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RNAi-dependent formation of heterochromatin and its diverse functions

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Abstract

Expression profiling of eukaryotic genomes has revealed widespread transcription outside the confines of protein-coding genes, leading to production of antisense and non-coding RNAs (ncRNAs). Studies in *Schizosaccharomyces pombe* and multicellular organisms suggest that transcription and ncRNAs provide a framework for the assembly of heterochromatin, which has been linked to various chromosomal processes. In addition to gene regulation, heterochromatin is critical for centromere function, cell fate determination as well as transcriptional and posttranscriptional silencing of repetitive DNA elements. Recently, heterochromatin factors have been shown to suppress antisense RNAs at euchromatic loci. These findings define conserved pathways that likely have major impact on the epigenetic regulation of eukaryotic genomes.

Sequencing of the human genome as well as those of model organisms has allowed the development of novel approaches to explore the complexities of eukaryotic genomes. A surprising finding is that large proportions of the genomes are transcribed, often on both DNA strands [1]. In addition to protein coding RNAs, there is widespread occurrence of so-called cryptic transcripts, antisense transcripts, and ncRNAs. An abundant source of ncRNAs is repetitive elements such as transposon-derived sequences. ncRNAs in some cases may represent “transcriptional noise” [2] of no particular biological significance. However, in many instances expression of ncRNAs is tightly controlled in response to specific environmental or developmental signals [3,4]. ncRNAs provide scaffolds for the recruitment of chromatin-modifying activities to assemble specialized chromatin structures [5,6].

Despite many functions associated with ncRNAs, their uncontrolled accumulation could lead to genomic instability [7]. Eukaryotes have evolved multiple strategies to prevent either the production of ncRNAs or to promote their processing. These pathways comprise conserved chromatin assembly factors [8,9], and/or RNA processing activities [10]. Factors involved in the assembly of heterochromatin are critical for suppressing ncRNAs [5]. Heterochromatin targeted to genomic regions containing high-density of repetitive DNA exerts repressive influence. These regions have distinct histone modification patterns compared to gene-rich euchromatin. An important characteristic of heterochromatin is its ability to spread and recruit various factors [11]. Heterochromatin mediates cell-type specific spreading of factors underlying key developmental choices [11]. It also targets

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activities involved in transcriptional and posttranscriptional silencing, as well as genome stability factors [11].

This review focuses on lessons learned from the *S. pombe* model system about heterochromatin assembly and its functions. I discuss findings suggesting that transcription of repeat elements provides scaffolds for assembly of heterochromatin, which suppresses repeat transcripts and promotes genome stability. I also highlight an emerging theme that silencing of different classes of repeat elements requires a common “toolkit” of repressor activities that are targeted via distinct mechanisms in different chromosomal contexts.

Formation of heterochromatin: a role for the RNAi machinery

The core of heterochromatin assembly pathway found in *Drosophila* and mammals is conserved in *S. pombe* [5]. It involves posttranslational modifications of histones and a common set of structural proteins. In addition to histone deacetylation, heterochromatin assembly requires methylation of histone H3 at lysine 9 (H3K9me) that provides binding site for the HP1 family chromodomain proteins [12]. In *S. pombe*, Chp1, Chp2 and Swi6 bind H3K9me through their chromodomain. Methylation of H3K9 is catalyzed by Clr4 – a homologue of *Drosophila* SU(VAR)3–9 and human Suv39h - which exists in a multisubunit E3 ubiquitin ligase complex containing Cullin 4 and Rik1 ([13] among others). Mapping of H3K9me and its associated factors has revealed extended chromosomal domains at centromeres, telomeres and the silent mating-type locus coated with heterochromatin [14]. A common feature shared by these loci is presence of repetitive DNA elements referred to as *dg* and *dh* repeats [11]. In addition, small heterochromatic islands are detected at several meiotic genes [14].

