RNA Interference and Epigenetic Control of Heterochromatin Assembly in Fission Yeast

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Large tracts of eukaryotic chromosomes contain no or relatively few genes and are often replete with repetitive elements. Many of these regions are heterochromatic in nature, being highly condensed and inaccessible to a variety of DNA-modifying factors, and therefore are generally thought to be synonymous with gene silencing. Importantly, a phenomenon of position effect variegation (PEV) first observed in flies, in which a euchromatic gene when juxtaposed to heterochromatin becomes repressed in a stochastic fashion but once silenced can be clonally inherited, suggests that cellular factors intimately associated with heterochromatin can stably maintain epigenetic gene silencing through multiple cell divisions without altering the underlying genetic sequence (Grewal and Elgin 2002).

PEV has also been observed in the fission yeast *Schizosaccharomyces pombe* whose genome contains large stretches of heterochromatin with properties similar to heterochromatin present in higher eukaryotes (Grewal 2000; Hall and Grewal 2003). Because of its rather compact genome and tractable genetic system, heterochromatin-mediated gene silencing is arguably best understood in this organism. In the last few years, great strides have been made in uncovering the molecular constituents of heterochromatin and how they effect epigenetic gene silencing. In particular, recent works from our lab and others have elucidated a number of molecules involved in heterochromatin formation, with the surprising discovery of the critical role of the RNA interference (RNAi) pathway in the targeting and assembly of heterochromatin at specific loci of the *S. pombe* genome. A basic picture of how heterochromatin assembles, propagates, and is stably maintained through mitosis and meiosis has begun to emerge.

HETEROCHROMATIC REGIONS IN *S. POMBE*

The three chromosomes of fission yeast contain several silenced domains that are constitutively associated with heterochromatin, namely the telomeres, centromeres, the mating-type (*mat*) region, and the ribosomal DNA (rDNA) tandem loci (Grewal 2000; Thon and Verhein-Hansen 2000). Unlike the small centromeres of the budding yeast *Saccharomyces cerevisiae*, which can comprise only a few hundreds base pairs (bp) of DNA, centromeres in *S. pombe* are relatively large complex structures (35–110 kilobases [kb]) that resemble higher

eukaryotic centromeres in structure and organization, having partitions of large inverted-repeat elements flanking the central core (*cnt*), a site of kinetochore assembly and microtubule attachment (Fig. 1A). Clusters of tRNA genes pepper within the inner (*imr*) domains, while tandem but variable copies of the *dg* and *dh* repeats, whose sequences are remarkably conserved among themselves, orient in an inverted symmetry within the centromeric outer (*otr*) domains surrounding both sides of the central core of each chromosome (Takahashi et al. 1992). Reporter genes ectopically placed at the *otr*, *imr*, and *cnt* domains are transcriptionally silenced (Allshire et al. 1994).

Although less is known about the nature of telomeres and rDNA loci, evidence of PEV, suppression of DNA recombination, and common heterochromatin-associated factors localized to these regions as to the centromeres indicate similar mechanisms of heterochromatin formation operating at these loci as well. In contrast, the mating-type region of fission yeast located on the right side of the centromere of chromosome II is a well-studied locus containing several *cis*-acting elements that are known to contribute to transcriptional silencing at the *mat* locus (Fig. 1B). Heterochromatin spanning across the *K* region, a 20-kb interval between *mat2* and *mat3* loci of the mating-type region, has been shown to be important for maintaining gene silencing and suppression of interchromosomal recombination. Interestingly, heterochromatin at the mating-type region has been implicated in the control of directionality of mating-type switching, in which mating-type information present at the upstream *mat1* locus is unidirectionally replaced with the opposite mating-type allele copied from donor cassettes present downstream at *mat2* and *mat3* loci. In fact, genetic screens looking for mating-type switching mutants identified factors that also participate in heterochromatin assembly (Egel et al. 1984; Grewal and Klar 1997).

