeric H3K9me2/3, resulting in derepression of major satellite transcripts (Pinheiro et al., 2012). Interestingly, Prdm3/Prdm16 depletion in *Suv39h* double null immortalized mouse embryonic fibroblasts resulted in the disruption of nuclear lamina structure (Pinheiro et al., 2012). The discovery of Prdm3 and Prdm16 suggest a sequential pathway for heterochromatic H3K9 methylation, although it is not clear whether the transition from mono- to di-/tri-methylation is regulated.

D. *Caenorhabditis elegans*

Unlike most model organisms where H3K9 methylation is normally found in distinct domains, such as pericentromeric and telomeric regions, *C. elegans* have large domains of H3K9me2/3 (but not DNA 5mC methylation) spanning the DNA repeat-rich chromosome arms of five pairs of autosomes and a pair of X chromosomes (Gu and Fire, 2010; Liu et al., 2011). In *C. elegans* embryonic nuclei, these chromosome arms are associated with the nuclear envelope, which is thought to provide a suppressive environment for gene expression (Mattout et al., 2011). The methyltransferases MET-2 (a SETDB1 homolog) and SET-25 (a G9A homolog) are

responsible for all forms of H3K9me (Towbin et al., 2012). MET-2, localized in the cytoplasm, deposits H3K9me1/2 on cytoplasmic free histones, and these marks facilitate anchoring of transgene DNA repeat arrays to the nuclear envelope, but not suppression of gene expression (Towbin et al., 2012). SET-25, devoid of a chromo domain, is nonetheless recruited to the nuclear periphery by H3K9me3, and converts H3K9me1/2 to H3K9me3, leading to transgene silencing (Towbin et al., 2012).

However, deletion of both H3K9 methyltransferases only resulted in minor loss of chromatin anchoring at the nuclear envelope (Towbin et al., 2012), suggesting that the main function of H3K9me in *C. elegans* is not the anchoring of chromosome arms to a repressive nuclear envelope, a phenomenon thought to be important for gene regulation during development. Zeller *et al.* used *met-2 set-25* double mutants, which lack all H3K9me, to confirm that H3K9me is not required for development (Zeller et al., 2016). These embryos develop normally, but have derepressed transposons and simple repeats in somatic and germline tissues, resulting in sterile adults due to extensive DNA-damage-driven apoptosis in the germline. Thus the main function of H3K9me in *C. elegans* is to protect repeat-rich sequences from replication stress. Repeat elements associated with H3K9me also support this conclusion. In *C. elegans* embryos, H3K9me2 and H3K9me3 have unique and overlapping distribution patterns. RNA transposons and transcriptionally silent genes were most strongly correlated with H3K9me3, while simple repeats were more likely to carry H3K9me2 alone.

Recruitment of SET-25 requires its own mark (H3K9me3). Given that SET-25 does not have a known domain that can recognize H3K9me to facilitate its recruitment to chromatin, it was speculated that chromo domain-containing HP1 homologs, HPL-1 and HPL-2, might facilitate its recruitment (Towbin et al., 2012). Surprisingly, deletion of both HP1 homologs does not affect SET-25 localization (Towbin et al., 2012). SET-25 does not fit the typical profile of H3K9 methyltransferases found at heterochromatin. From work in *S. pombe*, *Drosophila*, and mammalian cells, H3K9 methyltransferases are generally assumed to be recruited to H3K9me-

containing domains by directly binding to H3K9me or or via HP1 proteins bound to H3K9me. Given that SET-25 is not recruited via either mechanism, further investigation might reveal new modes of H3K9 methyltransferase recruitment to H3K9me. HPL-2 functions in vulval and germline development, and HPL-1 acts redundantly with HPL-2 in vulval development (Schott et al., 2006). While their molecular roles have not been established, the two HPL homologs have distinct genome distribution. HPL-1 co-localizes with foci enriched for H3K9me3 (Towbin et al., 2012), while HPL-2 localizes to pericentromeric regions enriched for H3K9me2 (Garrigues et al., 2015). Unlike *S. pombe* and mammalian chromosomes, where kinetochores are assembled at one location on each chromosome, *C. elegans* has even distribution of CeCENP-A, resulting in formation of diffuse kinetochores along the entire chromosome (Buchwitz et al., 1999). HPL-2 localizes to DNA repeats bordering CeCENP-A domains within the broad H3K9me2/3 domain on chromosome arms (Garrigues et al., 2015). These atypical pericentromeric regions are enriched for H3K9me2 as well as histone modifications associated with active transcription. Curiously, recruitment of HPL-2 is not dependent on its chromo domain (Garrigues et al., 2015). Given that other HP1 homologs can bind to RNA, it is quite possible that HPL-2 is recruited via sequence-based factors.

IV. Mechanisms of H3K9-mediated establishment of facultative heterochromatin

In this section, using three model organisms, I will describe two different mechanisms for H3K9me-mediated establishment of facultative heterochromatin for the purpose of silencing transposable elements. The first mechanism involves piRNA-mediated recruitment of H3K9 methyltransferase in the germline, while the second mechanism involves DNA binding factor-mediated recruitment of H3K9 methyltransferase in mouse embryonic stem cells.

E. *Drophila melanogaster*

piRNA-mediated silencing of transposons in the germline has been best studied in *Drosophila*. In contrast to siRNAs, piRNAs are not the result of Dicer activity. *Drosophila* piRNAs are derived from clusters of repeat sequences, termed “piRNA clusters,” located in pericentromeric and telomeric regions (Brennecke et al., 2007). Genomic location of piRNA clusters appears not to matter however, as insertion of a pericentromeric piRNA cluster into euchromatin does not affect its function (Muerdter et al., 2012). Clusters are transcribed either uni-directionally or bi-directionally, thus resulting in long piRNA precursors that are subsequently exported to the cytoplasm for processing and amplification via the “ping-pong cycle” (reviewed in (Guzzardo et al., 2013)). piRNAs are then loaded onto Piwi, targeted to transposon nascent transcript, where they recruit H3K9 methyltransferases to establish TGS. I will first describe the molecular features of piRNA clusters, and then discuss what is currently known for piRNA-mediated deposition of H3K9me and TGS.

While the *Drosophila* SETDB1 is broadly required for piRNA generation from both types of piRNA clusters (Rangan et al., 2011), these piRNA clusters have interesting differences in chromatin and genomic features, as well as molecular requirements for transcription. Bi-directional piRNA clusters are marked by H3K9me3, which recruits the HP1 homolog Rhino (Klattenhoff et al., 2009; Mohn et al., 2014; Rangan et al., 2011). Recruitment of Rhino is critical for transcription at this type of cluster, because Rhino recruits Deadlock and Cutoff, which are transcription termination cofactors. Cutoff is believed to prevent transcription termination by binding to uncapped 5’ ends and stabilizing cluster transcripts, as these transcripts are non-canonical by-products of convergent transcription of neighboring genes transcription termination (Mohn et al., 2014; Zhang et al., 2014). In contrast, uni-directional clusters have defined promoter and termination sequences as well as hallmarks of canonical pol II transcription, such as the presence of H3K4me2 (Mohn et al., 2014). Why SETDB1 is required for production of piRNAs at this type of cluster is unclear.

After piRNA amplification in the cytoplasm, piRNA-loaded Piwi scans, detects, and co-transcriptionally silences nascent transposon transcripts. Piwi is critical for TGS at transposons, as its depletion results in an increase of Pol II occupancy at promoters, elevated transposon transcript levels, and decrease in H3K9me3 at the transposon bodies, and ultimately male sterility (Sienski et al., 2012). The catalytic activity of Piwi is not required for TGS, highlighting its role as recruitment factor for TGS as opposed to being part of the piRNA generation ping-pong cycle (Sienski et al., 2012). Asterix, a zinc finger protein, and Panoramix both directly interact with Piwi, and are critical for TGS. Surprisingly, tethering of Panoramix to both RNA and DNA resulted in de novo deposition of H3K9me3 and TGS, bypassing requirements for Piwi and Asterix (Sienski et al., 2015; Yu et al., 2015). This silencing requires the presence of SETDB1, and its cofactor Windex for maximal silencing, as well as HP1. How TGS is established after H3K9me3 deposition is not known yet. The role of Panoramix is remarkably similar to Stc1 in *S. pombe*, which is an adaptor protein connecting Ago1 with the complex containing H3K9 methyltransferase Clr4. Like Panoramix, tethering of Stc1 was sufficient to induce TGS by recruitment of Clr4 in the absence of Ago1 (Bayne et al., 2010).

F. *Caenorhabditis elegans*

The majority of the >16,000 piRNA genes of *C. elegans* are found in two clusters on chromosome IV. Each piRNA is located downstream of a distinctive bipartite sequence motif (Ruby et al., 2006), which acts as an autonomous promoter for individual piRNA precursors (Billi et al., 2013; Cecere et al., 2012). Unlike *Drosophila*, where H3K9me promotes transcription at piRNA clusters to generate a very long piRNA precursor transcript, piRNA precursors in *C. elegans* are transcribed exactly 2 nt upstream of the mature piRNA to generate 28 or 29 nt precursors (Cecere et al., 2012; Gu et al., 2012b), independently of H3K9me3. The amplification of piRNA in the *C. elegans* germline also does not require the ping-pong cycle. Instead, secondary piRNAs are generated by RNA-dependent RNA polymerase (Bagijn et al., 2012; Das

et al., 2008; Lee et al., 2012). *C. elegans* secondary piRNA is bound by HRDE-1 (heritable RNAi-deficient 1), targeted to transposons and mediates TGS by initiating H3K9me3 and Pol II stalling (Ashe et al., 2012; Buckley et al., 2012; Luteijn et al., 2012). As in Drosophila, HPL-2, an HP1 homolog, is required for TGS, but the mechanism is not known (Ashe et al., 2012; Shirayama et al., 2012).

G. *Mus musculus*

Similar molecular features of piRNA amplification mentioned above have also been identified in the mouse germline, but in contrast to H3K9me3-mediated silencing, piRNA-mediated silencing is established via DNA methylation. Here I will describe a second mechanism for establishing H3K9me-mediated facultative heterochromatin in mouse embryonic stem cells.

Up to 10% of the mouse genome is made up of endogenous retroviruses (ERVs), some of which are capable of expression and replication (Stocking and Kozak, 2008). In mouse embryonic stem (mES) cells, two out of three classes of endogenous retroviruses (ERVs) are silenced by H3K9me3 deposited by the H3K9 methyltransferase SETDB1 and corepressor KAP1 (Matsui et al., 2010; Rowe et al., 2013). The complex is recruited by Krüppel-associated box zinc finger proteins (KRAB-ZFPs), which recognize ERV sequences (Wolf et al., 2015). Following the deposition of H3K9me3, DNA methyltransferase is recruited to stably silence ERVs. It is proposed that during genome reprogramming, KAP1 and SETDB1 are critical for establishing proviral silencing, but their silencing function is superseded by DNA methylation at later stages in development. It is not clear whether HP1 proteins facilitate silencing in this context.

V. H3K27-mediated facultative heterochromatin and epigenetic inheritance

A classic example of facultative heterochromatin is the maintenance of HOX gene repression by Polycomb-group proteins. Morphology of body segments is established by early transcriptional regulators acting on HOX genes, resulting in segment-specific HOX gene regulation. Polycomb-group proteins maintain the repressed HOX genes in a cell-type specific manner, even when the establishing signals are long gone, thus facultative heterochromatin is a means by which one embryo could differentiate into many cell types carrying the same genome.

Specific histone modifications, including H3K27me, are associated with Polycomb-mediated silencing. H3K27me is mediated by EZH2 of the PRC2 complex. Another component of the complex, EED, can bind H3K27me to stimulate EZH2 activity (Margueron et al., 2009). H3K27me recruits PRC1, which promotes chromatin compaction in vivo, an activity that is thought to contribute to TGS. The question of how the silenced gene expression is inherited in daughter cells and whether histone modifications are sufficient to mediate such inheritance has dogged the chromatin biology field for many years. Evidence against histone modification-mediate inheritance includes the loss of Polycomb mediated silencing upon deletion of Polycomb response element (PRE) in *Drosophila.* PRE is thought to recruit Polycomb group proteins via various DNA binding factors. During DNA replication, should histone modification be sufficient to maintain the silenced state, then the original nucleating sequence would no longer be required. However in *S. pombe*, H3K9me can be epigenetically inherited, but only in mutant cells that decrease the rate of H3K9 demethylation. We and others artificially tethered Clr4, the sole H3K9 methyltransferase responsible for me2 and me3, to a euchromatic reporter gene. Doing so allows us to examine whether the deposited H3K9me and silenced reporter can be maintained by a wild-type copy of Clr4 upon release of tethered Clr4. We observed epigenetic maintenance of H3K9me upon removal of Epe1, a putative demethylase (Audergon et al., 2015; Ragunathan et al., 2015). Furthermore, maintenance requires the chromo domain of Clr4, suggesting that upon release of tethered Clr4, wild-type Clr4 is recruited to the ectopic domain

via recognition of H3K9me. Our observations suggest that the cells have mechanism(s) in place to erase H3K9me, and slowing down the erasure kinetics enabled recruitment of Clr4 to further propagate H3K9me. Perhaps, the epigenetic maintenance of H3K27 is also opposed by a demethylase, providing an explanation for the continuous requirement for PRE-mediated establishment.

VI. Overview

In this chapter, I surveyed the function and mechanisms of H3K9me-mediated heterochromatin formation in various model organisms. While HP1 homologs and H3K9 methyltransferase(s) have been identified in most eukaryotic model organisms, the mechanistic role they play in heterochromatin establishment and how TGS is mediated is still unclear. The stepwise H3K9 methylation observed in *C. elegans* and mammalian cells suggest that H3K9 methylation states could be regulated. Also, the distinct genomic distribution of H3K9me2 versus H3K9me3 in *C. elegans* suggests that H3K9me2 and H3K9me3 are likely not performing the same function. In Chapter 2, I will present our work demonstrating the different roles of H3K9me2 and H3K9me3 in the context of pericentromeric heterochromatin establishment *in S. pombe*. In Chapter 3, I will present data from our investigation of the molecular requirements for epigenetic inheritance of H3K9me at pericentromeric repeats and at the ectopic locus. In particular, I will address whether H3K9 methylation states affect epigenetic maintenance.

VII. References

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Chapter 2

A transcriptionally permissive histone H3 lysine 9 methylation state enables RNAi-mediated heterochromatin assembly

Gloria Jih and Danesh Moazed

Chapter 2

This chapter is a part of an unpublished manuscript written by Gloria Jih and Danesh Moazed.

All experiments were performed by Gloria Jih, except for the following:
For the histone modification mass spectrometry analysis depicted in Figure 2.6, Mark Currie performed the histone purification, and Natarajan Bhanu and Benjamin Garcia (University of Pennsylvania) performed the mass spectrometry analysis.

For the peptide pull-down assay depicted in Figure 2.10, Nahid Iglesias performed the experiment, and Joao Paulo performed the quantitative mass spectrometry analysis.

