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Different means, same end – heterochromatin formation by RNAi and RNAi-independent RNA processing factors in fission yeast

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Abstract

The assembly of heterochromatin in eukaryotic genomes is critical for diverse chromosomal events including regulation of gene expression, silencing of repetitive DNA elements, proper segregation of chromosomes and maintenance of genomic integrity. Previous studies have shown that non-coding RNAs and the RNAi machinery promote the assembly of heterochromatin that serves as a multipurpose platform for targeting effectors involved in various chromosomal processes. Recent work has revealed that RNAi-independent mechanisms, involving RNA processing activities that utilize both non-coding and coding RNAs, operate in the assembly of heterochromatin. These findings have established that, in addition to coding for proteins, mRNAs also function as signaling molecules that modify chromatin structure by targeting heterochromatin assembly factors.

Emil Heitz first defined the concepts of euchromatin and heterochromatin in 1920s based on the differential compaction of chromatin during interphase. While euchromatin is less condensed, and more readily transcribed, heterochromatin is typically highly condensed and generally inhibitory to transcriptional machinery [1]. Heterochromatin can be classified into two subtypes: constitutive and facultative heterochromatin. Constitutive heterochromatin contains highly repetitive DNA sequences such as those found at centromeres and telomeres, and serves to stably silence transposable elements as well as facilitate chromosome dynamics [1]. In contrast, facultative heterochromatin is typically found associated with developmentally regulated genes, where the chromatin structure changes occur in response to cellular differentiation signals [2].

While initially described by its physical characteristics, heterochromatin is more recently defined by a variety of associated biochemical features. Heterochromatin regions are characterized by hypoacetylation of histones, and with the exception of *Saccharomyces cerevisiae*, histone H3 is methylated at lysine 9 (H3K9me) [1,3]. H3K9me mediates recruitment of the conserved HP1 protein family members that are essential for the assembly of heterochromatin [1,3–6]. Among other functions, HP1 proteins and other H3K9me-associated factors facilitate loading of chromatin modifiers and RNA processing activities to enforce transcriptional and posttranscriptional gene silencing (TGS and PTGS) across heterochromatin domains [1,7]. The H3K9me-HP1 platform also targets other factors including factors involved in chromosome segregation [1,8,9]. Defects in heterochromatin

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assembly cause increased recombination leading to genomic instability [1,10], highlighting the importance of heterochromatin assembly as a fundamental and essential cellular process.

Multiple strategies are utilized to nucleate heterochromatin [11]. In addition to DNA-binding proteins, RNAs also play pivotal roles in heterochromatin assembly (Figure 1A) [11–14]. RNAs can serve directly as molecular scaffolds for localizing chromatin-modifiers [11,15,16]. In addition, the processing of RNAs by RNA interference (RNAi) machinery [1,7,12] and nuclear RNA surveillance factors [14,17,18] is functionally coupled to the loading of heterochromatin factors. An emerging theme is that transcriptional activity and RNAs mediate heterochromatin formation, while heterochromatin factors prevent accumulation of potentially deleterious RNAs by facilitating their degradation or by assembling repressive chromatin. In addition to repeat elements, heterochromatin and RNA processing factors including RNAi machinery act broadly across the genome at gene-containing regions to suppress untimely expression of genes or aberrant RNAs [19–21].

In this review, we highlight insights gained from using the fission yeast *Schizosaccharomyces pombe* as a model system for the study of heterochromatin assembly mechanisms. We discuss how the production of RNAs and RNA processing activities are coupled to the recruitment of histone-modifying enzymes implicated in heterochromatin formation, and describe the RNA surveillance functions of heterochromatin factors. We also focus on recent developments linking RNA processing factors that target coding RNAs to the triggering of facultative heterochromatin assembly and modulation of developmentally regulated genes. Another key finding discussed is that heterochromatin can induce transcriptional silencing, at least in part by promoting nucleosome occupancy.