HETEROCHROMATIN ASSEMBLY FACTORS IN FISSION YEAST

Several *trans*-acting factors involved in effecting gene silencing at heterochromatic loci within the *S. pombe* genome have been identified (Table 1). These factors directly or indirectly affect heterochromatin assembly at these loci. Importantly, many of these factors have been shown to have homologs in higher eukaryotes that par-

Figure 1. Diagrammatic representation of the *S. pombe* centromeres and the mating-type region. (A) The central domain (cnt) at each centromere is flanked by a series of inner (imr) and outer (otr) repeat elements. The otr regions contain dg and dh repeats that are highly conserved among themselves and double-stranded RNA (dsRNA) transcripts have been detected from these regions. (B) The mating-type region contains three loci involved in mating-type switching—*mat1*, *mat2*, and *mat3*—and a number of silent *cis*-acting elements, among the most dominant is *cenH*, which lies within the 20-kb *mat2/3* interval, a heterochromatic region exhibiting both suppression of transcription and recombination. *cenH* possesses dg and dh repeats that are highly homologous to centromeric dg and dh repeats. dsRNA transcripts whose regulation is RNAi dependent have also been detected at *cenH*. The double black triangles between *cenH* and *mat3* indicate Atf1/Pcr1-binding sites.

take in heterochromatin formation as well. spSir2, Clr3, and Clr6 are histone deacetylases (HDACs), enzymes that remove acetyl group from histones' tails (Grewal et al. 1998; Bjerling et al. 2002; Nakayama et al. 2003; Shankaranarayana et al. 2003). spSir2 belongs to a special class of HDACs, present also in budding yeast and higher eukaryotes, that requires the cofactor nicotinamide adenine (NAD) for its catalytic activity (Shankaranarayana et al. 2003). Clr3 and Clr6 are homologous to mammalian HDACs class 1 (HDAC1 and HDAC2) and class 2 (HDAC2 and HDAC5), respectively. HDAC activity has been shown in many systems, including *S. cerevisiae*, to be a prerequisite for establishing a repressive chromatin state, and genome-wide analyses have indicated that heterochromatic regions tend to exhibit overall hypoacetylation relative to euchromatic regions (Kurdistan and Grunstein 2003; Schubeler et al. 2004).

Clr4 is homologous to Su(var)3-9 in *Drosophila* and SUV39H1 in mammals. Both loss of Clr4 and Su(var)3-9 suppress PEV in *S. pombe* and *Drosophila*, respectively

(Grewal and Elgin 2002). These proteins possess a conserved amino-terminal chromodomain and a carboxy-terminal SET domain. Clr4 has been shown to preferentially methylate lysine 9 residue on the amino tail of histone H3 (H3-K9) (Rea et al. 2000; Nakayama et al. 2001b). While the SET domain confers histone methyltransferase (HMT) activity on Clr4, the chromodomain is thought to be important for the localization of Clr4 to silenced loci (Nakayama et al. 2001b). Swi6 functions as a chromatin modifier and is structurally similar to heterochromatin protein 1 (HP1) of *Drosophila* and mammals (Lorentz et al. 1994). Swi6/HP1 contains a chromodomain at its amino terminus that has been shown to bind to H3-K9-methyl residues created by Clr4/Suv39H1, a variable hinge region, and the chromoshadow domain at its carboxyl terminus. The chromoshadow domain of Swi6/HP1 promotes Swi6/HP1 oligomerization, which is thought to be important for chromatin compaction and heterochromatin spreading (Brasher et al. 2000; Cowieson et al. 2000; Wang et al. 2000). Chp1 and Chp2