Abstract

Heterochromatic DNA domains play important roles in the regulation of gene expression and maintenance of genome stability by silencing repetitive DNA elements and transposons (Richards and Elgin, 2002). In organisms ranging from fission yeast to mammals, heterochromatin assembly at DNA repeats involves the activity of small noncoding RNAs (sRNAs) associated with the RNA interference (RNAi) pathway (Allshire and Ekwall, 2015; Aravin et al., 2008; Buckley et al., 2012; Castel and Martienssen, 2013; Chan et al., 2004; Holoch and Moazed, 2015a; Luteijn and Ketting, 2013; Reinhart and Bartel, 2002; Shirayama et al., 2012; Sienski et al., 2012; Volpe et al., 2002). Typically, sRNAs, originating from long noncoding RNAs transcribed from DNA repeats, guide Argonaute-containing effector complexes to complementary nascent RNAs to initiate histone H3 lysine 9 di- and tri-methylation (H3K9me2 and H3K9me3, respectively) and heterochromatin formation (Bayne et al., 2010; Buhler et al., 2006; Gerace et al., 2010b; Motamedi et al., 2004; Sienski et al., 2015; Verdel et al., 2004; Yu et al., 2015; Zhang et al., 2008a). H3K9me is in turn required for recruitment of RNAi to chromatin and promotes sRNA generation (Gerace et al., 2010b; Motamedi et al., 2004; Noma et al., 2004; Rangan et al., 2011). However, how heterochromatin formation, which silences transcription, can proceed by a co-transcriptional mechanism that also promotes sRNA generation remains paradoxical. Here, using Clr4, the fission yeast homolog of mammalian SUV39H H3K9 methyltransferases, we designed active site mutations, which allow H3K9me2 catalysis but block the transition to H3K9me3. We show that H3K9me2 defines a functionally distinct heterochromatin state that mediates RNAi-dependent co-transcriptional gene silencing (CTGS). Unlike H3K9me3 domains, which are transcriptionally silent, H3K9me2 domains are transcriptionally active, contain modifications associated with euchromatic transcription, and couple RNAi-mediated transcript degradation to the establishment of H3K9me domains. The two H3K9me states recruit reader proteins with different efficiencies, explaining their different downstream silencing functions. Our findings demonstrate that H3K9me2 and H3K9me3 define

functionally distinct heterochromatin states and uncover a mechanism for formation of transcriptionally permissive heterochromatin that is compatible with its broadly conserved role in RNAi-mediated genome defense.

Introduction

Histone post-translational modifications play crucial roles in the regulation and epigenetic memory of transcription in eukaryotes. In particular, the methylation of different lysine side chains in histone H3 is associated with transcriptionally active or repressed genes. Histone H3 lysine 4 (H3K4) and lysine 36 (H3K36) methylation are associated with active genes and mark promoters and transcribed regions. In contrast, histone H3 lysine 9 (H3K9) methylation is associated with transcriptionally silent heterochromatic regions of chromosomes (Jenuwein and Allis, 2001; Kouzarides, 2007; Shinkai and Tachibana, 2011; Zhou et al., 2011). Each of the above H3 lysines can be either mono-, di-, or tri-methylated (me1, me2, or me3, respectively), and each methylation state often signals a different functional outcome. For example, in *S. cerevisiae*, active genes are associated with a gradient of H3K4 methylation, with H3K4me3 enriched at transcription start sites and H3K4me2 enriched at downstream 5' regions (Li et al., 2007). While H3K4me3 recruits acetyltransferases to the promoter to help activate transcription (Hung et al., 2009; Taverna et al., 2006; Vermeulen et al., 2007), H3K4me2 recruits histone deacetylases to repress cryptic downstream transcription (Kim and Buratowski, 2009). Heterochromatic DNA domains, important for maintenance of genome stability in organisms from fission yeast to human, are associated with both H3K9me2 and H3K9me3 (Black et al., 2012), but it has remained unclear whether the different H3K9 methylation states carry out distinct tasks in heterochromatin assembly or function.

In many eukaryotes, the formation of heterochromatin is coupled to transcription- and RNA interference (RNAi)-based mechanisms that produce small RNAs (sRNAs) and the transcripts that serve as their targets (Castel and Martienssen, 2013; Malone and Hannon, 2009;

Moazed, 2009). The sRNA-mediated targeting of the nascent transcript is required to recruit H3K9 methyltransferases, but how the seemingly incompatible processes of repressive heterochromatin formation and transcriptional activity are coordinated is not understood. In the fission yeast *Schizosaccharomyces pombe*, H3K9 methylation and heterochromatin establishment at the pericentromeric DNA repeats require components of the RNAi machinery and are associated with the generation of high levels of small interfering RNAs (siRNAs) originating from noncoding pericentromeric transcripts (Allshire and Ekwall, 2015; Reinhart and Bartel, 2002; Volpe et al., 2003). Conversely, the generation of siRNAs requires both the RNAi machinery and H3K9 methylation (Gerace et al., 2010b; Motamedi et al., 2004; Noma et al., 2004; Verdel et al., 2004). This co-dependent relationship results from direct recruitment of the RNAi machinery to sites of heterochromatin assembly via distinct RNA- and chromatin-based signals. siRNAs direct the Argonaute-containing RNA-Induced Transcriptional Silencing (RITS) complex to nascent pericentromeric transcripts (Buhler et al., 2006; Holoch and Moazed, 2015a; Motamedi et al., 2004; Verdel et al., 2004). RITS in turn recruits the CLRC complex and its catalytic subunit, H3K9 methyltransferase Clr4, the sole *S. pombe* homolog of the mammalian SUV39H1 and SUV39H2 (Bayne et al., 2010; Gerace et al., 2010b; Hong et al., 2005; Horn et al., 2005; Jia et al., 2005; Zhang et al., 2008a). Clr4 recruitment ultimately leads to H3K9 di- and tri-methylation over several kilobases of pericentromeric chromatin. The resulting H3K9me further promotes association of the RITS complex with chromatin by providing a binding site for its chromo domain-containing subunit Chp1, an event that is required in order to recruit the RNA-Dependent RNA polymerase Complex (RDRC) and the Dicer (Dcr1) ribonuclease (Motamedi et al., 2004; Noma et al., 2004; Schalch et al., 2009; Sugiyama et al., 2005). RDRC uses the nascent transcripts targeted by RITS as templates to synthesize double stranded RNA that is processed into siRNA by Dcr1, resulting in co-transcriptional transcript degradation (Buhler et al., 2006; Colmenares et al., 2007). siRNA levels are thus diminished in the absence of H3K9me due to inefficient chromosomal localization of RITS/RDRC. Many features of this co-

dependent mechanism appear to be conserved in other systems. For example, in *Drosophila*, the generation of Piwi-associated small RNAs (piRNAs), which are required for H3K9 methylation and transposon silencing, requires the SETDB1 H3K9 methyltransferase (Rangan et al., 2011; Sienski et al., 2012). How silencing of transcription is coordinated with transcription- and nascent RNA-dependent RNAi, and whether H3K9 methylation states uniquely contribute to either mechanism remain unknown.

Both H3K9me2 and H3K9me3 have been associated with gene silencing in metazoans. However, uncovering potentially distinct roles for each methylation state has been hampered by the presence of multiple enzymes that catalyze mono-, di-, and tri-methylation. In mouse and human cells, five different SET domain methyltransferases mediate H3K9 methylation. The mammalian Clr4 homologs, SUV39H1 and SUV39H2 are responsible for heterochromatin formation at centromeric and pericentromeric DNA repeats, and each catalyzes both H3K9me2 and H3K9me3 (Fodor et al., 2010; Rea et al., 2000). Another enzyme, SETDB1 (also called ESET/KMT1E), catalyzes H3K9me2 and H3K9me3 to silence endogenous retrotransposons and other repeated sequences, whereas G9a (also called EHMT2/KMT1C) and GLP catalyze primarily H3K9me2 associated with repressed euchromatic DNA regions (Schultz et al., 2002; Shinkai and Tachibana, 2011; Tachibana et al., 2001). In *Arabidopsis thaliana*, the SUVH5/6 enzymes, which are required for siRNA-mediated DNA methylation and silencing, primarily di-methylate H3K9 (Du et al., 2015). Finally, in *C. elegans*, the MET-2 and SET-25 methyltransferases catalyze H3K9me1/2 and H3K9me3 methylation, respectively, which are associated with chromosome ends, transgene arrays, and nuclear RNAi-mediated silencing (Mao et al., 2015; Towbin et al., 2012; Towbin et al., 2013). Interestingly, silencing of transgene repeats in *C. elegans* requires both H3K9 methylation and components of the RNAi pathway (Buckley et al., 2012; Grishok et al., 2005; Gu et al., 2012a; Guang et al., 2010; Shirayama et al., 2012).

In order to understand whether the different methylation states of H3K9 make distinct contributions to heterochromatin formation, we took advantage of the known structural features of SET domain lysine methyltransferases to construct Clr4 enzymes with active site mutations that allow H3K9 di-methylation but impede or abolish H3K9 tri-methylation. Our results reveal that H3K9me2, in the absence of any detectable H3K9me3, is sufficient for RNAi recruitment, siRNA generation, and RNAi-mediated co-transcriptional RNA degradation. However, transition to H3K9me3 is required for efficient HP1 recruitment and transcriptional silencing. H3K9me2 and H3K9me3 thus define two functionally distinct types of repressive domains in *S. pombe*: transcription- and RNAi-permissive heterochromatin versus transcriptionally silent heterochromatin. H3K9 methylation states may therefore play a critical role in heterochromatin formation by coordinating transcription- and H3K9me-dependent RNAi with transcriptional silencing at DNA repeats. Given the conservation of sRNA-mediated H3K9 methylation, these findings may also inform mechanisms of sRNA-mediated chromatin silencing in other systems.

Results

Clr4 SET domain mutations that block H3K9me3 result in defective TGS.

To determine whether the different methylation states of H3K9 make distinct contributions to heterochromatin formation, we designed mutations in the active site of Clr4 that inhibit H3K9me3 catalysis. SET domain methyltransferases, including those associated with heterochromatin formation, contain a so-called switch position that determines how many methyl groups they add to their substrates (Figure 2.1a, highlighted green)(Collins et al., 2005). Phenylalanine (F) in the switch position, such as in the *N. crassa* DIM-5 and mammalian SUV39H1/2, allows the catalysis of di- and tri-methylated lysine, while tyrosine (Y) in the switch position, predominantly allows lysine mono- and di-methylation such as in the *A. thaliana* SUVH4/KYP (Figure 2.1a)(Collins et al., 2005; Du et al., 2015). The bulkier tyrosine residue is

thought to create a smaller catalytic pocket that cannot accommodate higher methylation states. Sequence alignment indicated that the Clr4 SET domain contains phenylalanine at the switch position (F449), equivalent to switch residues F294 in DIM-5 and F363 in the human SUV39H1 (Figure 2.1a, b; highlighted green)(Zhang et al., 2003). Besides the F/Y switch, the identity of the amino acid at the position equivalent to the *S. pombe* I418 was previously proposed to affect substrate methylation states, with bulkier residues such as proline in G9a correlating with di-methylation (Figure 2.1a, b; highlighted blue)(Collins et al., 2005; Xiao et al., 2003).

Figure 2.1 Clr4 SET domain mutations that block H3K9me3 result in defective TGS.
a, Diagram illustrating location of the Clr4 chromo (CD) and SET domains, and the mutations used in this study (top). Sequence alignment for the SET domain region containing these mutations in the indicated methyltransferases (bottom).
b, Crystal structure of *N. crassa* DIM-5 catalytic pocket in complex with a histone H3 N-terminal peptide (yellow), showing side chain of lysine 9 in the catalytic pocket. DIM-5 F294 (corresponding to *S. pombe* F449) is depicted in green; DIM-5 I263 (corresponding to *S. pombe* I418) is depicted in blue (PDB ID: 1PEG)(Zhang et al., 2003).
c, H3K9me2 ChIP-seq reads mapped to the pericentromeric repeat regions on the right arm of chromosome 1 in *clr4Δ*, *clr4*$^{+}$, *clr4*^{F449Y}, and *clr4*^{I418P} cells. The location of centromere 1 (*cen1*), innermost repeats (*imr1R*), outermost *dg* and *dh* repeats, and inverted repeat centromere (*IRC*) sequences are indicated. Chromosome 1 coordinates are indicated above the tracks and the sum of normalized reads mapping to chromosome 1 pericentromeric regions is indicated on the right. Data is presented as reads per million (rpm, Y axis).
d, Same as **c** but showing H3K9me3 ChIP-seq reads.
e, Left: Silencing of a *ura4*$^{+}$ transgene inserted within pericentromeric repeats (blue arrowheads below the map in **c** and **d**). Ten-fold serial dilution of cells with the indicated genotypes spotted on non-selective (N/S), minus uracil (- Ura) and 5-FOA-containing (+ FOA) medium. Right: qRT-PCR analysis of *otr1R::ura4*$^{+}$ transcript. Error bars, s.d.; n = 3 biological replicates.
f, Same as **c**, but showing pol II ChIP-seq reads.
g, qRT-PCR analysis of *dg* and *dh* transcripts. Values are shown as fold increase in RNA levels in mutant over *clr4*$^{+}$ cells. Error bars, s.d.; n = 3 biological replicates.

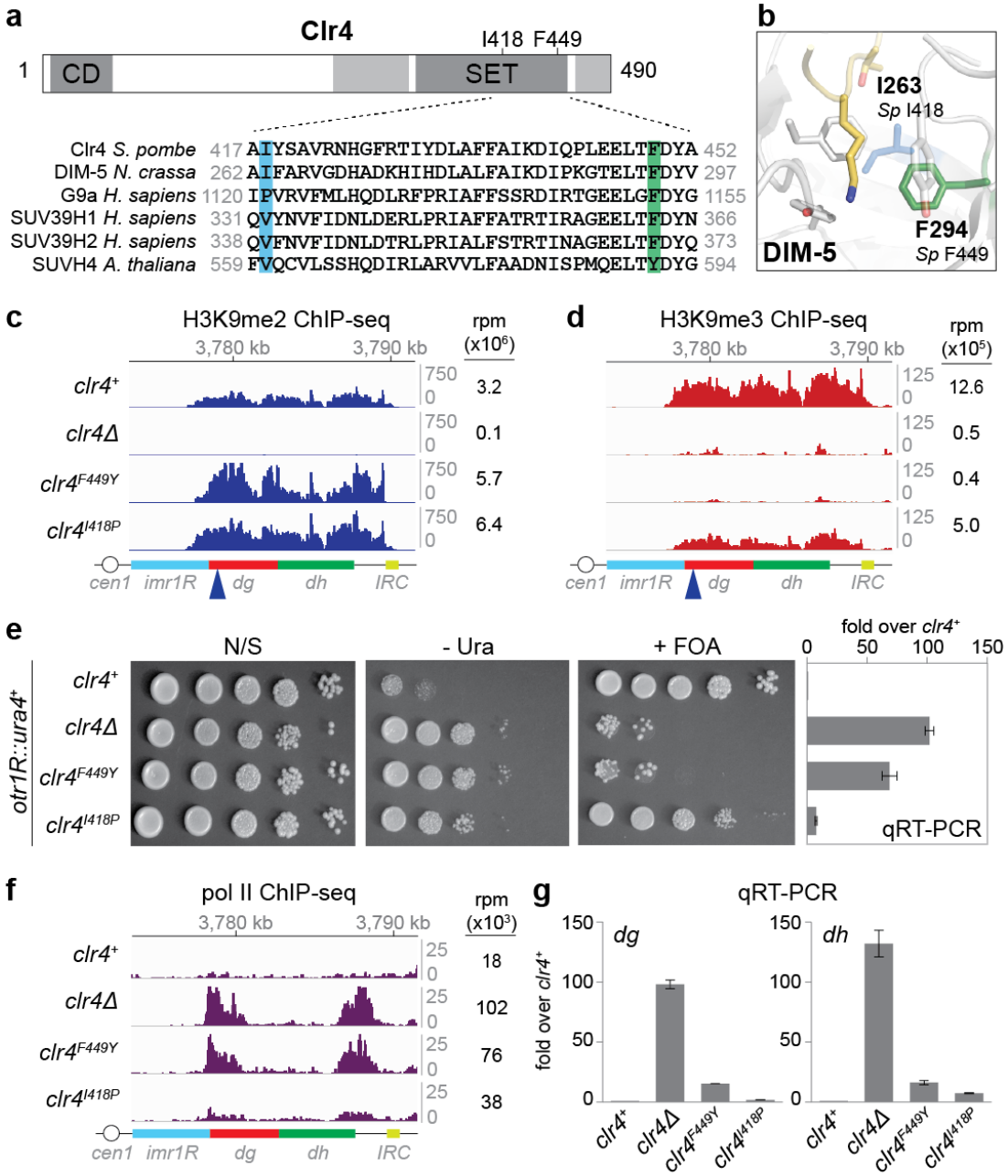


Figure 2.1 (Continued)

We constructed strains expressing endogenous levels of Clr4 protein with substitution of F449 to Y (*clr4*^{F449Y}) or I418 to P (*clr4*^{I418P}) (Figure 2.2) and performed ChIP-seq with well-characterized antibodies that recognize either H3K9me2 or H3K9me3 to test the effect of these substitutions on H3K9me states. While pericentromeric H3K9me2 levels were increased in *clr4* mutants relative to *clr4*$^{+}$ cells (Figure 2.1c), H3K9me3 was lost in *clr4*^{F449Y} cells and reduced in *clr4*^{I418P} cells (Figure 2.1d). The increase in H3K9me2 levels in the *clr4* mutants is likely due to available H3K9me2 that would otherwise be tri-methylated in *clr4*$^{+}$ cells. At the silent mating-type locus and telomeric DNA regions, both H3K9me2 and me3 were lost (Figure 2.3a-g), supporting previous findings suggesting that H3K9me3 is required for RNAi-independent spreading of heterochromatin away from nucleation sites at these regions (Al-Sady et al., 2013). We have therefore generated two *clr4* mutant alleles that affect H3K9me3 to different extents, providing us with tools to determine the role of histone H3K9me states in heterochromatin function.