Detailed investigations have provided insights into the multi-step process of heterochromatin formation. Heterochromatin targeted to certain genomic sites can spread across large domains [14–16]. Distinct mechanisms are responsible for the initial recruitment of heterochromatin in different chromosomal contexts. DNA binding factors can target heterochromatin proteins to specific sites [16–20]. As discussed above, however, major targets of heterochromatin are transposon-derived repeat elements. Heterochromatin nucleation mechanisms appear to recognize common features that transposons utilize to spread within their host genomes. Transcription of repeat elements and ncRNAs has been exploited via RNAi-based mechanisms to target heterochromatin to these loci [15,21](Figure 1).

S. pombe dg and *dh* pericentromeric repeats surrounding the site of kinetochore formation are transcribed but their transcripts are degraded by the RNAi machinery [21]. Core RNAi components Dicer (Dcr1), Argonaute (Ago1) and RNA-dependent RNA polymerase (Rdp1) are also important for heterochromatin formation at these repeat elements [15,21]. Biochemical studies have identified an RNA-induced transcriptional gene-silencing (RITS) complex that in addition to Chp1, Ago1, and Tas3 proteins contains siRNAs derived from *dg* and *dh* transcripts [22]. RITS localizes across heterochromatic domains in a manner dependent upon binding of Chp1 chromodomain to H3K9me [14,23–25], and facilitates targeting of Rdp1 [26,27]. Chromatin association of Rdp1 also requires another H3K9me binding protein Swi6 [26]. Loss of H3K9me in *clr4Δ* cells causes defective localization of RITS and its associated factors. This correlates with concurrent defects in siRNA production [23,25,26]. Together, these studies suggest that RNAi targets nascent centromeric transcripts in the context of heterochromatin. Indeed, RITS subunits can be crosslinked to transcripts that requires Dcr1 and Clr4 [27]. Clr4 appears to have dual functions in RNAi mediated suppression of repeat transcripts – in addition to creating the H3K9me binding site for RITS,

the association of Clr4 with RITS [28] may also facilitate the processing of centromeric transcripts into siRNAs.

RNAi-mediated heterochromatin assembly involves a self-reinforcing loop wherein siRNAs generated by the heterochromatin-bound RNAi factors feedback to facilitate recruitment of heterochromatin complexes [23,26](Figure 1). In this mechanism, primary siRNAs generated presumably through the processing of hairpin-like structures from centromeric RNAs [29] program RITS for the initial recruitment of heterochromatin. In turn, binding of Chp1 to H3K9me allows RITS to localize stably across heterochromatic domains. RITS engages nascent transcript, activating Rdp1 for generation of double stranded RNAs, which are then processed by Dcr1 into siRNAs [26,27]. A key question is how do siRNAs target heterochromatin. One possibility is that siRNA-primed RITS, tethered to nascent transcripts, mediates recruitment of histone modifying activities such as Clr4. In support of this model, artificial tethering of RITS to transcripts has been suggested to induce local heterochromatin formation [30]. However, this process still requires Dcr1, presumably for the production of siRNAs. Therefore, apart from the recruitment of RITS, additional siRNA-dependent steps appear to be critical for the RNAi-dependent nucleation of heterochromatin. Interestingly, loss of Clr4 causes defects in localization of RITS and vice versa, causing severe defects in processing of repeat transcripts into siRNAs. These results imply RITS and Clr4 complex loading onto chromatin in a cooperative manner via a process linked to Pol II transcription of the repeat elements [28].

S-phase transcription of centromeric repeats and heterochromatin assembly

An important aspect of RNAi-mediated heterochromatin assembly is that target repeats need to be transcribed. In addition to generating siRNA precursors, Pol II transcription appears to play a more direct role in heterochromatin assembly [28,31]. Mutations in Pol II subunits impair heterochromatic assembly [32,33]. Heterochromatic silencing also requires splicing factors known to associate with elongating Pol II [34,35]. An apparent paradox is that Pol II must access sequences assembled into repressive heterochromatin. Multiple mechanisms counter the repressive influence of heterochromatin [36,37]. Interestingly, Swi6-mediates recruitment of an antisilencing factor [38,39], that is critical for Pol II transcription of centromeric repeats [38](see below). Moreover, it has been shown that heterochromatin is dynamically controlled during cell cycle progression that has implications for repeat transcription [31,40].