Table 1. Factors Involved in Heterochromatin-mediated Silencing

Locus	Function	Motifs/similarity
spSir2	NAD-dependent histone deacetylase	<i>S. cerevisiae</i> , <i>Drosophila</i> , human Sir2
clr1	Putative DNA-binding protein	Three zinc fingers
clr2	Unknown	No similarities in database
clr3	Histone deacetylase	Human HDAC4 and HDAC5, <i>S. cerevisiae</i> Hda1
clr4	Histone methyltransferase	SET domains and chromodomains/ <i>Drosophila</i> Su(var)3-9 and Polycomb, human SUV39H1
clr6	Histone deacetylase	Human HDAC1 and HDAC2, <i>S. cerevisiae</i> Rpd3
swi6	Chromatin modifier	Chromodomains and shadow domains/ <i>Drosophila</i> and human HP-1 and Polycomb
chp1	Chromatin modifier	Chromodomain, homologous to Swi6
chp2	Chromatin modifier	Chromodomains and shadow domains, homologous to Swi6
rik1	Putative RNA/DNA-binding protein	11 WD40-like repeats

are also chromodomain proteins. Chp2 is similar to Swi6 in structure possessing a chromodomain as well as a chromoshadow domain. Loss of Chp1, Chp2, or Swi6 results in derepression at a number of heterochromatic regions including at the centromeres and the rDNA tandem repeat loci (Thon and Verhein-Hansen 2000). Rik1, which shares homology to Ddb1, a group of evolutionarily conserved proteins involved in cullin-mediated ubiquitination and nucleotide excision repair (Tuzon et al. 2004), has been shown to be essential for Clr4 methylation of H3-K9 at heterochromatic loci (Nakayama et al. 2001b).

HISTONE CODE AND HETEROCHROMATIN ASSEMBLY

The basic unit of eukaryotic chromatin is nucleosome, an octamer of histone proteins made up of two molecules each of H2A, H2B, H3, and H4. A stretch of 146 bp of genomic DNA coils tightly around the carboxy-terminal two-third of the core histones, leaving the amino-terminal tails of histones to freely interact with other chromosomal factors. Certain key amino acid residues on the histone tails are targeted by a number of enzymes to a variety of posttranslational modifications such as acetylation, methylation, phosphorylation, ADP-ribosylation, and ubiquitylation. These modified residues have been shown to serve as markers that can recruit chromatin modifier proteins. Accumulating evidence suggests these covalent modifications on histone tails together may constitute a “histone code” that is read by nonhistone chromosomal proteins to effect changes ranging from a few nucleosomes at a locus to large-scale global organization of an entire chromosome (Strahl and Allis 2000; Jenuwein and Allis 2001). Recent findings in fission yeast and higher eukaryotes have led to a model of heterochromatin assembly based on a sequential series of histone modifications involving concerted hypoacetylation by HDACs and methylation by Clr4/Suv39H1 and other HMT proteins.

An important advance in which a molecular marker could be identified with heterochromatin occurred when work performed in our lab by Jun-ichi Nakayama, in collaboration with David Allis’s group, provided the first in vivo evidence of H3-K9 methylation associated with heterochromatin (Nakayama et al. 2001b). Nakayama et al. detected H3-K9 methylation at the fission yeast’s centromeres and the mating-type region, and this methylation pattern depends on the presence of Clr4: Loss of Clr4 completely abolishes H3-K9 methylation at these loci (Nakayama et al. 2001b). Moreover, point mutations in the SET domain as well as the carboxy-terminal post-SET motif severely hinder the ability of Clr4 to methylate H3-K9 (Nakayama et al. 2001b). The discovery of stable methylation marks on heterochromatin-associated histone H3-K9 reveals that this may be part of the histone code, signaling to recruit heterochromatin assembly factors. Indeed, one protein that is capable of binding to methyl H3-K9 marks is Swi6/HP1. Swi6/HP1 can bind to methylated H3-K9 in vitro, but with preference for dimethyl and trimethyl H3-K9, which is thought to correspond to the degree of gene silencing (Bannister et al.

Figure 2. Stepwise model of heterochromatin assembly. Heterochromatin assembly is thought to require concerted deacetylation and methylation on the histones’ tails. Deacetylation by the HDACs’ enzymes creates a repressive chromatin state and allows histone methyltransferases such as Clr4/Suv39h1 to methylate lysine 9 on histone H3 (H3-K9 Me). Methylation of H3-K9 provides the binding sites for Swi6/HP1 proteins, which, once bound to chromatin, can facilitate heterochromatin spreading to adjacent sequences.