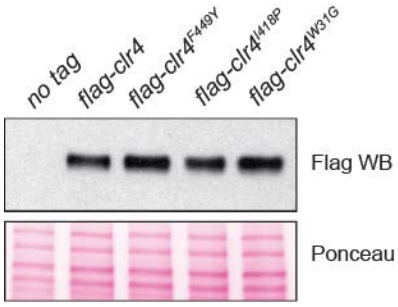


Figure 2.2 Mutant Clr4 proteins are stably expressed.
Western blot of N-terminal 3xFlag-tagged Clr4 showing SET mutations (F449Y or I418P) or a chromo domain mutation (W31G) do not affect Clr4 protein stability.

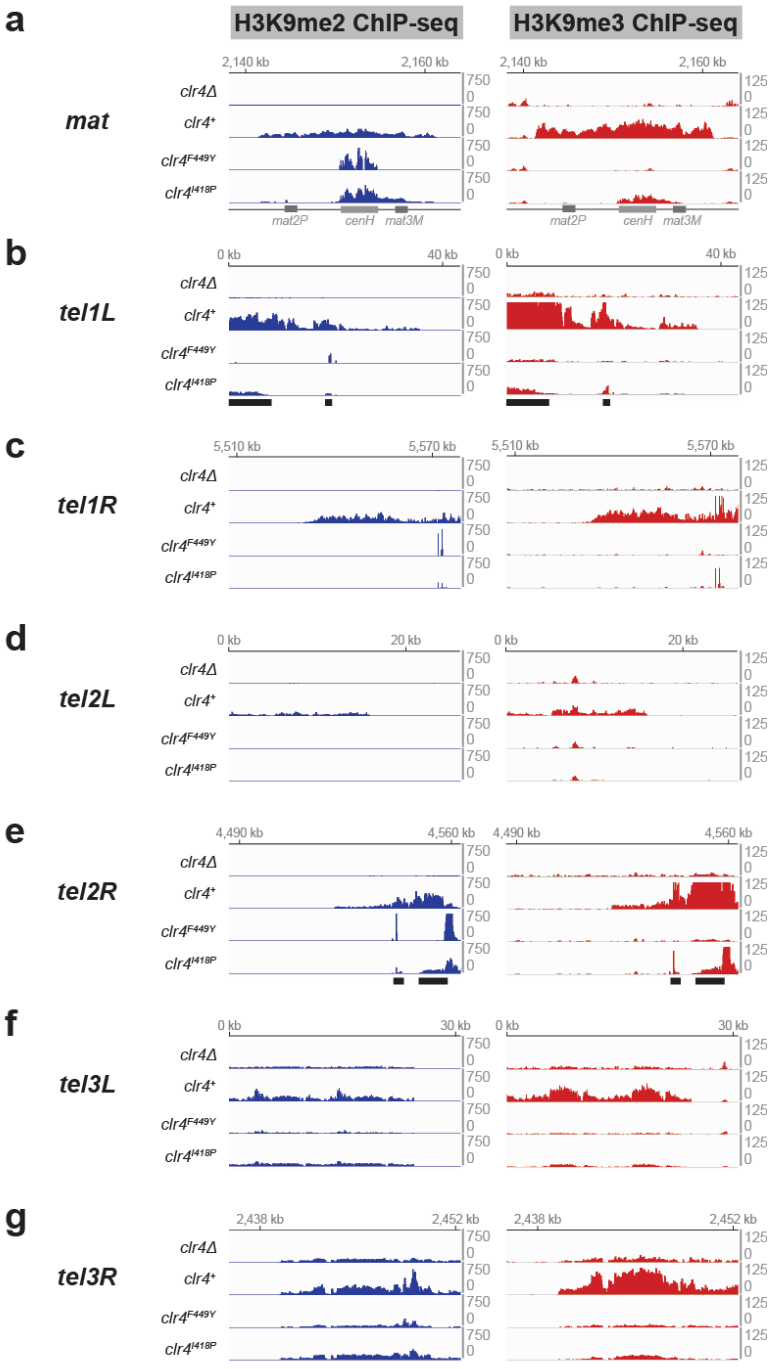


Figure 2.3 Clr4 mutants have reduced H3K9me spreading at mating type and telomeric regions.
a-g, ChIP-seq data showing changes in H3K9me2 and H3K9me3 levels outside of RNAi-dependent nucleation regions (indicated by solid black bars below tracks) at mating type (*mat*) and telomeric DNA regions (*tel1L, tel1R, tel2L, tel2R, tel3L, and tel3R*) in *clr4Δ*, *clr4*$^{+}$, *clr4*^{F449Y}, and *clr4*^{I418P} cells. *tel3L* and *tel3R* represent reads from the rDNA repeats. Data is presented as reads per million (Y axis).

To investigate the effects of H3K9me states on gene silencing, we examined the expression of a *ura4*$^{+}$ reporter gene inserted at the pericentromeric outer repeats of chromosome 1 (*otr1R::ura4*$^{+}$) in *clr4*$^{+}$, *clr4*Δ, *clr4*^{F449Y} or *clr4*^{I418P} cells. Silencing of *otr1R::ura4*$^{+}$ was monitored by growing cells on medium lacking uracil (– Ura) or containing 5-fluoroorotic acid (+ FOA), which is toxic to cells expressing *ura4*$^{+}$, and quantitative RT-PCR (qRT-PCR). Similar to *clr4*Δ cells, *clr4*^{F449Y} cells displayed increased growth on – Ura plates and grew poorly on + FOA plates (Figure 2.1e, left) indicating a complete loss of *otr1R::ura4*$^{+}$ silencing. *clr4*^{I418P} had a milder silencing defect, which was consistent with partial loss of H3K9me3 in this mutant (Figure 2.1d, e). In agreement with the growth silencing assay, *otr1R::ura4*$^{+}$ transcripts were derepressed in *clr4*^{F449Y} and to a lesser extent in *clr4*^{I418P} cells (Figure 2.1e, right). Furthermore, we observed greatly increased RNA polymerase II (pol II) occupancy at the endogenous pericentromeric *dg* and *dh* repeats with *clr4*^{F449Y} cells having similar levels of pol II occupancy to that of *clr4*Δ cells (Figure 2.1f). Surprisingly, despite the observed loss of TGS at the *dg* and *dh* repeats in *clr4*^{F449Y} cells, dg and *dh* transcript levels were 10- to 15-fold lower in *clr4*^{F449Y} cells than in *clr4*Δ cells (Figure 2.1g). Together with the absence of detectable H3K9me3 in *clr4*^{F449Y} cells (Figure 2.1d), this result suggests that H3K9me2 may be sufficient for promoting co-transcriptional gene silencing by RNAi.

Transcription-permissive H3K9me2 recruits RNAi and precedes H3K9me3 establishment

To directly test the aforementioned possibility (Figure 2.4a), we examined the levels of pericentromeric siRNAs as well as the pericentromeric recruitment of the RITS complex subunit Chp1 in the Clr4 SET domain mutants. Consistent with our hypothesis, in both *clr4*^{F449Y} and *clr4*^{I418P} cells, *dg* siRNA levels were similar or higher than in *clr4*$^{+}$ cells and *dh* siRNA levels were only reduced by about 2-fold, while, as expected, a drastic loss of *dg* and *dh* siRNAs was observed in *clr4*Δ (Figure 2.4b)(Buhler et al., 2006; Motamedi et al., 2004). Furthermore, Chp1 association with pericentromeric DNA repeats was increased at all three chromosomes in both

clr4^{F449Y} or *clr4*^{I418P} cells, presumably due to increased H3K9me2 (Figure 2.4c and Figure 2.5a, b). Together these results demonstrate that H3K9me2 is sufficient for the activation of chromatin-dependent RNAi (Figure 2.4a), and suggest that the silencing of endogenous pericentromeric *dg* and *dh* transcripts we observed in cells lacking H3K9me3 (Figure 2.1g) is due to H3K9me2- and RNAi-dependent co-transcriptional degradation of RNAs transcribed from pericentromeric repeats.

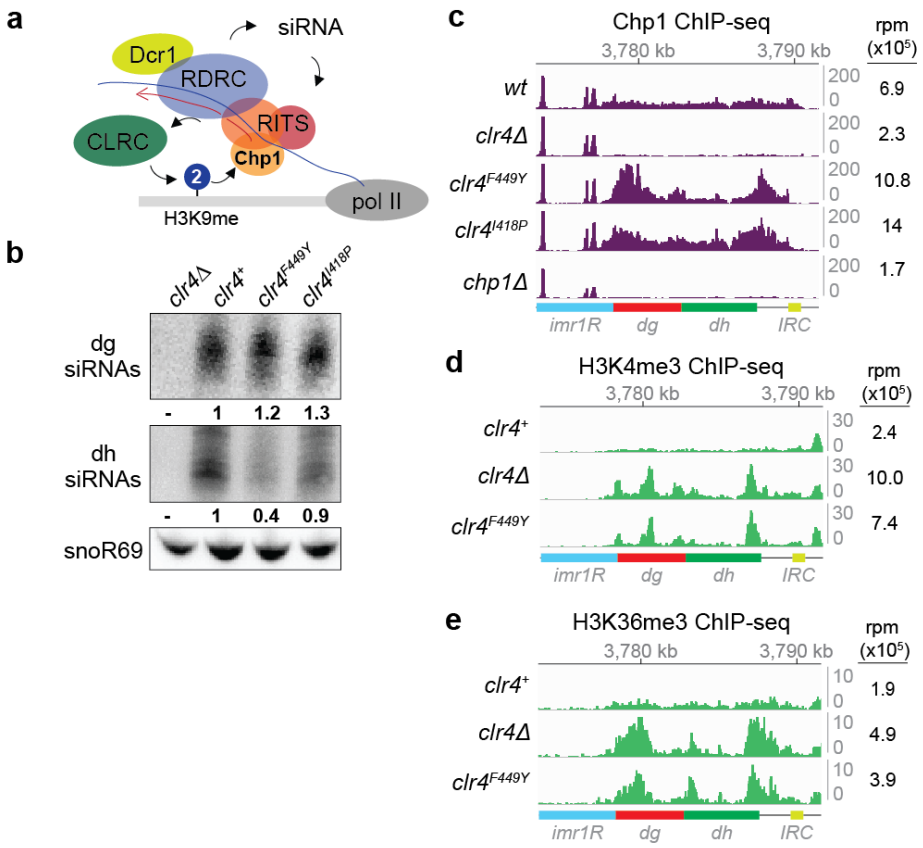


Figure 2.4 Transcription-permissive H3K9me2 recruits RNAi.
a, Model for recruitment of the RNAi machinery (RITS, RDRC, and Dcr1) to a nascent pericentromeric transcript to mediate siRNA amplification and Clr4-dependent H3K9 methylation.
b, Northern blot of *dg* and *dh* siRNAs in cells with the indicated genotypes. Ratios are determined by fold increase in siRNA levels in mutants over wild-type (*wt*) cells. Northern blot of snoR69 was used as an internal control.
c, Chp1 ChIP-seq reads mapped to the pericentromeric repeat regions on the right arm of chromosome 1. The sum of normalized reads mapped to pericentromeric region to the right of cen1 is indicated on the right. Data is presented as reads per million (rpm, Y axis).
d, e, Same as **c**, but showing H3K4me3 (**d**) and H3K36me3 (**e**) ChIP-seq reads.

Since increased pol II occupancy was associated with high levels of H3K9me2 (Figure 2.1c, f), we tested whether H3K9me2 heterochromatin domains contain histone modifications that are linked to transcription(Li et al., 2007). We observed increased levels of transcription-associated modifications, H3K4me3, H3K36me3, H3K14 acetylation (H3K14ac), and H4K16 acetylation (H4K16ac) in *clr4*^{F449Y} relative to *clr4*$^{+}$ cells (Figure 2.4d, e and Figure 2.5c, d). Additionally, using quantitative mass spectrometry (MS) analysis of purified nuclear histones to directly assess histone H3K9me states, we observed a nearly 1-to-1 ratio of H3K9me2 and H3K9me3 in *clr4*$^{+}$ cells (Figure 2.6a, b). Moreover, while 30% of the H3K9me2 peptide contained K14ac, no K14ac was associated with H3K9me3 peptides (Figure 2.6b). We note that high levels of H3K9me1 were detected in both *clr4*$^{+}$ and *clr4*Δ cells (Figure 2.6b), suggesting that fission yeast harbor another enzyme(s) that catalyzes H3K9me1. These results demonstrate that H3K9me2 domains are associated with transcription-coupled histone modifications commonly found in euchromatic domains and raise the possibility that transcriptionally permissive H3K9me2 domains may precede H3K9me3 and transcriptional gene silencing during heterochromatin establishment.