RNAi-dependent targeting of heterochromatin

RNAi is a conserved cellular process involved in posttranscriptional silencing [22,23]. RNAi also plays a key role in the assembly of heterochromatin in several organisms [1,12,24–26]. In *S. pombe*, the core RNAi factors Argonaute (Ago1), Dicer (Dcr1), and RNA-dependent RNA polymerase (Rdp1) target transcripts generated by *dg* and *dh* repeats embedded in major heterochromatin domains at centromeres, subtelomeres and the silent *mat* locus [27–29]. Dcr1 processes double stranded RNA (dsRNA) into small interfering RNAs (siRNAs), which are loaded onto the RNA-induced transcriptional silencing (RITS) complex. In addition to Ago1, RITS consists of the chromodomain protein Chp1 and Tas3 [30]. RITS facilitates the localization of Rdp1 [31], a component of the RNA-dependent RNA polymerase complex (RDRC) that also contains the polyA polymerase Cid12 and the putative helicase Hrr1 [32]. RDRC requires Swi6/HP1 for its localization [31], and generates dsRNA [31,32] in a process involving polyadenylation of transcripts by Cid12 [32].

siRNAs are believed to target RITS to nascent repeat transcripts via interaction with Ago1 (Figure 1B) [30]. RITS localization to heterochromatic loci also depends on the binding of Chp1 to H3K9me [29,33–35]. RITS processes repeat transcripts and mediate the localization of Clr4/Suv39h [36], a methyltransferase involved in methylation of H3K9 [5,37]. Clr4 forms a multisubunit E3 ubiquitin ligase complex (ClrC) that contains Cullin 4 and Rik1, among other factors [38,39]. ClrC interaction with RITS [36] requires an adaptor protein Stc1 that is implicated in H3K9me at *dg/dh* repeats [40]. Like RITS, ClrC and Stc1 are required for the production of siRNAs [33,40]. H3K9me by ClrC also recruits the HP1 family proteins Swi6 and Chp2. HP1 proteins are implicated in various heterochromatin functions such as TGS [9,41–44]. Clr4 contains chromodomain that also binds H3K9me [36]. The ability of Clr4 to methylate H3K9 and bind to H3K9me is critical for spreading of heterochromatin [36].

A fascinating aspect of RNAi-dependent heterochromatin formation is that repeats assembled in repressive chromatin must be transcribed to generate siRNA precursors. Swi6/HP1 recruits Epe1, a JmjC domain-containing anti-silencing factor that facilitates RNAPII transcription of heterochromatic repeats [45]. Heterochromatin is also dynamically regulated during the cell cycle [46,47]. Chromatin modifiers silence heterochromatic repeats throughout most of the cell cycle, with RNAPII accessibility increased only during a short window in S phase. Both strands of repeats are transcribed, and preferential loading of Rik1 and Ago1, components of the ClrC and RITS complexes, respectively, happens during S phase [46]. The findings that RITS and its associated factors affect localization of ClrC (and vice versa) [36,40] imply that cooperative recruitment of these factors occurs via a transcription-coupled mechanism [46]. Mutations in RNAPII transcription machinery and associated splicing factors affect heterochromatin assembly [48–52]. It is possible that RNAPII transcription also plays a more direct role in heterochromatin assembly that is independent of RNAi (see below).

RNAi and heterochromatin factors suppress antisense RNAs

Transcriptome analyses have revealed that extensive transcription occurs, often on both DNA strands, across the *S. pombe* genome [53–55]. There is widespread generation of cryptic, antisense, and read-through RNAs [19,53–56]. Histone deacetylases (HDAC) and RNA processing activities silence heterochromatic repeat elements [1,7]. HDACs also suppress transcripts initiating from cryptic promoters within open reading frames [56], and silence retrotransposons [57]. Surprisingly, while Clr4 and RNAi were initially thought to mainly silence heterochromatic repeats, recent evidence suggests that they act broadly across the genome to suppress read-through antisense transcripts [19,20,58]. Studies also indicate the presence of functional redundancy in the pathways that contribute to suppression of aberrant RNAs. For instance, the loss of either Clr4 or RNAi causes only minor defects in antisense suppression. However, loss of these factors together with a variant histone, H2AZ, causes widespread cumulative increase in antisense RNAs [19,20]. These studies indicate that while RNAi and heterochromatin factors act broadly to suppress aberrant RNAs, functional redundancies can mask their individual roles.