2001; Lachner et al. 2001; Jacobs and Khorasanizadeh 2002; Nielsen et al. 2002). In fission yeast, localization of Swi6 to centromeric and the mating-type loci is entirely dependent on the presence of H3-K9 methylation (Nakayama et al. 2001b). Deletion of Clr4 or loss of Clr4 HMT activity delocalizes Swi6 from heterochromatic loci suggesting that the binding of Swi6 to chromatin is primarily dependent on Clr4 to methylate H3-K9 (Nakayama et al. 2001b). Interestingly, loss of Swi6 prevents the spreading of H3-K9 methylation throughout the *mat* locus while mutations in Clr4 attenuate Swi6 association with heterochromatin (Nakayama et al. 2001b; Hall et al. 2002). Together, these data support a model of heterochromatin assembly in which Swi6 initially localizes to chromatin via its binding to methylated H3-K9 established by Clr4 (Fig. 2). Once bound to methylated H3-K9, Swi6 can stimulate self-oligomerization through its chromoshadow domain and the recruitment of additional Clr4 proteins to methylate H3-K9 residues on adjacent nucleosomes (G. Xiao and S.I.S. Grewal, unpubl.), thereby creating more binding sites for Swi6. The result is a feedback loop that allows heterochromatin to spread to neighboring sequences (Fig. 2).

Although methylation of H3-K9 by Clr4 seems to be the critical step in the assembly of heterochromatin, it is likely to be preceded by a series of deacetylation steps, by Clr3, spSir2, and Clr6 HDACs, because loss of any of these proteins either reduces or abolishes H3-K9 methylation altogether (Nakayama et al. 2001b, 2003; Shankaranarayana et al. 2003).

RNAi-MEDIATED HETEROCHROMATIN FORMATION

Repetitive DNA elements have long been known to play an important role in inducing heterochromatin (Selker 1999; Hsieh and Fire 2000; Hall and Grewal 2003). In fission yeast, a *cenH* element homologous to centromeric *dg* and *dh* repeats present within the *mat2/3* interval contributes to epigenetic silencing of the mating-type region (Grewal and Klar 1996). Mutant strain carrying a deletion of *cenH* fails to efficiently establish heterochromatin at the *mat* locus (Grewal and Klar 1996, 1997). Insertion of *cenH* confers repression on a reporter gene and induces heterochromatin at an ectopic euchromatic site (Ayoub et al. 2000). Recent findings suggest *cenH* and similar repetitive DNA elements collaborate with the RNAi machinery to induce heterochromatin formation at loci containing these repetitive elements (Hall et al. 2002; Hall and Grewal 2003).

The fission yeast contains three evolutionarily conserved components of the RNAi machinery—namely, Ago1 (a PAZ/Piwi family Argonaute), Dcr1 (a RNaseIII-like enzyme Dicer), and Rdp1 (an RNA-dependent RNA polymerase)—and all have been found to be necessary for heterochromatin formation in *S. pombe* (Hall et al. 2002; Volpe et al. 2002). In RNAi mutants lacking either Ago1, Dcr1, or Rdp1, there is a complete loss of H3-K9 methylation and Swi6 localization at centromeric repeats and at *cenH* ectopic site (Hall et al. 2002; Volpe et al. 2002). Interestingly, H3-K9 methylation and Swi6 localization are apparently normal at the mating-type region in these RNAi mutants (Hall et al. 2002). However, in contrast to wild-type strains, RNAi mutants grown in the presence of a deacetylase inhibitor trichostatin A (TSA), which has been shown to remove epigenetic imprint of transcriptional silencing at the *mat* locus, fail to efficiently reestablish heterochromatin upon the removal of TSA (Hall et al. 2002). These results distinguish heterochromatin formation into two distinct phases: the initial targeting and nucleation of heterochromatin, and the spreading and maintenance of heterochromatin. Importantly, the results support the critical role for RNAi in the initial phase of heterochromatin formation, while at the mating-type region RNAi becomes dispensable at the subsequent phase of heterochromatin maintenance.