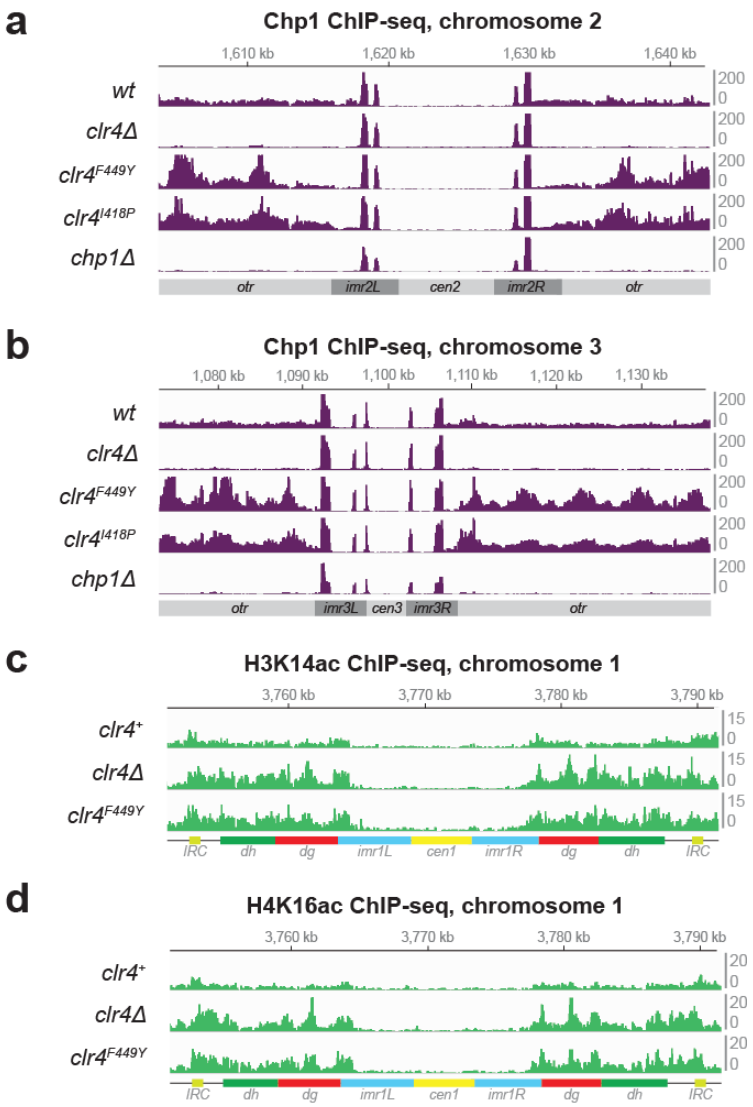
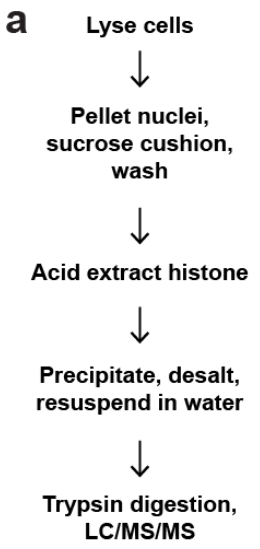


Figure 2.5 Clr4 mutant cells have increased levels of Chp1, H3K14ac, and H4K16ac at pericentromeric DNA repeats.
a, ChIP-seq data showing increased Chp1 reads mapping to pericentromeric regions of chromosome 2 in *clr4*^{F449Y} and *clr4*^{I418P} mutants compared to wild-type (*wt*) cells.
b, Same as **a**, but showing pericentromeric regions of chromosome 3.
c, ChIP-seq data showing increased H3K14ac mapped reads at pericentromeric regions of chromosome 1 in *clr4*^{F449Y}.
d, ChIP-seq data showing increased H4K16ac mapped reads at pericentromeric regions of chromosome 1 in *clr4*^{F449Y}. Data is presented as reads per million (Y axis).



b

H3 9-17: KSTGGKAPR	Normalized % *wt*	*clr4*Δ
unmodified	83.36	74.60
K9me1	6.40	15.06
K9me2	0.66	0
K9me3	1.02	0
K9ac	3.94	4.68
K14ac	2.38	2.84
K9me1, K14ac	1.86	2.63
K9me2, K14ac	0.30	0.06
K9me3, K14ac	0	0
K9ac, K14ac	0.09	0.14

Figure 2.6 Quantitative mass spectrometry of histone H3 tail modifications.
a, Scheme of histone purification
b, Quantitative mass spectrometry analysis of modifications associated with the indicated H3 tail peptide in wild-type (*wt*) and *clr4Δ* cells.

To test this hypothesis, we examined whether H3K9me2 precedes H3K9me3 during *de novo* RNAi-dependent heterochromatin establishment in wild-type cells. We treated *S. pombe* cells with Trichostatin A (TSA), a histone deacetylase inhibitor previously shown to disrupt heterochromatin (Ekwall et al., 1997), and harvested them at several time points after TSA removal (Figure 2.7a). Successful re-establishment of heterochromatin was demonstrated by gradual and partial restoration of silencing at the *otr1R::ura4*$^{+}$ transgene over a period of several hours (Figure 2.7b). Consistent with our hypothesis, H3K9me2 levels were rapidly restored to the same levels as untreated cells by 3 to 5 hours after TSA removal (Figure 2.7c, e; area highlighted in darker blue indicate the region with highest loss of H3K9me2 resulting from TSA treatment). In contrast, H3K9me3 levels displayed slower recovery kinetics and, like transgene silencing (Figure 2.7b), were not fully restored even 7 hours after TSA removal (Figure 2.7d, e). These results are consistent with a model wherein the establishment of H3K9me2 domains, which are transcriptionally permissive (Figure 2.1c, f; 3rd row), precedes H3K9me3 and transcriptional silencing. Temporal separation between H3K9me2 and H3K9me3 may allow the

establishment of large domains of transcription- and RNAi-dependent H3K9me throughout several kilobases of pericentromeric chromatin prior to silencing of transcription – which, if it occurred too early, could interfere with siRNA-mediated spreading.

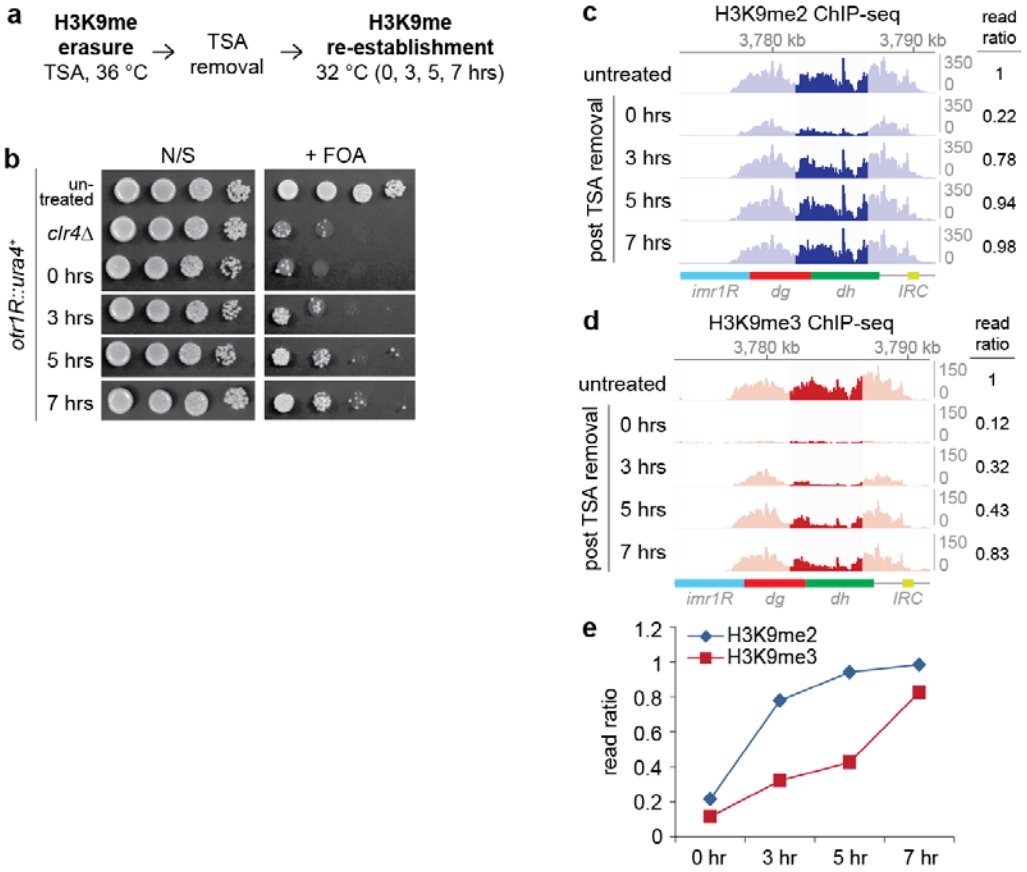


Figure 2.7 Transcription-permissive H3K9me2 precedes H3K9me3 establishment.
a, Experimental strategy for *de novo* H3K9me establishment.
b, Spotting of 10-fold serial diluted cells on non-selective (N/S) and FOA-containing (+ FOA) medium to evaluate re-establishment of *otr1R::ura4*$^{+}$ silencing at 0, 3, 5, 7 hours after TSA removal. Untreated and *clr4Δ* cells serve as positive and negative control for *otr1R::ura4*$^{+}$ silencing, respectively.
c, H3K9me2 ChIP-seq reads mapped to pericentromeric repeats to the right of *cen1* in untreated and TSA-treated cells at the indicated time points following TSA removal. The highlighted region displayed the greatest loss of H3K9 methylation resulting from TSA treatment. Read ratio (indicated on the right) was obtained by normalizing the sum of reads mapping to the highlighted region for TSA-treated compared to untreated cells.
d, Same as **c**, but showing H3K9me3 ChIP-seq reads.
e, Graph showing plotted read ratios of **h** and **i**, colored in blue and red for H3K9me2 and H3K9me3, respectively.

H3K9me states regulate the recruitment of HP1 proteins and Clr4.

Efficient silencing at pericentromeric repeat regions requires the recruitment of heterochromatin protein 1 (HP1) proteins, Swi6 and Chp2, which bind to H3K9me and promote TGS at least in part by recruiting the Clr3 histone deacetylase complex (Motamedi et al., 2008; Sugiyama et al., 2007). We next explored how H3K9me states affect the recruitment of those downstream factors that, unlike Chp1, are not required for H3K9me establishment (Figure 2.8a, b). In *clr4*^{F449Y} cells, Swi6 recruitment to pericentromeric DNA regions was reduced by about 3-fold (Figure 2.9a), while Chp2 recruitment was slightly impaired (Figure 2.9b). Therefore, loss of H3K9me3 reduced Swi6 and Chp2 recruitment to pericentromeric DNA but increased Chp1 recruitment (Figure 2.4c). To determine whether this differential recruitment of chromo domain proteins was due to their differing affinities for H3K9me2 and H3K9me3 for chromo domain proteins, we examined the ability of unmodified H3 (H3K9me0), H3K9me2, and H3K9me3 peptides to associate with chromo domain proteins in *S. pombe* extracts in which binding can be assessed in the context of each protein's association with its native binding partners. Consistent with the ChIP-seq data (Figure 2.4c and Figure 2.9a, b), quantitative mass spectrometry using isobaric mass tags showed that Chp1 and Chp2 were bound to H3K9me2 and H3K9me3 peptides with similar efficiency, while Swi6 associated with H3K9me3 peptides more efficiently than with H3K9me2 peptides (Figure 2.10a-c). Together with previous findings(Schalch et al., 2009), these results suggest that efficient recruitment of Chp1/RNAi and reduced recruitment of Swi6 to pericentromeric DNA repeats in *clr4*^{F449Y} cells is due to their different affinities for H3K9me2 relative to H3K9me3, and provide an explanation for efficient H3K9me2-dependent RNAi versus H3K9me3-dependent TGS. Furthermore, consistent with the reduced Swi6 and Chp2 recruitment to pericentromeric DNA repeats in *clr4*^{F449Y} cells, we observed a reduction in Clr3 recruitment to the same regions in *clr4*^{F449Y} cells (Figure 2.9c). Therefore, defective TGS, associated with the loss of H3K9me3, results at least in part from a defect in HP1 and Clr3 recruitment.

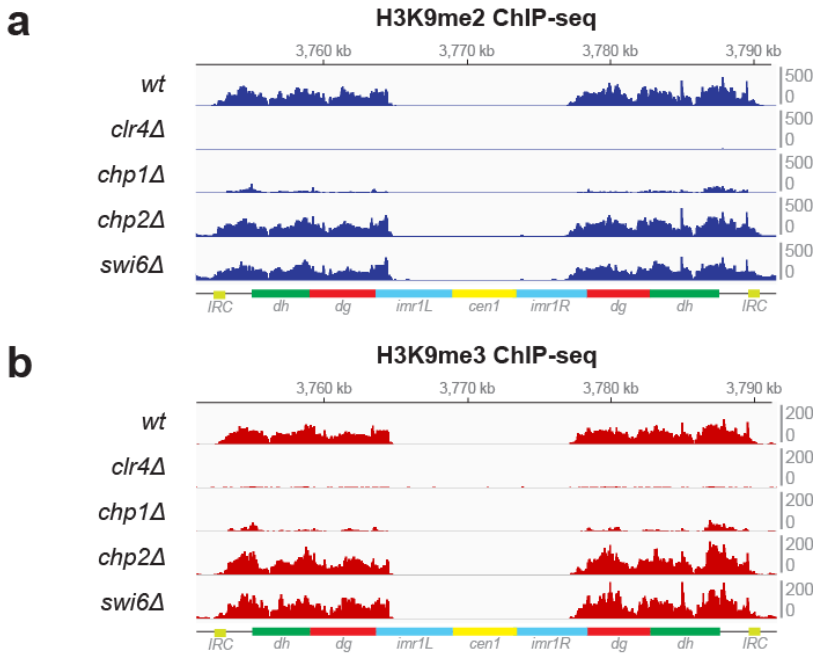


Figure 2.8 Chp2 and Swi6 are not required for the formation of H3K9me2 or me3 domains at pericentromeric DNA repeats.
a-b, ChIP-seq data showing that unlike Chp1, Chp2 and Swi6 are not required for RNAi-mediated H3K9 methylation, as indicated by similar levels of H3K9me2 (**a**) and H3K9me3 (**b**) mapped reads at pericentromeric regions of chromosome 1 in wild-type (*wt*), *chp2Δ*, and *swi6Δ* cells. *clr4Δ* serves as a control for specificity of the anti-H3K9me antibodies. Data is presented as reads per million (Y axis).

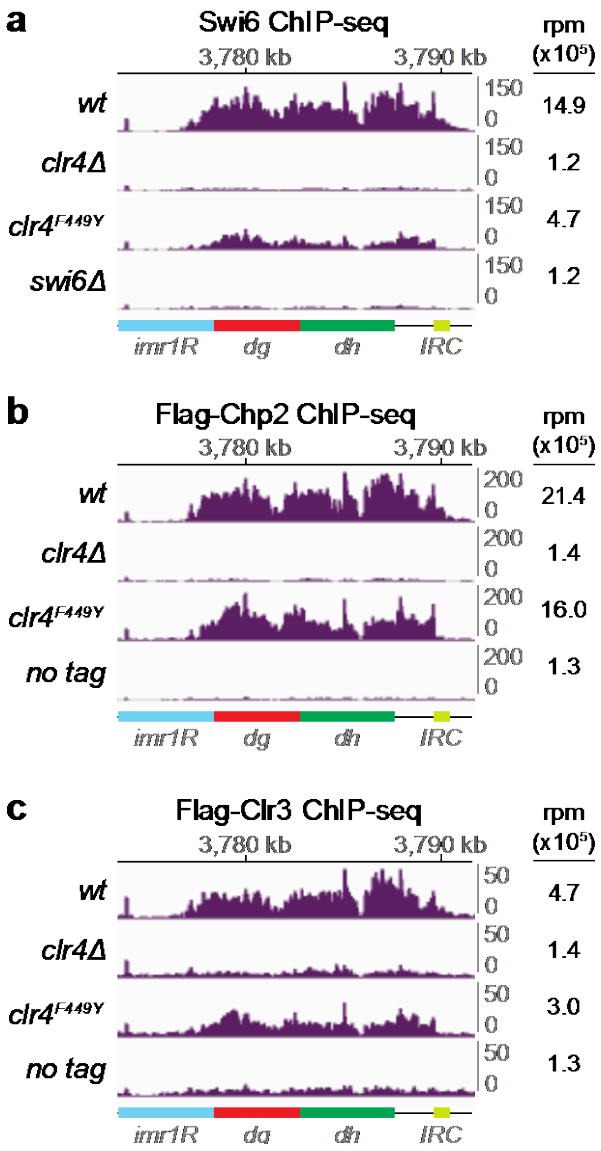


Figure 2.9 H3K9me states regulate the recruitment of HP1 proteins and Clr4.
a-c, Swi6 (**a**), Flag-Chp2 (**b**), and Flag-Clr3 (**c**) ChIP-seq reads mapped to pericentromeric repeats on the right arm of chromosome 1. The sum of normalized reads is indicated on the right. Data is presented as reads per million (rpm, Y axis).
d-f, H3K9me3 (**d**), H3K9me2 (**e**), and Flag-Clr4 (**f**) ChIP-seq reads mapped to pericentromeric repeats on the right arm of chromosome 1.