How RNAi and heterochromatin factors suppress antisense RNAs is a focus of ongoing research. Heterochromatin is believed to target cohesin, which in turn promotes transcription termination at convergent genes [21]. RNAi and heterochromatin factors also promote RNA degradation via a mechanism that involves the nuclear exosome [19,36]. Structural analysis reveals a PIN domain in the RITS subunit Chp1, which together with Tas3 is believed to provide a platform for RNA processing activities [59]. RITS and Clr4 also interact with Mlo3, an RNA quality control and export factor [20]. Loss of Clr4 impairs RITS association with Mlo3, which is required for antisense suppression and centromeric siRNA production [20]. In addition, Mlo3 forms a complex with the RNA surveillance complex TRAMP [20], of which the Trf4 family poly(A) polymerase Cid14 is a member [20,60]. Cells lacking Mlo3 or TRAMP accumulate antisense RNAs that are targeted by Clr4 and RNAi [19,20]. These analyses have led to the suggestion that Clr4 and Mlo3 serve as a hub that connects heterochromatin machinery to RNA processing through the cooperative actions of TRAMP, RNAi and the exosome. It is possible that the polyadenylation of target RNAs by Mlo3-associated TRAMP “tags” RNAs for degradation [20]. The targeted RNAs can be degraded directly by the exosome, or alternatively, the Clr4-mediated interaction between Mlo3 and RITS can result in the shuttling of targeted RNAs into the RNAi pathway [20]. The processing of transcripts by Ago1/RITS creates entry sites for the 3′-5′ exonuclease activity of the exosome, and also recruits RDRC and Dicer to generate siRNAs. This model implies competition between the exosome and RNAi degradation activities, such that high levels of

RITS and RDRC at heterochromatic loci might favor processing of transcripts into siRNAs, while the exosome pathway would be favored at other genomic loci.

RNAi-independent but transcription-dependent heterochromatin assembly

Despite a prominent role for RNAi in heterochromatin assembly, the loss of RNAi factors does not completely abolish H3K9me [18,41,61,62]. It has been suggested that DNA polymerase- ϵ subunit Cdc20 interacts with ClrC to target H3K9me [63]. In addition, Dcr1-independent small RNAs, called primal RNAs, have been proposed to target H3K9me at centromeres via a mechanism requiring Ago1 [64], however decreased-levels of H3K9me are detected in *ago1 Δ* cells [18,61,62]. Recent evidence for an RNAi-independent mechanism that utilizes transcription and RNAs to nucleate heterochromatin has been uncovered [18]. A key finding is that cells lacking Mlo3 or TRAMP assemble functional centromeric heterochromatin in RNAi deficient cells. *mlo3 Δ* restores Rik1/ClrC localization and triggers *de novo* heterochromatin assembly in RNAi mutants. Furthermore, *mlo3 Δ* causes H3K9me at selected euchromatic loci that show elevated levels of antisense RNAs in mutant cells [18]. The failure to process aberrant RNAs by Mlo3-TRAMP, which act co-transcriptionally, might generate specific RNA structures recognized by the ClrC. Additional degradation activities could be involved, which like RNAi, couple RNA processing to heterochromatin nucleation [14]. Indeed, the nuclear exosome subunit Rrp6 acts in parallel with RNAi to promote heterochromatin formation [18]. Whether the exosome serves as a scaffold or its enzymatic activity is critical for RNAi-independent loading of heterochromatin factors is unknown. It is possible that a RNAi-independent, transcription-dependent mechanism is activated during S-phase, when transcription of both strands of centromeric repeats mimics upregulation of bidirectional transcripts in *mlo3 Δ* , correlating with targeting of Rik1/ClrC [46].

The loss of either the RNAPII processivity factor TFIIS or the histone acetyltransferase Mst2 also rescues the heterochromatin defects of RNAi mutants [18,65]. However, the exact mechanism responsible for these effects is not known. Defective RNAPII elongation could bypass RNAi by affecting the processing of transcripts and/or the release of RNAPII. Alternatively, impaired transcription may stabilize residual H3K9me in RNAi mutants by precluding elongation-coupled turnover of histones and/or their modification state.

Facultative heterochromatin assembly by mRNA processing factors

The *S. pombe* genome contains small blocks of heterochromatin dispersed throughout the genome [14,29]. In wild-type cells, these heterochromatin islands encompass ~30 genes. However, in cells lacking the anti-silencing factor Epe1, additional H3K9me peaks covering ~100 genes are detected [14], suggesting that this model eukaryotic genome harbors numerous heterochromatin nucleation sites normally hidden by negative regulators.