A multiprotein RNAi effector complex called RITS (RNA-induced silencing complex) has been biochemically characterized in *Drosophila* (Hammond et al. 2001; Murchison and Hannon 2004). RITS contains Argonaute and small interfering RNA (siRNA) molecules of 21–24 nucleotides (nt) cleaved by the enzyme Dicer from longer double-stranded RNA (dsRNA) transcripts (Bernstein et

al. 2001). These siRNAs are thought to provide the specificity for RISC-targeted degradation or translational inhibition of mRNA and transcriptional repression. In fission yeast, siRNAs corresponding to centromeric *dg* and *dh* repeats have also been detected in wild-type cells (Reinhart and Bartel 2002). Interestingly, dsRNA transcripts derived from centromeric *otr* repeats and from *cenH* of the mating-type region, while not present in wild-type strains, could be detected in RNAi mutants, suggesting that in wild-type cells, dsRNA transcripts are actively being processed into siRNAs (Volpe et al. 2002; K. Noma and S. Grewal, unpubl.). However, the connection between siRNAs and heterochromatin formation was not readily understood until the recent purification of RITS (RNA-induced initiation of transcriptional gene silencing) in fission yeast (Verdel et al. 2004).

RITS is an RNAi effector complex containing Chp1, a heterochromatin-associated chromodomain protein that is required for H3-K9 methylation and Swi6 localization at the centromeres (Partridge et al. 2002), a novel protein Tas3, and importantly Ago1 and siRNAs (Verdel et al. 2004). RITS can localize to centromeric *otr* repeats in an RNAi-dependent manner. In the absence of Dcr1, RITS can still assemble but without siRNAs and can no longer localize to the centromeres, suggesting RITS-associated siRNAs might provide the locus-specific sequence that can guide RITS to designated genomic site. Loss of any of the RITS components abolishes H3-K9 methylation and Swi6 localization at the centromeres. These results

Figure 3. Model of RNAi-mediated heterochromatin formation in *S. pombe*. Double-stranded RNAs (dsRNAs) produced (possibly aided by Rdp1) from regions containing repetitive elements are recognized and converted into siRNAs by Dcr1. siRNAs contain the locus-specific sequence and, in complex with RITS or yet unknown protein(s), may facilitate the recruitment of heterochromatin assembly factors such as Clr4 to site with homologous sequence. Once H3-K9 has been methylated by Clr4, Swi6 can localize to the target site and stimulate the spreading and establishment of heterochromatin.

implicate RITS as the complex that links the RNAi pathway to heterochromatin assembly, providing a cellular mechanism for how RNAi can induce heterochromatin formation at specific genomic loci. A basic picture of how the RNAi machinery can cooperate with chromatin modifier factors to assemble heterochromatin at regions producing dsRNAs can now be delineated as follows (Fig. 3).

The first step in RNAi-mediated heterochromatin assembly involves the synthesis of double-stranded transcripts from genomic regions containing highly repetitive elements. These transcripts are recognized and converted by Dcr1 into siRNAs. siRNAs in association with RITS facilitate in guiding RITS to genomic loci with homologous sequences. Once bound to chromatin, RITS can mediate the recruitment of histone modifier proteins including Clr4. Alternatively, RITS presumably recruits the RNAi machinery to the target site to promote the generation of siRNAs that in turn stimulates the recruitment of heterochromatin assembly factors. However, once Clr4 is recruited and the initial methylation of H3-K9 is established, Swi6 can abet in the spreading of heterochromatin in *cis* into neighboring sequences. The persistent presence of RITS on chromatin once heterochromatin has already been assembled suggests a posttranscriptional role for RITS (K. Noma and S. Grewal, unpubl.). Perhaps RITS participates in posttranscriptional repression and, conceivably in collaboration with Rdp1 and Dcr1, processes dsRNA into siRNAs, similar to the function of RISC seen in other systems (Murchison and Hannon 2004).