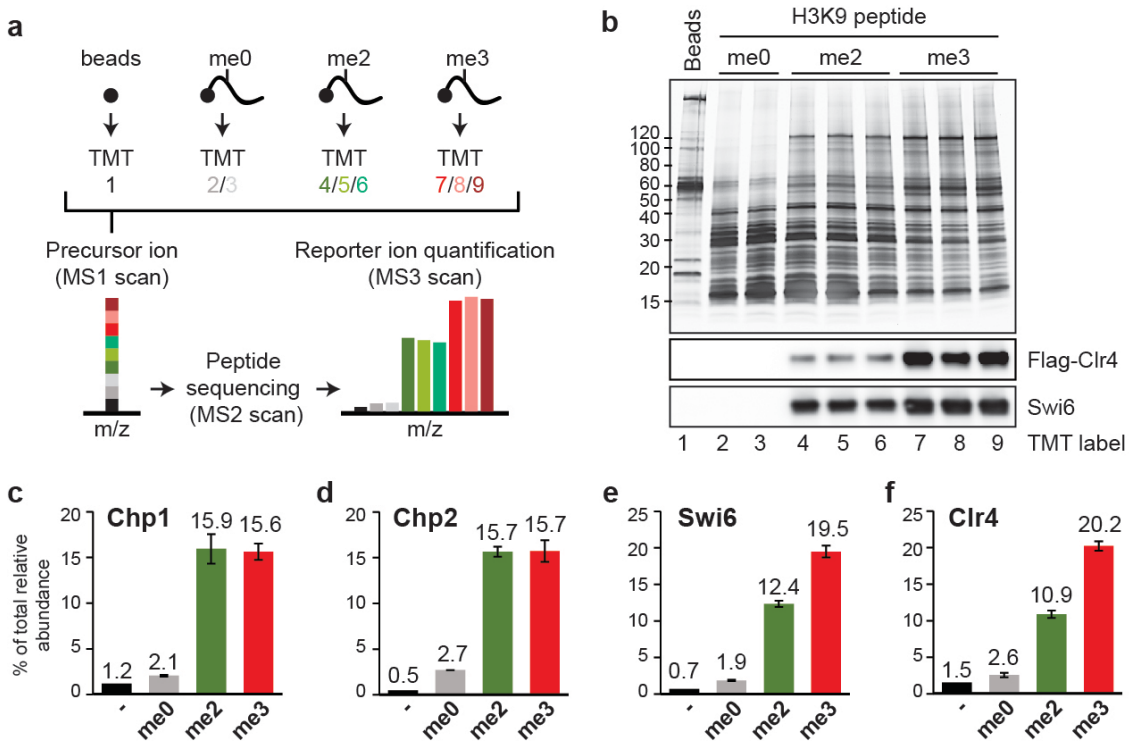


Figure 2.10 Association of chromo domain proteins with differentially methylated H3K9 peptides.
a, Scheme of peptide pull down assay followed by quantitative mass spectrometry.
b, Silver stain, Flag-Clr4 and Swi6 western blot showing protein mixture isolated by H3 tail peptides carrying H3K9me0, me2, or me3.
c, Identification and quantification of proteins associated with different H3 tail peptides using quantitative mass spectrometry.

We next investigated the mechanism that governs the transition from H3K9me2 to H3K9me3, and therefore TGS, by examining factors that contribute to Clr4 recruitment. Clr4 localizes to pericentromeric DNA repeats by interaction with the RNAi machinery as well as through the interaction of its chromo domain with H3K9me(Bayne et al., 2010; Gerace et al., 2010b; Zhang et al., 2008a). Moreover, Clr4^{W31G}, containing a chromo domain mutation, has lower affinity for H3K9me2 and H3K9me3 and cells carrying this mutation have reduced levels of H3K9me3[16,27]. The chromo domain of Clr4 may therefore increase Clr4 residence time on chromatin to promote the transition from H3K9me2 to H3K9me3[27]. Consistent with previous findings, we observed a drastic loss of H3K9me3 but not H3K9me2 in *clr4*^{W31G} cells, relative to

clr4^{+} cells, throughout pericentromeric DNA repeats (Figure 2.11a, b), as well as greatly reduced association of Clr4^{W31G} itself with chromatin (Figure 2.11c). In contrast, Clr4^{F449Y} associated with pericentromeric DNA repeats at levels close to that of wild-type Clr4 (Figure 2.11c). Since *clr4*^{F449Y} cells, like *clr4*^{W31G} cells, lack H3K9me3 but have high levels of H3K9me2 (Figure 2.11a, b), we conclude that H3K9me2 is sufficient for RNAi-mediated recruitment of Clr4 to pericentromeric DNA repeats. Together, these results suggest that following the establishment of H3K9me2 by RNAi, the association of the chromo domain of Clr4 with H3K9me2 is required for transition to H3K9me3 and TGS.

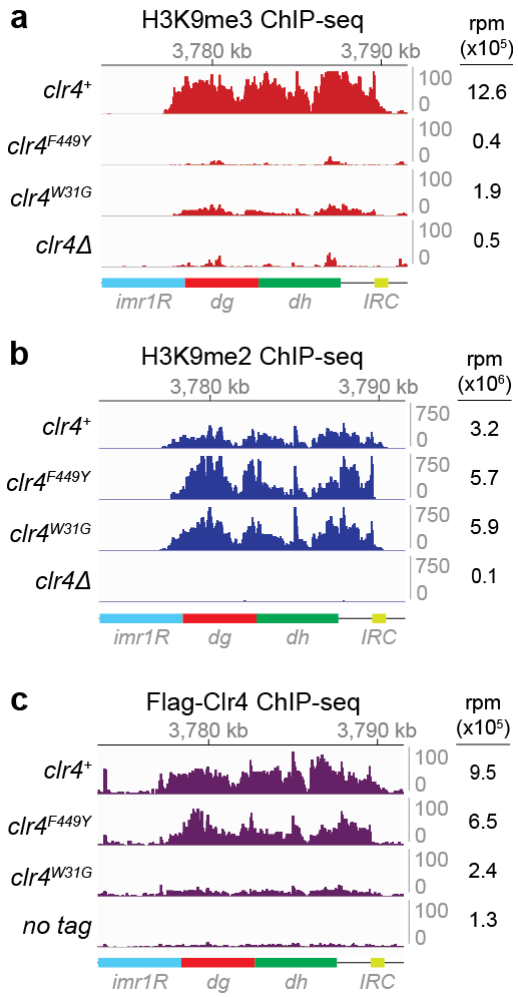


Figure 2.11 H3K9me states regulated by the recruitment of Clr4.
a-c, H3K9me3 (**a**), H3K9me2 (**b**), and Flag-Clr4 (**c**) ChIP-seq reads mapped to pericentromeric repeats on the right arm of chromosome 1. The sum of normalized reads is indicated on the right. Data is presented as reads per million (rpm, Y axis).

Discussion

In this study we have investigated whether histone H3K9me2 and H3K9me3 states play distinct roles in heterochromatin function. Our findings reveal that H3K9me2 is sufficient for the stable association of RNAi with pericentromeric DNA repeats and for efficient siRNA amplification, leading to degradation of target nascent transcripts and co-transcriptional gene silencing (CTGS). Thus, rather than a mere intermediate in the synthesis of H3K9me3, H3K9me2 defines a chromatin state that is transcription- and RNAi-permissive and mediates silencing by promoting the co-transcriptional degradation of target RNA. On the other hand, transition to H3K9me3 is required for achieving the most stringent levels of silencing, involving the efficient recruitment of HP1 proteins and transcriptional gene silencing (TGS). These results uncover the coordination of two silencing mechanisms to achieve heterochromatin assembly in S. pombe (Figure 2.12) and have implications for mechanisms of gene silencing that rely on H3K9me, a broadly conserved histone modification in eukaryotes.

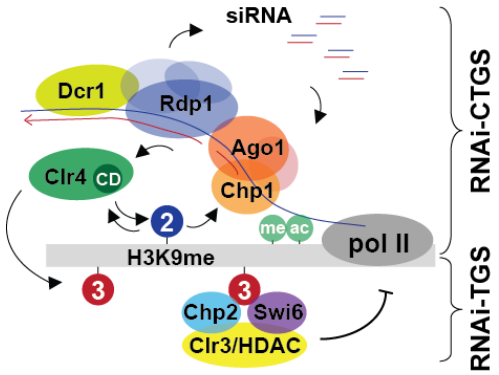


Figure 2.1 Model for RNAi-mediated heterochromatin assembly.
Model highlighting the role of H3K9 methylation states in RNAi-mediated silencing and heterochromatin assembly. Top: Initial RNAi-mediated recruitment of Clr4 promotes formation of extended H3K9me2 domains, which are transcriptionally permissive and contain transcription-associated histone marks (green circles labeled with ac, me). Bottom: The association of the chromo domain (CD) of Clr4 with H3K9me2 is required for the formation of H3K9me3 domains, which efficiently recruit HP1 proteins to promote TGS.

H3K9 methylation states mediate distinct heterochromatic function

Chromatin immunoprecipitation studies in a broad range of organisms that contain H3K9 methylation indicate the presence of both H3K9me2 and H3K9me3 in heterochromatic DNA domains, although H3K9me3 appears to be the predominant form associated with constitutive heterochromatin (Barski et al., 2007; Hawkins et al., 2010; Peters et al., 2003; Zhu et al., 2013). TGS is generally thought to be the major mechanism of silencing within these domains. However, the discovery that RNAi mediates heterochromatin formation, together with the observation that the RNAi machinery is localized to sites of heterochromatin formation, raised the possibility that RNAi-mediated degradation of nascent transcripts also contributes to silencing (Motamedi et al., 2004; Verdel et al., 2004; Volpe et al., 2002). Support for this co-transcriptional mechanism of silencing (CTGS) came from the observation that ectopic silencing induced by tethering the RITS complex to RNA of the *ura4*$^{+}$ reporter gene, which required both RNAi components and H3K9 methylation, was accompanied by little or no reduction in RNA polymerase II occupancy the targeted *ura4*$^{+}$ locus (Buhler et al., 2006). In this study, our ability to create a Clr4 enzyme that can perform H3K9 di-methylation, but not tri-methylation, led to the discovery that H3K9me2 is sufficient for full chromosome-associated RNAi and furthermore, promotes RNAi-dependent silencing of target pericentromeric *dg* and *dh* transcripts independently of TGS. Remarkably, in the absence of Clr3-mediated TGS, H3K9me2- and RNAi-dependent silencing results in a 10- to 15-fold reduction in *dg* and *dh* transcript levels (Figure 2.1g). The ability of H3K9me2 to promote RNAi suggests that it can efficiently recruit the Chp1 subunit of the RITS complex and promote its localization on chromatin. Consistent with this hypothesis, Chp1 from cell extracts binds to histone H3K9me2 and H3K9me3 peptides with similar efficiency and is efficiently recruited to pericentromeric repeat regions in the absence of detectable H3K9me3. We therefore conclude that H3K9me2 is transcriptionally permissive and when coupled to RNAi, can mediate CTGS.

Our studies reveal a specific requirement for the transition from H3K9me2 to H3K9me3 in TGS. This is likely due to the higher affinity of Swi6, and possibly Chp2, for H3K9me3 relative to H3K9me2. Consistent with previous studies (Schalch et al., 2009), our affinity pull-down experiments indicate that Swi6 binds more efficiently to H3K9me3 than H3K9me2 peptides. Swi6 and Chp2 mediate the recruitment of the Clr3-containing HDAC complex, SHREC, which is required for TGS, and their lower affinity for H3K9me2 relative to H3K9me3 provides an explanation for the observed loss of TGS in in clr4F449Y cells. Our findings also provide unambiguous support for the previously proposed role of H3K9me3 in RNAi-independent spreading, based on diminished spreading of H3K9 methylation in *clr4*^{W31G} mutant cells, which have reduced H3K9me3 levels (Al-Sady et al., 2013; Zhang et al., 2008). This spreading defect likely explains the dramatic loss of H3K9me at the mat locus, outside of the siRNA-producing cenH region, and at the telomeres, in *clr4*^{F449Y} cells. Together, these studies demonstrate two distinct roles for Clr4-mediated H3K9 tri-methylation, involving transcriptional gene silencing and spreading of H3K9me, and suggest that these roles are mediated by recruitment of the chromo domain-containing HP1 proteins, Swi6 and Chp2, and Clr4 itself. Histone H3K9me is recognized by multiple readers that include HP1 proteins and other chromo domain-containing proteins, and readers containing other conserved domains such as the PHD, BAH, and ankyrin repeats (Patel and Wang, 2013). Fine-tuning of the affinities of these readers for different H3K9 methylation states, together with their coupling to different effector modules, may allow H3K9 methylation states to bring about distinct organism- and cell type-dependent silencing mechanisms.

Implications for the mechanism of transcription- and sRNA-dependent histone H3K9 methylation

Transcription serves two critical functions in RNAi-mediated heterochromatin assembly in *S. pombe*. First, it provides a source for the generation of siRNAs, which feedback on the

chromosome and specifically mediate heterochromatin assembly at complementary chromosome regions. The transcription-dependent mechanisms that produce the initial trigger small RNA have been defined and involve the activity of the Dicer ribonuclease on double stranded RNA generated from base pairing of sense and anti-sense transcripts to generate siRNAs, and other ribonucleases that act on single stranded transcripts and generate degradation products that form priRNAs (Halic and Moazed, 2010; Marasovic et al., 2013; Yu et al., 2014). Second, transcription produces the nascent noncoding pericentromeric *dg* and *dh* transcripts (cenRNAs) that acts as templates or scaffolds for recruitment of the RITS complex, which then mediates H3K9 methylation by recruiting Clr4, and siRNA amplification by recruiting RDRC and Dicer (Castel and Martienssen, 2013; Holoch and Moazed, 2015a). TGS may need to be tightly regulated to allow enough transcription for siRNAs to be continuously replenished in order to reestablish H3K9me domains after DNA replication and cell division. Furthermore, the de novo formation of large domains of transcription- and siRNA-dependent H3K9 methylation may necessitate a period permissive to transcription, and may therefore be impeded by rapid or premature TGS. Pulses of transcription during the S phase have been proposed to provide the siRNA source for heterochromatin re-establishment during cell division (Chen et al., 2008; Kloc et al., 2008). We propose that the slower kinetics of de novo H3K9me3 establishment relative to H3K9me2 establishment (this study) allow the siRNA- and cenRNA-dependent spreading of H3K9me prior to the onset of H3K9me-dependent TGS.

The separation of H3K9me-dependent siRNA generation from TGS may also be critical for generation of large amounts of siRNAs to act as trans-acting genome-wide defense agents. Although *S. pombe* does not contain active dispersed transposons that are targeted by RNAi, the pericentromeric repeat regions are thought to be transposon remnants (Rhind et al., 2011). These regions may therefore have evolved from DNA repeats that produced siRNAs with general genome defense functions, akin to Drosophila and mammalian unique piRNA clusters, which produce piRNAs that target dispersed transposons (Czech and Hannon, 2016). In this

regard, the Drosophila SETDB1 methyltransferase is broadly required for piRNA generation from both the uni-strand and dual-strand piRNA clusters (Rangan et al., 2011). The relationship between H3K9 methylation and sRNA biogenesis from the dual-strand clusters are complex as SETDB1 as well as an HP1 protein, called Rhino, are required for transcription of the piRNA precursors (Klattenhoff et al., 2009; Mohn et al., 2014; Rangan et al., 2011). However, the situation at the uni-strand clusters may be similar to what we have described here in S. pombe as SETDB1 is required for piRNA generation but not transcription at these clusters (Rangan et al., 2011). Uni-strand clusters appear to contain very low levels of H3K9me3 and are enriched for RNA pol II and H3K4me2 (Mohn et al., 2014), raising the possibility that, similar to S. pombe pericentromeric DNA repeats in clr4F449Y cells, they may be enriched for H3K9me2. Therefore, despite major differences in the mechanisms of siRNA biogenesis in S. pombe and piRNA generation in Drosophila and mammals, the coupling of sRNA precursor transcription to histone H3K9 methylation states and the proteins that recognize these states, is likely to be an evolutionarily conserved feature. In this regard, Windei, the Drosophila homolog of mAM, a human protein required for SETDB1-mediate H3K9 tri-methylation in vitro, is required for piRNA-mediated transposon silencing (Sienski et al., 2015; Yu et al., 2015), raising the possibility that H3K9 methylation states may be actively regulated in Drosophila and mammals.

Materials and methods

Strain construction

Strains used in this study are listed in Table 2.1. All Flag-tagged genes were expressed under the control of their endogenous promoters and terminators.

Table 2.1. List of strains used in this chapter.