A distinctive feature of heterochromatin islands is their preferential association with meiotic genes that are silenced during vegetative growth [14]. Notably, heterochromatin islands are functionally important. H3K9me-associated HP1 and silencing effectors (HDACs and RITS) localize to target meiotic loci. In addition, Dcr1 and Rdp1 localize to certain heterochromatin islands [58]. These blocks of heterochromatin are akin to facultative heterochromatin and are regulated dynamically in response to developmental signals. Specifically, nutritional signals (such as nitrogen starvation) that induce sexual differentiation and meiosis cause disassembly of heterochromatin islands [14]. Little is known about the cascade of events signaling the remodeling of heterochromatin islands, except that this process involves Epe1 [14]. Epe1 opposes the effects of chromatin modifiers that promote nucleosome occupancy at target loci [45].

How do cells assemble heterochromatin islands? Although multiple mechanisms are likely at work, an important observation is that transcription of target meiotic loci is essential for heterochromatin formation [14]. Blocking transcription and production of mRNAs abolishes H3K9me at meiotic loci, while the loss of RNAi by itself has no major effect. These results suggest that RNAi-independent mechanism(s) utilize meiotic gene mRNAs to nucleate heterochromatin [14].

Meiotic mRNAs are processed by a mechanism involving Mmi1, a protein that binds RNAs containing “determinant of selective removal” (DSR) sequences and mediates their degradation by the exosome [66]. This process also requires the conserved protein Red1, which interacts with Mmi1 and the exosome [67] (Figure 1B). Deletion of DSR from meiotic genes abolishes heterochromatin nucleation at these loci [14]. Moreover, the artificial expression of DSR induces H3K9me at an ectopic site [14]. Together with results showing that the loss of Mmi1, Red1 or Rrp6 abolishes H3K9me at meiotic loci, these findings implicate elimination machinery in transcription-coupled assembly of heterochromatin islands [14]. Consistent with this, the elimination machinery localizes to meiotic genes. In particular, the localization of Red1, a component of a protein network that recruits Clr4 to specific meiotic genes correlates with its requirement for H3K9me at individual loci [14]. The processing of meiotic mRNAs also requires pre-mRNA 3'-processing machinery, in particular the poly(A) polymerase Pla1 [67–69]. Current evidence suggests that Mmi1 and Red1 cooperate with 3'-processing machinery to promote the polyadenylation of mRNAs, effectively coupling RNA processing to heterochromatin assembly. Thus, in addition to coding for proteins, mRNAs perform regulatory functions and modulate gene expression by nucleating heterochromatin. Such RNAi-independent mechanisms likely mediate the transcription- and RNA-dependent heterochromatin formation observed in higher eukaryotes [11,13,15,70].

Nucleosome occupancy and heterochromatic transcriptional silencing

In contrast to the significant progress made in understanding heterochromatin assembly, the mechanisms of heterochromatic transcriptional silencing remain poorly understood. Loss of factors such as Clr4 result in elevated levels of RNAPII at heterochromatic loci [9,42,46], suggesting that heterochromatin inhibits transcriptional machinery. Clr4 is essential for the recruitment and spreading of the HP1 proteins Chp2 and Swi6, both of which are critical for the assembly of repressive heterochromatic structures [9,41,42]. The oligomerization of HP1 bound to methylated H3K9 provides a mechanism for bridging nucleosomes, which may promote chromatin condensation [43,71,72]. However, recent studies suggest that chromatin-associated HP1 proteins also provide a recruiting platform for repressive chromatin-modifying factors that are required for promoting TGS [41,42,73,74](Figure 2).

At least three effector protein complexes that act as critical determinants of heterochromatic TGS have been identified in genetic and biochemical studies. These include the Snf2-HDAC repressor complex (SHREC), a Clr6 HDAC complex, and the Asf1-HIRA histone chaperone (Figure 2)[42,56,73]. SHREC (containing the HDAC Clr3 and the Snf2 family protein Mit1) associates with Chp2 and Swi6, both of which are required for its localization across heterochromatic domains [9,42,44]. Swi6 also associates with a Clr6 HDAC complex and Asf1-HIRA histone chaperone [73]. While these effectors can be recruited to specific loci independent of heterochromatin, their association with HP1 proteins allows them to spread and act broadly across silenced domains [41,73].