RNAi-INDEPENDENT HETEROCHROMATIN FORMATION AT THE MATING-TYPE REGION

While RNAi mutants display a complete loss of heterochromatin at the centromeres, heterochromatin and gene silencing are not affected at the mating-type region (Hall et al. 2002). This rather paradoxical result suggests that other *cis*-acting silent elements besides *cenH* could compensate for the loss of RNAi to maintain heterochromatin at the *mat* locus. Indeed, since RNAi and *cenH* act in the same pathway and *cenH*-deleted mutant strains containing a reporter gene inserted at the *mat2/3* interval could still acquire an epigenetic *off* state, these observations suggest the existence of RNAi-independent factors that can initiate heterochromatin formation at the mating-type region in the absence of RNAi (Grewal and Klar 1997; Hall et al. 2002). Songtao Jia from our lab analyzed the *mat2/3* interval and identified two heptamer binding sites for the transcription factor Atf1/Pcr1 located near the *mat3* locus (Jia et al. 2004). Whereas RNAi mutants retain normal epigenetic silencing at the mating-type region, RNAi mutants lacking either Atf1 or Pcr1 exhibit a dramatic reduction in silencing, accompanied by significant decreases in H3-K9 methylation and Swi6 level at the *mat* locus (Jia et al. 2004). Chromatin immunoprecipitation (ChIP) assay confirms Atf1 and Pcr1 localization to *mat3* region containing the Atf1/Pcr1-binding sites. Deletion of those sites negates the binding of Atf1 and Pcr1 to *mat3* locus. Interestingly, Atf1/Pcr1 localization

to the mating-type region is not affected in the absence of Swi6. Taken together, these results suggest that Atf1/Pcr1 can bind directly to the *mat3* region, and it acts probably upstream of Swi6 and is capable of recruiting heterochromatin assembly factors such as HDACs and Clr4 to nucleate heterochromatin formation independent of the RNAi pathway (Jia et al. 2004).

PROPAGATION AND INHERITANCE OF HETEROCHROMATIC STRUCTURES

Results from our studies of the fission yeast have revealed multiple parallel pathways that could lead to heterochromatin formation. Once heterochromatin is established, its propagation and stability need to be maintained with fidelity through multiple cell divisions without being “diluted” by macrocellular processes such as DNA replication and chromosome condensation and segregation. For example, during DNA replication, epigenetic imprint from one DNA molecule has to be transferred to the newly synthesized molecule. Since heterochromatin propagates in *cis* (Nakayama et al. 2000), this probably requires heterochromatin assembly factors to collaborate with the DNA replication machinery to reestablish epigenetic marks (imprinting) on the new DNA molecule as it is being synthesized. Alternatively, components of the DNA replication machinery such as proliferating cell nuclear antigen (PCNA) and chromatin assembly factor-1 (CAF-1) can recognize epigenetic imprints at heterochromatic loci and direct heterochromatin assembly factors to newly duplicated regions marked for heterochromatin reassembly (Shibahara and Stillman 1999; Zhang et al. 2000).

We have proposed a chromatin-replication model in which modified histones and histone-associated proteins segregate semiconservatively to each DNA strand during DNA replication that could promote self-reassembly of heterochromatin on both duplicated chromosomes (Grewal and Klar 1996; Hall and Grewal 2003). Swi6 is stably associated with heterochromatin throughout the mitotic cell cycle and the entire period of meiosis (Nakayama et al. 2000). Also, Swi6 can associate with DNA polymerase α , a component of the DNA replication machinery (Ahmed et al. 2001; Nakayama et al. 2001a), and mouse HP1 has been shown to interact with CAF-1 (Murzina et al. 1999). These findings suggest that components of heterochromatin such as Swi6/HP1 can collaborate with the DNA replication apparatus to mediate heterochromatin reassembly during DNA replication. The simplest scenario of the chromatin-assembly model entails methylated histone H3-K9 being stochastically redistributed to each of the two DNA strands as the replication fork passes through a heterochromatic site. Next, Swi6 can bind to the “diluted” methylated histone H3-K9 on the newly duplicated DNA molecules and helps to recruit HDACs and HMT proteins such as Clr4 to fully restore the epigenetic marks and reestablish heterochromatin to the original locus on both duplicated chromosomes. Alternatively, Swi6 and methylated histone H3-K9 are randomly transferred to the newly duplicated DNA molecules together as a unit. Interestingly, a