Strain	Genotype	Source
SPY137	*h$^+$ leu1-32 ade6-M210 ura4-DS/E otr1R(SphI)::ura4$^+$*	2
SPY815	SPY137 *clr4Δ::kanMX6*	3
SPY1098	SPY137 *swi6Δ::natMX6*	5
SPY1368	SPY137 *chp2Δ::kanMX6*	4
SPY2421	SPY137 *chp1Δ::TAP-kanMX6*	1
SPY4719	SPY137 *ago1Δ::kanMX6*	1
SPY4636	SPY137 *hphMX6-5'(1kb)-3xflag-clr4*	1
SPY4642	SPY137 *hphMX6-5'(1kb)-3xflag-clr4^{F449Y}*	1
SPY5562	SPY137 *kanMX6-5'(1kb)-3xflag-clr4^{W31G}*	1
SPY6537	SPY137 *hphMX6-5'(1kb)-3xflag-clr4^{I418P}*	1
SPY5378	SPY137 *hphMX6-5'(700bp)-3xflag-chp2*	1
SPY5380	SPY137 *hphMX6-5'(700bp)-3xflag-chp2 clr4Δ::kanMX6*	1
SPY5382	SPY137 *hphMX6-5'(700bp)-3xflag-chp2 kanMX6-5'(1kb)-clr4^{F449Y}*	1
SPY5593	SPY137 *hphMX6-5'(900bp)-3xflag-clr3*	1
SPY5595	SPY137 *hphMX6-5'(900bp)-3xflag-clr3 clr4Δ::kanMX6*	1
SPY5597	SPY137 *hphMX6-5'(900bp)-3xflag-clr3 kanMX6-5'(1kb)-clr4^{F449Y}*	1

1 = this study; 2 = Karl Ekwall; 3 = Hong *et al.*, 2005; 4 = Motamedi *et al.,* 2008; 5 = Li *et al.,* 2009

Growth Assays

Cells were grown in YES to log phase, 1 x 10^7 cells were pelleted, suspended in 100 µl of water, and then serially diluted 10 fold. 3 ul of each dilution was spotted on the appropriate growth medium. FOA was used at 1 mg/ml. Plates were incubated at 32 °C for 3-4 days.

H3K9me Erasure by TSA and Recovery

Cells were inoculated in 50 ml YEA containing 25 µg/ml TSA and grown at 36 °C for 15 hours. Cells were then pelleted and washed to remove TSA, suspended in YEA at 32 °C to reestablish heterochromatin, and harvested at 0, 3, 5, and 7 hour for ChIP analysis.

Chromatin Immunoprecipitation (ChIP)

ChIP was performed as previously described (Yu et al., 2014). Conditions for fixation and immunoprecipitation (IP) are briefly described here. 50 ml cultures of cells were grown overnight in YES to an optical density (OD) of 2 and fixed as follows. For H3K9me2, H3K9me3, and Chp1 ChIP, cell were fixed with 1% formaldehyde for 15 min. For Swi6 and Flag-protein ChIP, we used a modified dual-crosslinking protocol in which cells were first incubated at 18 °C for two hours, resuspended in 5 ml of room temperature PBS, and fixed with 1.5 mM ethylene glycol bis-succinimidyl succinate (EGS, Thermo Scientific #21565) for 30 min, followed by addition of formaldehyde to 1% final concentration for another 30 min (Zeng et al., 2006). For each IP, ~ 2 µg of antibody was pre-incubated with 30 µl of Invitrogen Dynabeads Protein A or Protein G: anti-H3K9me2 (Abcam 1220) with Protein A, anti-H3K4me3 (Millipore 04-745) with protein G, anti-H3K36me3 (Abcam 9050) with protein A, anti-H4K16ac (Active Motif 39167) with protein A, anti-Chp1 (Ab18191) with protein A, anti-Flag (Sigma F1804) with protein G, anti-Swi6 (rabbit polyclonal) with protein A, anti-RNA pol II 8WG16 (BioLegend MMS-126R-500) with protein A. For IP with H3K9me3 antibody, Dynabeads M-280 Streptavidin beads were first incubated with 1 µg of anti-H3K9me3 (Diagenode C15500003), followed by blocking with 5 µM biotin. 500 µl (for ChIP-seq) or 100 µl (for ChIP-qPCR) of sheared chromatin lysate was added to the antibody-bead mixture and incubated for 2 hr at 4 °C on a rotating device. After reversing cross-links and DNA clean-up, qPCR was performed using primers listed in Table 2.2. *fbp1*$^{+}$ was used as internal control in pol II ChIP-qPCR.

Library preparation and high throughput sequencing

For ChIP-seq, immunoprecipitated DNA was cleaned up using the Qiagen PCR Purification Kit after reversing cross-links, RNaseA, and proteinase K treatment. Immunoprecipitated DNA was eluted from column with the provided elution buffer (50 µl x 2), subjected to additional shearing in a Qsonica water bath sonicator at 20% amplitude for 15

minutes of total shearing time (each cycle is 15" on + 15" off), followed by vacuum centrifugation to reduce the volume to 30 µl. DNA concentration was measured using Qubit dsDNA HS Kit.1 to 10 ng of immunoprecipitated DNA was used for standard Illumina library construction using barcoded adapters and protocol described previously (Wong et al., 2013). Libraries were pooled and sequenced on the Illumina HiSeq2000. Raw reads were mapped to the *S. pombe* genome using Bowtie's default parameters. Mapped reads were normalized to reads per million, tiled with igvtools, and visualized with IGV. The raw and processed ChIP-seq data are publicly available at the NCBI Gene Expression Omnibus under accession number GSE83495.

RNA Extraction and Quantitative Reverse Transcription PCR

RNA extraction and RT-qPCR were performed as previously described (Yu et al., 2014). Briefly, total RNA was extracted with hot phenol followed by ethanol precipitation. The precipitated nucleic acid was treated with DNase I (Roche) and cleaned up with Qiagen RNeasy Mini Kit. cDNA was prepared from 200 ng of RNA using SuperScript III Reverse Transcriptase (Invitrogen) with primers listed in Table S3. *act1*$^{+}$ cDNA was used as internal control.

Small RNA (sRNA) Extraction and Northern Blot

sRNA was purified from 50 ml of cells (OD = ~1.5) as previously described (Holoch and Moazed, 2015b). Briefly, total sRNA was purified with the mirVana miRNA Isolation Kit (Ambion), loaded on 17.5% polyacrylamide / 7M urea gel, transferred to positively charged nylon membrane (Roche #1417240), and probed with a mixture of ^{32}P-labeled DNA probes corresponding to *dg* or *dh* siRNA sequences described previously (Holoch and Moazed, 2015b).

Quantitative mass spectrometry assay for association of silencing complexes with histone H3 peptides

Exponentially growing wild-type (SPY137) cells (2.4 x 10^{10} cells) were harvested by centrifugation, transferred to a 50 ml tube, washed twice in TBS (50 mM Tris [pH 7.6], 150 mM NaCl), and frozen at -80°C (approximately 1.6 g cells). All subsequent steps were performed at 4°C. The frozen cells were resuspended in 1 volume of ice-cold lysis buffer (20 mM HEPES [pH 7.5], 150 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA [pH 8.0], 0.5 mM DTT, 10% glycerol, 0.25% Triton X-100, 1 mM phenylmethylsulfonyl fluoride [PMSF], complete protease inhibitor cocktail [Roche]) and lysed by glass-bead beating for 5x 45 sec at 5000 rpm with 1 ml 0.5 mm glass beads per 800 µl of yeast resuspension (MagNa Lyser, Roche). Tubes were punctured and the crude lysate was collected in a fresh tube by centrifugation. After two consecutive rounds of centrifugation at 12,000 x g for 3 min and 15 min, respectively, the cleared lysates were pooled, diluted to a protein concentration of 25 mg/ml with ice-cold lysis buffer, and divided into 4 aliquots of 800 µl in which 1500 units of Benzonase (#71205-3, Novagen) was added. Biotinylated peptides histone H3 1-21 K9me0 (#AS-61702, Anaspec), H3 1-21 K9me2 (#AS-64359-1, Anaspec), H3 1-21 K9me3 (#AS-64360-1, Anaspec) were pre-incubated with washed Streptavidin myOne C1 (#65002, Invitrogen) for 4 h, and washed once with ice-cold lysis buffer. For each immunoprecipitation, 10 µg of biotinylated peptides coupled to 50 µl beads was added to 800 µl lysate (approximately 25 mg/ml). Samples were rotated for 3 h at 4°C, the beads were collected on magnetic stands, and washed 4 times with 1 ml ice-cold lysis buffer and eluted twice with 200 µl of 500 mM NH_4OH at 37°C for 20 min. The elutions were combined and 10% of eluted protein was analyzed by silver staining. The remaining 90% of the eluted protein was analyzed by multiplexed quantitative mass spectrometry at the Thermo Fisher Scientific Center for Multiplexed Proteomics at Harvard Medical School. Sample processing steps included tandem protein digestion using LysC and trypsin, peptide labeling with Tandem Mass Tag reagents and peptide fractionation. Multiplexed quantitative mass spectrometry data were collected on an Orbitrap Fusion mass spectrometer operating in a MS3 mode using synchronous precursor selection for MS2 fragment ion selection (McAlister et al., 2014). MS/MS

data were searched against a Uniprot *S. pombe* database with both the forward and reverse sequences using the SEQUEST algorithm. Further data processing steps included controlling peptide and protein level false discovery rates, assembling protein groups, and protein quantification from peptides.

Analysis of histone post-translational modifications

Fission yeast total histones were prepared by modifications of a previously described protocol (Recht et al., 2006). One liter of culture (SPY137 or SPY815) grown to an OD600 of 0.9 - 1.0 was harvested by centrifugation and washed once with water, once with Buffer A (1.0 M Sorbitol 50 mM potassium phosphate, pH 6.8, 14 mM 2-mercaptoethanol), and resuspended in Buffer A containing 0.1 mg/ml Zymolase. Cells were incubated at 30°C with gentle shaking until >90% were converted to spheroplasts. The suspension was pelleted at 3200 x g for 5 min at 4°C, washed in cold Buffer A, resuspended in cold Buffer B (18% Ficoll-400, 20 mM potassium phosphate, pH 6.8, 1.0 mM $MgCl_2$, 0.5 mM EDTA, and complete EDTA-free protease inhibitor cocktail (Roche)), and dounced using a prechilled douncer. The sample was then centrifuged at 3200 x g for 5 min. at 4°C. The supernatant was centrifuged again at 3200 x g for 5 min. at 4°C. Nuclei were pelleted by spinning at 50,000 x g in a Beckman SW40 Ti rotor. The pellet was resuspended in Buffer C (0.34 M Sucrose, 20 mM Tris-HCl, pH 7.4, 50 mM KCl, 5 mM $MgCl_2$, and complete EDTA-free protease inhibitor cocktail (Roche)), layered on a 2 ml sucrose cushion at the bottom of the tube, and centrifuged at 30,000 x g for 30 min at 4C°. Nuclei were resuspended in Buffer D (10 mM Tris, pH 8.0, 0.5% NP-40, 75 mM NaCl, and complete EDTA-free protease inhibitor cocktail (Roche)) and pelleted by centrifuging at 3200 x g at 4°C for 5 min. a total of three times. Nuclei were resuspended in Buffer E (10 mM Tris-HCl, pH 8.0, 400 mM NaCl, complete EDTA-free protease inhibitor cocktail (Roche)). Cold H_2SO_4 was added slowly to the suspension with mixing to a final concentration of 1.5 M, followed by incubation for 30 min at 4°C with mixing. The nuclei were centrifuged at 20,000 x g at 4°C for 5 min, and acid

extraction was repeated as before. The supernatant from the acid extractions were pooled and TCA precipitated. The pellet was washed once with cold 0.1% HCl Acetone, twice with cold Acetone, and the pellet was resuspended in water. The sample quality and yield were determined by SDS-PAGE and Bradford assay, respectively. The remaining histones were chemically derivatized using propionic anhydride, trypsin-digested and derivatized again as described previously (Sidoli et al., 2016). The desalted peptides were resuspended in 0.1N acetic acid and about 1ug was analyzed using using an EASY-nLC nanoHPLC (Thermo Scientific, Odense, Denmark) coupled with a Q-Exactive mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Nano-liquid chromatography was carried out in a gradient of 0-35% solvent B (A = 0.1% formic acid; B = 95% MeCN, 0.1% formic acid) over 30 min and from 34% to 100% solvent B in 20 minutes at a flow-rate of 250 nL/min. Full scan MS spectrum (m/z 290−1650) was performed in the Orbitrap with a resolution of 30,000 (at 400 *m/z*) with an AGC target of 1x10e6. To facilitate MS/MS-based quantification, the MS/MS events included both data-dependent acquisition and targeted acquisition for isobaric peptides. The relative abundance of histone H3 and H4 peptides were calculated by using EpiProfile.

Data reporting

No statistical methods were used to predetermine sample size. The investigators were not blinded to allocation during experiments and outcome assessment. All quantitative experiments are presented as means ± s.d. of three independent biological experiments.

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Chapter 3

Clr4 recruitment via chromo domain interaction with tri-methylated H3K9 mediates epigenetic maintenance of H3K9 methylation

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Chapter 3

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All experiments were performed by Gloria Jih.

Abstract

In *S. pombe*, the RNAi machinery is required for efficient recruitment of histone H3 lysine 9 (H3K9) methyltransferase Clr4 to the pericentromeric repeats to mediate heterochromatic silencing. However, in RNAi mutants, the level of pericentromeric H3K9 di- and tri-methylation (designated as “H3K9me” in this chapter) is decreased to around 5 – 20% of wild-type level, suggesting that Clr4 is still localized to the pericentromeric repeats, albeit inefficiently (Halic and Moazed, 2010; Sadaie et al., 2004). One possibility is the existence of a minor pathway for recruiting Clr4 to the chromatin. We provide evidence showing that the residual H3K9me observed in RNAi mutants is not due to an alternative mechanism for recruiting Clr4, but rather a mechanism of maintenance, in which the chromo domain of Clr4 becomes important for recognizing and maintaining the histone modification. In addition, the ability of Clr4 to catalyze H3K9me3 is required for the maintenance of residual H3K9me. We speculate that because the Clr4 chromo domain has higher affinity for H3K9me3, this interaction becomes critical for epigenetic maintenance in the absence of RNAi-mediated Clr4 recruitment. Our study provides mechanistic insights into the inheritance of histone modifications and epigenetics states.

Introduction

During development, transcriptional states are established and inherited, allowing for a single embryo to differentiate into many cell types carrying the same genome (Ringrose and Paro, 2004). This epigenetic phenomenon is thought to be facilitated by transmission of DNA and histones carrying chemical modifications from mother to daughter cells, resulting in maintenance of the transcriptional states. During DNA replication, parental histones are randomly distributed onto the two newly synthesized daughter DNA strands (Jackson and Chalkley, 1974; Probst et al., 2009; Radman-Livaja et al., 2011; Sogo et al., 1986). It is therefore been generally assumed that histone modifications play a role in epigenetic memory of transcriptional states. Here we use *S. pombe* to investigate molecular requirements for the maintenance of H3K9me, a hallmark histone modification of heterochromatin.

In *S. pombe* pericentromeric regions, the RNAi machinery is required for wild-type levels of H3K9me and for its spreading into inserted reporter genes, which is required for silencing (Verdel et al., 2004; Volpe et al., 2002). In the absence of the RNAi machinery, H3K9me is lost from the reporter gene, but persists at low levels throughout the pericentromeric regions (Figure 3.1a). In contrast, RNAi is not required for maintenance of H3K9me at the mating type locus and telomeres (Figure 3.1a, right). One possible explanation is that Clr4 is weakly recruited via factors that recognize pericentromeric sequence to continuously establish low levels of H3K9me. Another possibility is that Clr4 can bind to its own mark and catalyze low levels of H3K9me, resulting in maintenance of this mark. Clr4 and its mammalian homologs have chromo domain, which binds methylated H3K9 via an aromatic cage (Ivanova et al., 1998) (Melcher et al., 2000; Zhang et al., 2008b). Mutation of any aromatic cage residue results in loss of binding to methylated peptide *in vitro*, as well as loss of Clr4 recruitment at the mating type. These results suggest that the chromo domain of Clr4 is important for recruiting Clr4 to H3K9me domain (Zhang et al., 2008b).