Asf1-HIRA facilitates histone deacetylation by Clr6, which together with SHREC is essential for hypoacetylation of histones [73]. However, an important observation is that loss of SHREC activities (Clr3 and Mit1) affect nucleosome occupancy at heterochromatic

regions, resulting in the appearance of hypersensitive sites [42]. Similarly, factors involved in SHREC localization, such as Ctr4 and HP1 proteins, prevent the appearance of nucleosome free regions within heterochromatin regions [74]. SHREC acts in overlapping manner with Af1-HIRA because *clr3 asf1* double mutant show substantial reduction in nucleosome occupancy [73]. These analyses have revealed a critical aspect of heterochromatic silencing: specifically that HDACs and histone chaperones associated with the H3K9me-HP1 docking platform function in promoting nucleosome occupancy and the assembly of repressive chromatin.

Concluding Remarks

Recent studies have yielded the surprising finding that the transcription of loci assembled in repressive chromatin is critical for targeting of heterochromatin assembly machinery. Since heterochromatin formation occurs at several loci dispersed across the genome, a fundamental question remains: what makes these loci preferential targets of heterochromatin modifications? Given the role of transcription and RNAs in this process, it is likely that special features of target RNAs serve as signals that are recognized by the heterochromatin machinery. Changes in RNAPII transcription of these loci may also provide a mechanical signal that is recognized by heterochromatin factors.

Heterochromatin assembly represents a powerful genomic defense mechanism and is essential for the maintenance of genome integrity and gene regulation. Importantly, abnormal heterochromatin assembly has been linked to human diseases such as cancer. Therefore, deciphering the nature of signals that trigger heterochromatin formation is critical, both for our understanding of the roles that this specialized chromatin plays in epigenetic reprogramming of eukaryotic genomes, and for the potential to apply this knowledge to the prevention and treatment of human diseases.

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* of special interest

** of outstanding interest

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Figure 1.

(A) Heterochromatin nucleation is mediated by RNA-based and DNA sequence-based mechanisms RNA-based mechanisms to establish heterochromatin include both RNAi-dependent and RNAi-independent pathways. DNA-sequence based mechanisms rely on sequence specific DNA binding factors to recruit chromatin-modifying activities. **(B) RNA-based heterochromatin nucleation pathways.** The RNAi-dependent pathway (left) involves RITS, RDRC and Dicer. Guided by siRNAs, the RITS complex localizes to nascent transcripts where it interacts with Stc1, a scaffold that bridges RITS to Clr4. Clr4 methylates H3K9 to assemble heterochromatin. Repeat transcripts are polyadenylated by Mlo3-associated TRAMP, a factor that mediates processing of RNAs by exosome and the RNAi machinery. The RNAi-independent pathway (right) requires the RNA elimination machinery to assemble facultative heterochromatin at meiotic genes. The RNA binding protein Mmi1, along with factors involved in pre-mRNA 3'-end processing and the RNA elimination machinery, recognize specific meiotic RNAs. Red1, a protein that interacts with the exosome, may form a specialized complex that recruits Clr4 required for the assembly of heterochromatin at specific meiotic genes.

Figure 2. Heterochromatin promotes transcriptional silencing through histone deacetylation and changes in nucleosome occupancy

H3K9me that is initially targeted to nucleation sites by DNA- or RNA-based mechanisms can be spread to surrounding sequences via a process that involves Clr4 binding to methylated H3K9. HP1 proteins (Chp2 and Swi6) bound to H3K9me provide a recruiting platform for loading of the histone deacetylase complexes SHREC and Clr6-complex, and the histone chaperones Asf1-HIRA. Clr6 and SHREC have overlapping functions in limiting RNAPII occupancy at heterochromatic loci [9]. Asf1-HIRA facilitates deacetylation of histones by Clr6 HDAC. Asf1-HIRA and SHREC also promote nucleosome occupancy and eliminate nucleosome-free regions (NFRs) that are thought to prevent access to the transcriptional machinery and enforce transcriptional gene silencing.