number of studies have used fluorescence recovery after photobleaching (FRAP) analysis to closely monitor the kinetics of Swi6 and HP1 association with heterochromatin in real time, and all have found that the turnover rates for Swi6 and HP1 proteins are quite rapid, suggesting that the binding of Swi6/HP1 proteins to methylated H3-K9 is a dynamic event and that under conditions that could temporarily disrupt heterochromatin Swi6/HP1 can rapidly reassociate with H3-K9 methyl marks to reestablish heterochromatin (Cheutin et al. 2003, 2004; Festenstein et al. 2003).

In fission yeast, RNAi mutants are unable to retain heterochromatin at the centromeres, which implies the requirement for RNAi in stably maintaining heterochromatin. Indeed, our recent data suggest that stable association of RITS with chromatin is critical for the generation of RITS-associated siRNAs and the maintenance of heterochromatin at centromeres (K. Noma and S. Grewal, unpubl.). Therefore, even though heterochromatin once established is relatively stable, it tends to attenuate over time because of macrocellular processes such as DNA replication and chromosome condensation that could disrupt long-lived chromosomal structures such as heterochromatin. Consequently, there would be a continual need for heterochromatin nucleation factors, such as the RNAi machinery and/or Atf1/Pcr1, to recruit heterochromatin assembly factors, such as Clr4 and Swi6, to maintain heterochromatin so as to faithfully preserve epigenetic imprints across multiple cell divisions.

BOUNDARY DETERMINANTS OF HETEROCHROMATIN

The existence of constitutive heterochromatin domains in fission yeast and higher eukaryotes suggests the need for robust cellular mechanisms that can demarcate clear boundaries partitioning heterochromatic regions from eu-

chromatic regions. In addition, chromatin domains have to be organized in a way that gives deference to a chromosome's structural constraints imposed by diverse cellular processes impinging upon the entire chromosome. For instance, chromatin surrounding the central core (*cnt*) and inner repeats (*imr*) of *S. pombe* centromeres differ in structure and organization from the *outer* (*otr*) repeats regions (Partridge et al. 2000). These two chromatin domains are epigenetically silenced and are enriched with Mis6 and CENP-A centromeric proteins, which are essential for proper biorientation of sister centromeres during mitotic metaphase (Saitoh et al. 1997; Partridge et al. 2000; Takahashi et al. 2000). Surprisingly, Swi6 is virtually excluded from the *cnt* and most of the *imr* regions and instead is confined to the outer repeats (*otr*) regions (Partridge et al. 2000). Evidently, a cluster of tRNA genes situated at the exterior region of *imr* domains act as barriers separating one silenced domain from another silenced domain with disparate functions (Partridge et al. 2000). Interestingly, tRNA genes that act as boundary elements as well have also been demonstrated in *S. cerevisiae* (Donze et al. 1999).

The embedded heterochromatic *K*-region of the *mat* locus within a larger euchromatic domain also implies the existence of boundary elements. Whereas marker genes placed within the *mat2/3* interval or *K*-region are subject to epigenetic silencing, marker genes inserted outside the *K*-region are not repressed, suggesting elements present on both sides of the *K*-region could act to insulate the heterochromatic *mat2/3* interval from the surrounding euchromatin. In fact, two 2-kb identical inverted repeats termed IR-L and IR-R flank the left and right sides of the *K*-region, respectively. While H3-K9 methylation is associated with transcriptional silenced domains, H3-K4 methylation correlates with active transcribed regions (Strahl et al. 1999; Noma et al. 2001). Ken-ichi Noma in our lab performed a high-resolution mapping of H3-K9

Figure 4. Distinct and mutually exclusive histone H3-K4 and H3-K9 methylation patterns respectively delimit euchromatic and heterochromatic domains at the *mat* locus. Distribution of H3-K4 and H3-K4 methylation patterns and Swi6 levels at the mating-type region shown in alignment with a physical map of the *mat* locus (top). IR-L and IR-R flanking the left and right side of the *mat2/3* interval, respectively, act as boundary elements partitioning heterochromatin spanning the *mat2/3* interval from surrounding euchromatin regions. Loss of Swi6 prevents the spreading of H3-K9 methylation beyond the left side of *cenH* (bottom).