In this chapter, we first demonstrate that in the absence of RNAi, H3K9me2 cannot be established *de novo*, thus excluding the possibility of an RNAi-independent mechanism for Clr4 recruitment. Then we show that the residual H3K9me in RNAi mutants is epigenetically maintained by Clr4's ability to recognize and write its mark, specifically tri-methylated H3K9. We further confirm these observations by utilizing an ectopic system designed to examine epigenetic maintenance of the silenced state.

Results

H3K9me cannot be established de novo in the absence of RNA

To test for RNAi-independent recruitment of Clr4, we determined whether H3K9 methylation could be established *de novo* at the pericentromeric repeats in cells with deletions of RNAi factors *dcr1*$^+$ or *ago1*$^+$. To perform this experiment, we reintroduced *clr4*$^+$ into *clr4Δ*, *clr4Δ ago1Δ*, or *clr4Δ dcr1Δ* cells (Figure 3.1b) and quantified H3K9me2 levels using ChIP-seq and ChIP-qPCR. As shown in Figure 3.1c, the reintroduction of *clr4*$^+$ into *clr4Δ* cells fully restored H3K9me2 at the pericentromeric *dg* and *dh* repeats of chromosome 1. In contrast, *clr4*$^+$ reintroduction into *clr4Δ ago1Δ* or *clr4Δ dcr1Δ* double mutant cells failed to promote any H3K9me2 at the pericentromeric repeats (Figure 3.1c, d). At telomeres, where Clr4 can be recruited by Taz1 (Kanoh et al., 2005), *clr4*$^+$ reintroduction into double mutants restored H3K9me2. These results show that RNAi is the primary mechanism for establishment of pericentromeric H3K9me domains and suggests that the residual H3K9me observed at these repeats after deletion of RNAi components results from epigenetic maintenance.

Figure 3.1 RNAi-independent H3K9 methylation at pericentromeric repeats is epigenetically inherited.

a, ChIP-seq experiments showing the persistence of residual histone H3K9me2 at the pericentromeric *dg* and *dh* repeats of chromosome 1 in *ago1Δ* and *dcr1Δ* cells. Libraries were sequenced on the Illumina HiSeq2500 platform and normalized to read per million (y axis). Chromosome coordinates are indicated above the plots.
b, Scheme for the reintroduction of *clr4*$^{+}$ into *RNAiΔ*, *clr4Δ* cells to test the requirement for RNAi in H3K9me establishment. *clr4*$^{+}$ was reintroduced to the native locus to avoid overexpression.
c, ChIP-seq experiments showing that the re-introduction of *clr4*$^{+}$ into *clr4Δ* cells, but not *clr4Δ ago1Δ* or *clr4Δ dcr1Δ* cells, restores H3K9me2 at the pericentromeric repeats of chromosome 1 (left). Reads for H3K9me2 at the telomeres of chromosome 1 (*tel1L*) on the right side show that, unlike the centromeres, establishment of telomeric H3K9me does not require RNAi.
d, ChIP-qPCR experiments verify that RNAi is required for the reestablishment of H3K9me2 at the pericentromeric dg repeats.
e, ChIP-qPCR experiments show that a mutation in the chromodomain of Clr4 (Clr4^{W31G}) abolishes the maintenance of H3K9me2 at *dg* repeats.
f, ChIP-qPCR experiments show that an additional copy of wild type *clr4*$^{+}$, but not *clr4*^{W31G}, boosts residual H3K9me2 levels at *dg*. Error bars represent standard deviations.

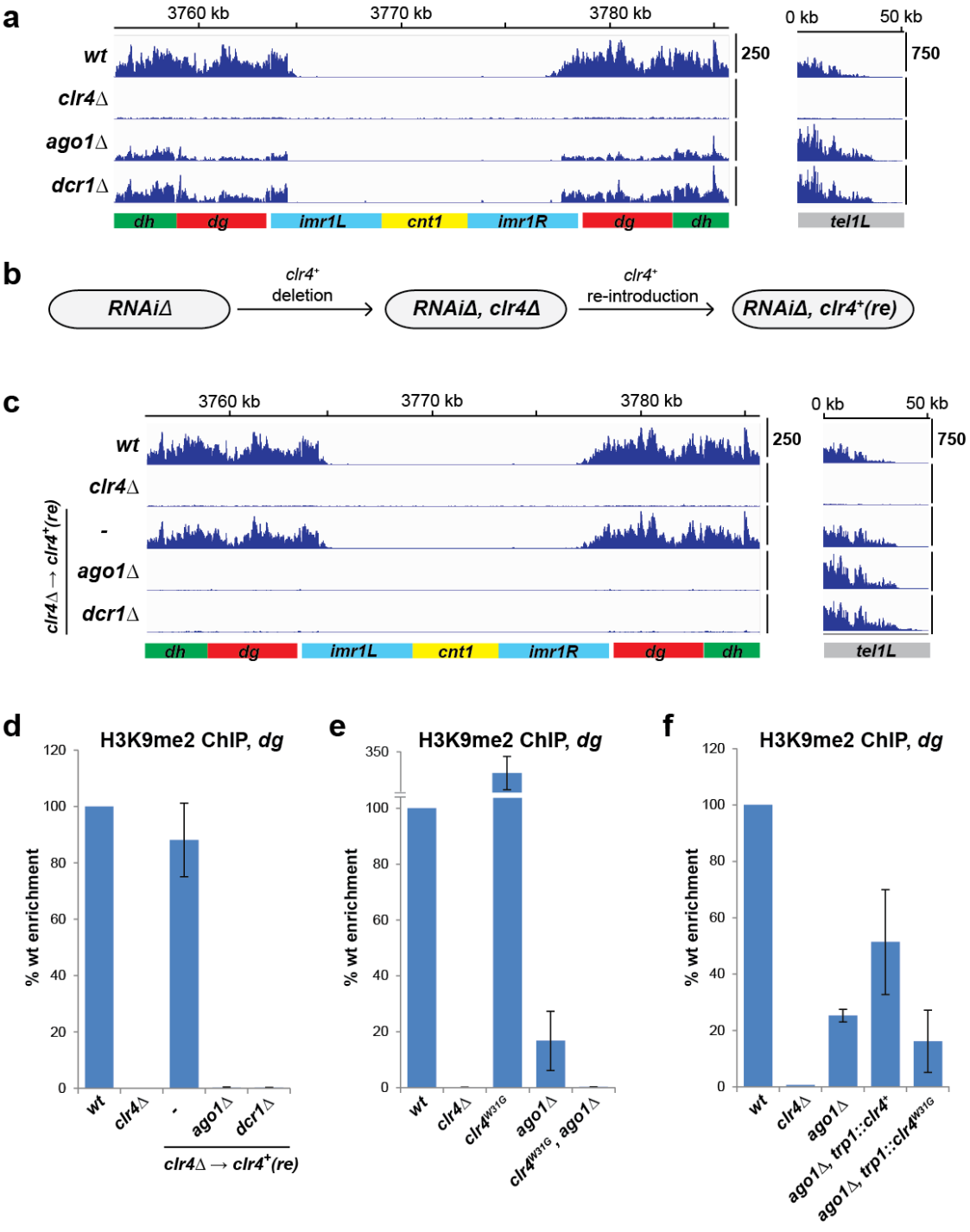

Figure 3.1 (Continued)

RNAi-independent H3K9 methylation at pericentromeric repeats is epigenetically maintained

To test whether the residual H3K9me observed in RNAi mutants is epigenetically maintained by Clr4 itself, we combined *ago1Δ* with *clr4*^{W31G} to form a double mutant. Clr4^{W31G} contains a mutation in the chromo domain that attenuates binding to H3K9me. Despite its inability to bind H3K9me, it is still capable of robust H3K9 di-methylation when recruited by the RNAi machinery, indicated by high levels of H3K9me2 (Figure 3.1e). However, once the RNAi machinery is removed, Clr4^{W31G} could no longer be recruited, resulting in the complete loss of H3K9me in *clr4*^{W31G} *ago1Δ* cells (Figure 3.1e). The complete loss of H3K9me in *ago1Δ clr4*^{W31G} double mutant cells suggests that the residual H3K9me marks are maintained by direct recruitment of Clr4 via binding of its chromo domain to pre-existing marks. In further support of this hypothesis, the introduction of an additional copy of wild type *clr4*$^{+}$, but not the *clr4*^{W31G} mutant, into *ago1Δ* cells boosted the residual H3K9 methylation levels by about 2-fold (Figure 3.1f). Our results support a direct "read-write" mechanism in which Clr4 binds to pre-existing H3K9 methylated nucleosomes and catalyzes the methylation of H3K9 on newly deposited nucleosomes, resulting in epigenetic maintenance of H3K9 methylation.

H3K9 tri-methylation is critical for epigenetic maintenance of RNAi-independent H3K9 methylation at pericentromeric repeats

Previous work showed that Clr4 has higher affinity for H3K9me3 over H3K9me2 (Figure 2.10) (Schalch et al., 2009; Zhang et al., 2008b). In RNAi mutants, given that the chromo domain plays a role in maintaining residual H3K9me at endogenous pericentromeric repeats, we asked whether H3K9 methylation states affect Clr4 recruitment. We combined *ago1*Δ with *clr4*^{I418P}, a *clr4* mutant allele that has decreased H3K9me3 at pericentromeric regions, but high levels of H3K9me2. Cells carrying this allele have mild defect in transcriptional gene silencing (see Chapter 2). A complete loss of pericentromeric H3K9me2 was observed in *clr4*^{I418P} *ago1*Δ

cells (Figure 3.2), suggesting that in the absence of RNAi-mediated recruitment, H3K9me3 becomes important for Clr4 to self-recruit to its own mark and epigenetically maintain it.

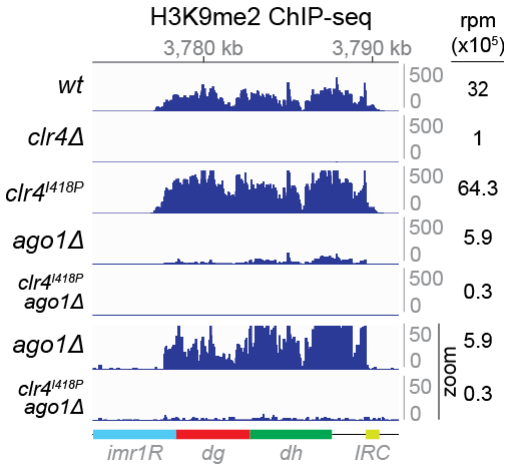

Figure 3.2 H3K9me3 is critical for maintaining residual H3K9me at pericentromeric repeats.
H3K9me2 ChIP-seq reads mapped to pericentromeric repeats on the right of chromosome 1 in wild-type and the indicated mutant cells. The bottom two tracks have a 10-fold expanded Y axis scale to highlight the complete loss of H3K9me2 in the *clr4*^{I418P} *ago1Δ* double mutant cells.

Clr4 chromo domain and H3K9me3 catalysis are required for the maintenance of ectopic H3K9me

The above conclusions regarding molecular requirements for epigenetic maintenance were made from observations at pericentromeric DNA repeats. We wanted to further investigate the mechanism of epigenetic inheritance by testing the requirement for the Clr4 chromo domain and H3K9me3 in an ectopic system. An ectopic heterochromatic domain can be induced by artificially tethering Clr4 to a euchromatic locus (Kagansky et al., 2009). In this system, a Clr4 protein lacking its chromo domain is fused to the bacterial TetR protein (TetR-Clr4-I) and, in the absence of tetracycline (- tet), binds to an array of *tet* operator (*10xtetO*) sites located upstream of an *ade6*$^{+}$ reporter gene (Figure 3.3a). Recruitment of TetR-Clr4-I results in *ade6*$^{+}$ silencing and formation of red colonies due to accumulation of an intermediate in adenine biosynthesis

when cells are grown on medium with limiting adenine (Figure 3.3b, left) (Audergon et al., 2015; Ragunathan et al., 2015). When TetR-Clr4-I is released by addition of tetracycline (+ tet), heterochromatin cannot be maintained by endogenous *clr4*$^{+}$ (Figure 3.3b, 1st row, right) (Kagansky et al., 2009). However, in cells where the putative histone demethylase Epe1 is deleted, the ectopic heterochromatin can be maintained upon release of tethered Clr4 (Audergon et al., 2015; Ragunathan et al., 2015). This is likely due to a decreased rate of H3K9me erasure, providing endogenous Clr4 with the opportunity to read-write its mark. The observed maintenance is epigenetic because 1) the endogenous DNA sequence at ectopic locus cannot recruit Clr4 and 2) in this particular system, ectopic heterochromatin establishment is RNAi-independent.

To test whether the chromo domain is important for maintaining heterochromatic silencing at the reporter gene after TetR-Clr4-I release, we replaced *clr4*$^{+}$ with *clr4*^{W31G} in *epe1Δ* background. Consistent with previous observations at pericentromeric repeats, the replacement resulted in the loss of heterochromatin maintenance, indicated by white colonies on plates containing tetracycline (Figure 3.3b, third row, right). Replacement of *clr4*$^{+}$ with *clr4*^{I418P} also resulted in the loss of heterochromatin maintenance, further confirming that H3K9me3 is critical for recruiting Clr4 to maintain epigenetic inheritance. Consistent with the silencing assay, ChIP-seq and ChIP-qPCR experiments showed the establishment of broad domains of H3K9me2 by TetR-Clr4-I in *clr4*^{I418P}, *clr4*^{W31G}, and *clr4*$^{+}$ cells (Figure 3.3c). However, 24 hours after the release of TetR-Clr4-I by growth in + tet medium, H3K9me2 levels were maintained in *clr4*$^{+}$ cells, as expected, but declined to background levels in *clr4*^{I418P} and *clr4*^{W31G} cells, similar to the levels observed in *clr4*Δ cells (Figure 3.3d). These results confirm the importance of Clr4 chromo domain and H3K9me3 in the epigenetic maintenance of heterochromatin.