and H3-K4 methylation across the entire 47 kb *mat* locus (Noma et al. 2001). Consistent with evidence of the *K*-region being silenced, there was considerable enrichment of H3-K9 methylation throughout the *mat2/3* interval while H3-K4 methylation was virtually absent within this region (Fig. 4). Noma et al. also mapped the level of Swi6 proteins across the *mat* locus and found its localization pattern identically paralleled H3-K9 methylation pattern throughout the mating-type region, confirming the idea that H3-K9-methyl marks act as binding sites for Swi6 proteins (Noma et al. 2001). Unexpectedly, there was a sudden sharp concomitant decline of H3-K9 methylation and Swi6 levels on both sides of the *K*-region where the two IR elements reside (Noma et al. 2001). Outside of the *K*-region, there was a gradual increase in H3-K4 methylation on both sides where a number of transcriptionally active loci lie. These results indicate that methylation of H3-K4 or H3-K9 is associated in a mutually exclusive manner with euchromatin and heterochromatin, respectively (Noma et al. 2001). Intriguingly, ChIP mapping across the 53-kb chicken β -globin locus also found similar patterns of H3-K4 methylation and H3 acetylation that are negatively correlated with the pattern of H3-K9 methylation (Litt et al. 2001).

The high level of methylated H3-K9 seen at the two IR elements on the side of the *K*-region while its level precipitously dropped at the immediate outside region of the *mat2/3* interval suggest IR-L and IR-R may act as boundaries for the heterochromatic *mat2/3* interval. Indeed, removal of either IR element allows heterochromatin to spread outside of the *mat2/3* interval and weakens heterochromatin at the *K*-region (Noma et al. 2001; Thon et al. 2002).

CONCLUDING REMARKS

While it has become increasingly clear that cells utilize heterochromatin to impose transcriptional silencing constitutively to a particular genomic region, the larger roles that heterochromatin plays in other cellular processes—such as chromosome segregation, suppression of recombination, and maintenance of genomic integrity—have only begun to be appreciated. For instance, the condensed structures of heterochromatin in *S. pombe* have been shown to be important for proper functioning of the centromeres and telomeres. Heterochromatin loss at the centromeres affects proper orientation and assembly of the kinetochore (Pidoux and Allshire 2000; Sullivan et al. 2001). In addition, components of heterochromatin such as Swi6 have been reported to recruit cohesin proteins to the centromeres as well as other heterochromatic regions along the chromosome arms, including the *mat2/3* interval (Hall and Grewal 2003). In RNAi mutants in which heterochromatin is absent at the centromeres, centromeric cohesion was compromised, accompanied by severe mitotic and meiotic segregation defects (Hall et al. 2003). Last, at the telomeres, telomere-specific silencing factors, such as Taz1 and Rap1, and RNAi components contribute to heterochromatin formation. The presence of heterochromatin at the telomeres is important for the

maintenance of proper telomere length and the clustering of telomeres at the spindle pole body during meiosis (Cooper et al. 1998; Nimmo et al. 1998; Hall et al. 2003).

The many commonalities shared between heterochromatic regions of the *S. pombe* genome and genomes of other higher eukaryotes—from homologous molecular components of heterochromatin to similar processes of heterochromatin assembly and, even still, to similar higher-order chromatin organization of the genome, such as the structures of centromeres and telomeres—suggest that insights gained from the studies of heterochromatin in *S. pombe* will be equally applicable to the studies of heterochromatin in organisms with larger and more complex genomes. Particularly, in view of the fact that a relatively small portion of mammalian genomes consists of euchromatin, with euchromatic “islands” often scattered within larger heterochromatic domains, the role of heterochromatin in these systems will be vital for the normal functioning of the cell and the biology of the organism as a whole.

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