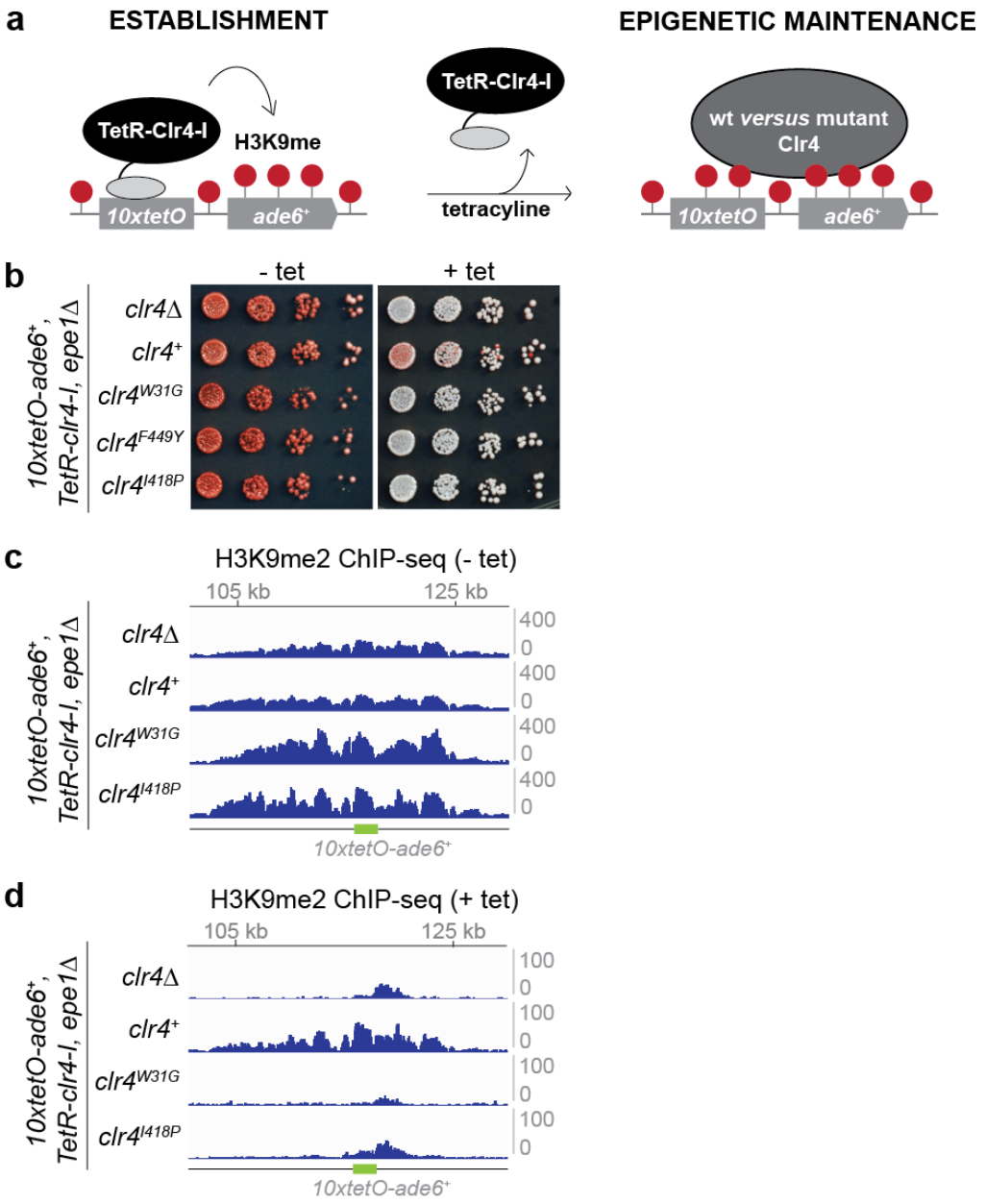

Figure 3.3 H3K9me3 is required for epigenetic inheritance
a, Experimental strategy for testing requirements for epigenetic inheritance.
b, 10-fold serial dilutions of cells plated on low-adenine medium lacking tetracycline (- tet) or containing tetracycline (+ tet) to assess establishment and maintenance of *10xtetO-ade6*$^{+}$ silencing.
c, H3K9me2 ChIP-seq reads mapped to chromosomal region containing the *10xtetO-ade6*$^{+}$. Data is presented as reads per million (rpm, Y axis).
d, Same as **c**, but after 24 hour growth in tetracycline-containing medium (+ tet).

Discussion

In this chapter, we established that residual H3K9me at pericentromeric repeats is maintained by Clr4 itself. Our findings indicate that the Clr4 chromo domain and H3K9me3 are required for the epigenetic inheritance of H3K9me domains following their establishment by either endogenous RNAi or artificial recruitment of Clr4 to an ectopic site. We speculate that the requirement for H3K9me3 is due to higher affinity of the Clr4 chromo domain for H3K9me3 relative to H3K9me2 (Schalch et al., 2009; Zhang et al., 2008b). However, it is also possible that the loss of H3K9me3 results in increased histone turnover due to higher transcriptional activity (as described in Chapter 2), and therefore H3K9me domains cannot be maintained. Indeed, inhibition of pericentromeric transcription in RNAi mutants by the deletion of *mst2*$^{+}$, a gene encoding for H3K14 acetyltransferase, results in increased H3K9me levels and rescues defective silencing in RNAi mutants (Reddy et al., 2011). However, given that in RNAi mutants, maximal transcriptional activity, as compared to that of *clr4Δ*, is already observed at pericentromeric repeats, it is more likely that H3K9me3 plays a role in the weak but stable recruitment of the Clr4 chromo domain. We note that the chromo domain of Suv39h1, the mouse homolog of Clr4, is required for its localization to pericentromeric heterochromatin (Melcher et al., 2000). It is unknown whether this localization involves an interaction with H3K9me2 or H3K9me3 and whether it is required for epigenetic maintenance of heterochromatin in mammals. Nevertheless, the requirement of the chromo domain for Suv39h1 heterochromatin localization raises the possibility that it plays a similar role in epigenetic inheritance as that described for the Clr4 chromo domain.

In development, epigenetic maintenance refers to the inheritance of gene expression or repression, not the inheritance of chromatin molecular features, though the latter provides a means for the former. While our work on residual H3K9me at the pericentromeric repeats focuses solely on the inheritance of chromatin mark H3K9me (in RNAi mutants with defective

silencing), observations made at the ectopic locus demonstrate that H3K9me3 and a functional Clr4 chromo domain are important for epigenetic inheritance of the transcriptional state.

Regulation of HOX genes by Polycomb proteins is critical for body patterning. In this context, memory of silencing has to be propagated through cell divisions to ensure cell remember its identity (Zhang et al., 2008b). H3K27me is a hallmark of Polycomb-mediated silencing, and is thought to repress gene expression by preventing the acetylation of H3K27 and/or promoting chromatin compaction (Pasini et al., 2010; Tie et al., 2009). H3K27me is deposited by EZH2 of the PRC2 complex, and the activity of EZH2 is stimulated upon EED recognition of H3K27me (Margueron et al., 2009). Since EZH2 and EED are in the same complex, the situation is analogous to Clr4 being able to read and write its own mark. Indeed, when aromatic cage residues of EED are mutated, global levels of H3K27me2 and H3K27me3 are diminished. Interestingly, in vitro activity assays with reconstituted EZH2-containing PRC2 complex showed that EZH2 activity was significantly more robust when H3K27me3 peptide was added the reaction compared to H3K27me2 (Margueron et al., 2009). These observations suggest that similar to what I have described here for the methylation states of H3K9me, the methylation states of H3K27me may be critical for epigenetic inheritance of Polycomb silencing, although in vivo tests are required to test this possibility.

Materials and methods

Growth Assays

Cells were grown in YES to log phase, 1 x 10^7 cells were pelleted, suspended in 100 µl of water, and then serially diluted 10 fold. 3 ul of each dilution was spotted on YE or YE + tetracycline (2.5 µg/ml). Plates were incubated at 32 °C for 3-4 days, followed by 4 °C overnight incubation(to enhance the color red) prior to imaging.

ChIP

See the materials and methods section in Chapter 2 for details.

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Chapter 4

Perspectives

Using the fission yeast *S. pombe*, I have demonstrated that H3K9me2 and H3K9me3 mediate different silencing functions through differential recruitment of chromo domain proteins. The methylation status coordinates two different modes of silencing at the pericentromeric repeats during heterochromatin establishment. My findings show that H3K9me2 is a chromatin state that is permissive for transcription, indicated by increased levels of Pol2 as well as histone marks associated with active transcription. This transcriptionally permissive environment, when combined with stable recruitment of the Chp1- and siRNA-containing RITS complex, results in co-transcriptional gene silencing (CTGS), effectively allowing for robust processing of pericentromeric transcripts into siRNAs. I also observed that during the establishment of pericentromeric heterochromatin, H3K9me2 accumulates at a faster rate compared to H3K9me3, and proposed that the H3K9me2 chromatin state is a critical step during the heterochromatin establishment process. Transcriptionally permissive chromatin environment enables efficient recruitment of H3K9 methyltransferase Clr4 through nascent pericentromeric transcripts, which serve as a chromatin scaffolds onto which the RNAi machinery assembles. In addition, nascent transcripts provide the template for the synthesis of siRNAs, which further enables RNAi recruitment to the chromatin via base-pairing of siRNAs with nascent transcript. Finally, the appearance of H3K9me3 confers transcriptional gene silencing (TGS) through the recruitment of Clr3, a histone deacetylase, via Swi6 (and possibly Chp2), both of which are HP1 proteins that contain the chromo domain. I also showed that in the absence of mechanisms that strongly recruits Clr4 to the chromatin, H3K9me3 availability becomes essential for the self-recruitment of Clr4 and epigenetic inheritance of H3K9me.

Regulation of histone methylation states as a possible mechanism for controlling epigenetic transmission

The last observation mentioned above provides interesting perspectives beyond mechanistic insights for Clr4 self-recruitment. First, only H3K9me3, rather than both H3K9me2

and H3K9me3, has the potential to confer epigenetic transmission of the silenced state. Second, cells are likely to actively regulate the methylation status of H3K9 to prevent erroneous epigenetic transmission of silencing. Clr4 is a distributive methyltransferase, unlike its *Neurospora crassa* homolog DIM-5, an H3K9 tri-methyltransferase that acts in a processive manner (Zhang et al., 2003). Western blot for histones purified from DIM-5 showed that all H3K9 is tri-methylated (Collins et al., 2005). In contrast, histone purify from *S. pombe* have all three forms of H3K9 methylation (Chapter 2, Figure 2.6). The various forms of H3K9 methylation suggest that regulation can be exerted on Clr4 itself (discussed later), but also directly upon various methylation states by histone demethylases. Epe1 is a putative demethylase that contains the JmjC domain. Although the activity of Epe1 has not been demonstrated biochemically, many observations regarding its anti-silencing function have been reported. For one, deletion of Epe1 in RNAi mutants resulted in rescue of defective silencing and significant increase in the level of H3K9me (Trewick et al., 2007). Another report demonstrates that when *epe1Δ* is combined with *mst2Δ* (an acetyltransferase that promotes transcription), ectopic heterochromatin formation can occur (Wang et al., 2015). All these observations strongly suggest that cells actively regulate H3K9me3 to prevent erroneous silencing of essential genes.

The regulation of histone marks conferring gene silencing is likely to be also present in the development of multicellular organisms, where it is critical during cell division to maintain patterns of gene expression through faithful transmission of not only the DNA sequence but also chromatin features. H3K27 tri-methylation is a hallmark modification in HOX gene silencing, which prevents H3K27 acetylation (Pasini et al., 2010; Tie et al., 2009). If the prevention of H3K27 acetylation were the key function of methylation, why do the HOX genes have to be H3K27 tri-methylated rather than mono- or di-methylated? H3K27 is methylated by EZH2, which is found in the PRC2 complex, along with the beta propeller (WD) domain-containing EED. The enzymatic activity of EZH2 is stimulated upon EED binding to H3K27 methylation (Margueron et

al., 2009). In mouse embryonic stem cells, EZH2 is also responsible for domains of H3K27me1 and H3K27me2 found at regions distinct from H3K27me3-regulated genes (Ferrari et al., 2014). One possible explanation for why H3K27 is tri-methylated at the HOX genes is that the activity of EZH2 is highest when EED is binds to H3K27me3-containing peptides in vitro (Margueron et al., 2009), and thus only H3K27me3 may facilitate additional H3K27 trimethylation. This possible mechanism for the regulation of H3K27me3, along with regulation of H3K9me3 described in the previous paragraph, suggests that inheritance of histone modifications associated with silencing are likely to be tightly regulated.

Another means of preventing erroneous silencing marks from being established is possible through regulation the mono-methylation state. As mentioned earlier, DIM-5 acts in a processive manner and can rapidly convert a methylated substrate to the tri-methylated form. This is not the case for Clr4 (Nakayama et al., 2001) or its mammalian homologs SUV39H1 (Rea et al., 2000) and G9a (Patnaik et al., 2004). In vitro methyltransferase assay for these proteins showed that mono-methylated H3K9 tail peptide (or di-methyl in the case of SUV39H1) serves as a much weaker substrate for receiving a second and/or third methyl group, in comparison with a non-methylated histone tail peptide (see addition notes below). Given that these H3K9 methyltransferases have less substrate affinity/activity in vitro, mono-methylated K9 could be a way for the cells to safeguard histones from higher levels of H3K9 methylation to prevent errant silencing. *C. elegans* and mammalian cells both have cytosolic mono-/di-methyltransferase that deposits H3K9me1/2 prior to chromatin incorporation. In *C. elegans*, MET2 resides in the cytosol to deposits H3K9me1/me2, and in mammalian cells, Prdm3 and Prdm16 deposits H3K9me1 on cytoplasmic histones (Pinheiro et al., 2012; Towbin et al., 2012). In *S. pombe*, H3K9 mono-methyltransferase has yet to be identified, while Clr4 is the sole H3K9 methyltransferase for H3K9me2/3. Mass spectrometry analysis showed that a moderate amount of H3K9me1 is present in histones purified from *clr4Δ* cells (Chapter 2, Figure 2.6). This observation, along with examples from *C. elegans* and mammalian cells, suggests that histone

H3 is mono-methylated on K9 before incorporation into chromatin. In support of this speculation, H3K9me1 appears to be distributed genome-wide. This observation is based on ChIP-seq experiment, in which I was not able to detect H3K9me1 signals after normalization to total reads, but was able observe H3K9me1 enrichment using ChIP-qPCR, after normalizing to an H3K9R mutant that cannot be mono-methylated. Given that the H3K9R mutation is not lethal the cells, it is unlikely that H3K9me1 plays a specific role in transcriptional regulation. Thus H3K9me1 could be a means by which cells regulate the levels of the silencing mark, H3K9me3, simply by making mono-methylated histone tail a less desirable substrate for the Clr4 methyltransferases. Identification of the H3K9 mono-methyltransferase would be required to test this hypothesis holds true. It would be interesting to perform H3K9me1 ChIP-qPCR to determine whether H3K9me1 is lost in any of the eight SET domain-encoding candidates with known or putative histone methylation activity. After trying a few antibodies, I finally found one that was specific and showed H3K9me1 enrichment in wild-type cells over H3K9R. So the experiment can now be easily done.

My methyltransferase assays with recombinant Clr4 show that its substrate preference is for H3K9me0 over H3K9me1 over H3K9me2 peptide. With H3K9me0 peptide, I obtained the highest level of incorporated radioactive label, but I do not have any insight into the distribution of methylation states, which can range anywhere from me1 to me3. My guess is that the signal for incorporated radioactive label constitutes mostly the addition of a single methyl group to the H3K9me0 peptide. This experiment would have to be repeated as a time course and using mass spectrometry to obtain methylation kinetics for recombinant Clr4. I also attempted to stimulate Clr4 activity with H3K9me3 peptide and mono-nucleosome, but did not observe the enhancement in activity previously reported for EZH2 upon EED binding to H3K27me3 peptide. It remains to be tested whether an array of H3K9me3 nucleosomes could stimulate Clr4 activity. Such *in vitro* experiments are required to gain full insight into how the methylation activity of Clr4 may be regulated to assemble H3K9me3 heterochromatin that can be epigenetically inherited.

Finally, another layer of regulation over H3K9 methylation states is exerted at the level of the histone methyltransferase itself. Clr4 containing a chromo domain mutation, which reduces binding affinity for H3K9me, can perform H3K9me2, but not H3K9me3 even when recruited to the chromatin by RNAi machinery (Chapter 2, Figure 2.11). This observation suggests that the association of Clr4 with its own mark determines its ability to catalyze H3K9me3. In a related example, one of the mammalian homologs of Clr4, SETDB1 can deposit H3K9me2, but requires a co-factor to deposit H3K9me3, resulting in transcriptional inhibition (Wang et al., 2003).

Conclusions

To this day, I can still vividly recall learning about position effect variegation (PEV) in college. At the time, I found it cool and frustrating. Frustrating because I could not fathom what was going on at the molecular level. Little did I know I would be spending a great many years studying the hallmark histone modification associated with PEV in *Drosophila*, showing that in fission yeast a little chemical modification can change the expression and heritability of the underlying DNA sequence.

This dissertation highlights the different roles that H3K9me2 and H3K9me3 play in RNAi-mediated heterochromatin establishment in fission yeast. During the course of this work, I also discovered that H3K9me3 has the potential to confer epigenetic inheritance of a silenced gene. It remains to be determined whether H3K9me2 and H3K9me3 play different roles in other eukaryotes and whether the methylation state is regulated. It will also be of great interest to investigate whether the histone methylation states confer protection from epigenetic misregulation as speculated above.

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