During the G2 phase, heterochromatin factors either bind directly to H3K9me or interact with H3K9me-bound proteins [11]. H3K9me bound HP1 preferentially recruits histone deacetylases (HDACs), to assemble repressive chromatin [19,20,41,42]. As cells enter mitosis, phosphorylation of histone H3 serine 10 remodels heterochromatin by interfering with the binding of chromodomain proteins to H3K9me [20,31,36,40]. This dramatic alteration in heterochromatin correlates with preferential recruitment of condensin, which in addition to chromosome condensation also participates in centromeric silencing [31]. However, centromeric sequences are amenable to transcription during S phase [31].

An interesting finding is that transcription of repeats in S-phase correlates with chromatin loading of heterochromatin factors. Recruitment of RITS subunit Ago1 and Clr4 complex subunit Rik1 (which is critical for loading Clr4 onto chromatin [43]) is coupled to Pol II transcription of repeats [28,31]. The preferential loading of Rik1 is restricted to transcribed centromeric repeats and is not observed at non-transcribed region within a heterochromatic domain [31], supporting a causal relationship between Pol II transcription and recruitment of Rik1. Indeed, increased transcription of repeats is coupled to enhanced Rik1 loading in an

RNAi-dependent manner [28]. Elongating Pol II also targets the H3K36me modification, and H3K36 methyltransferase Set2 acts in a pathway parallel to Clr4 to promote targeting of RITS and heterochromatin machinery [31]. These studies suggest dual modes of targeting heterochromatin whereby Pol II transcription of repeats or chromodomain proteins bound to H3K9me recruit silencing factors. Coupling S-phase transcription with the recruitment of heterochromatin factors may contribute to the recapitulation of heterochromatin during chromosome replication.

Spreading and maintenance of heterochromatin

Heterochromatin nucleated at specific sites can spread to the surrounding regions. This process, impacted by the chromosomal context [44], involves a complex interplay between histone-modifying activities and proteins that bind to modified histones [5]. Spreading of heterochromatin has been reported to require RNAi [45]. RITS subunit Tas3, that connects RNAi and heterochromatin factors [46,47], contains an alpha helical motif, which oligomerises to promote *cis* spreading of RITS at centromeres [48]. However, RNAi is dispensable for heterochromatin spreading at other genomic loci [16,23] (M. Zofall and S. Grewal, unpublished). Long-range spreading of heterochromatin requires a unique characteristic of Clr4. Clr4 not only methylate H3K9, but also bind to H3K9me via its chromodomain [28] (Figure 1). Mutations in Clr4 that abolish its binding to H3K9me *in vitro* affect spreading of heterochromatin *in vivo*. The ability of Clr4 to both “read” and “write” H3K9me presumably allows it to modify adjacent nucleosomes, which lead to spreading of heterochromatin. Heterochromatin propagation beyond original nucleation sites also requires Swi6 [15]. Swi6 may stabilize chromatin binding of histone-modifying activities (including HDACs implicated in the spreading [20]) but it also forms oligomers [41,49], which could promote heterochromatin spreading by facilitating higher-order chromatin organization.

The “epigenetic loop” mechanism in which factors bound to H3K9me facilitate the propagation of this mark might also account for *in cis* inheritance of heterochromatic structures [50]. Indeed, in cells defective in *de novo* heterochromatin assembly, inheritance of preassembled H3K9me through successive cell divisions requires Clr4 binding to H3K9me [28] and Swi6 [15]. These factors may bind to the parental H3K9me distributed to sister chromatids during DNA replication to modify newly assembled nucleosomes. Transcription-coupled heterochromatin assembly during S phase may aid this process, which also requires histone chaperones (such as CAF1) associated with HP1 and replication proteins [51–53].

Posttranscriptional and transcriptional heterochromatic silencing

Heterochromatin spreading has been described in different species. However, the biological significance of this conserved feature of heterochromatin has not been extensively studied. Mapping of heterochromatin-associated factors by ChIP in *S. pombe* has unraveled a new theme in which heterochromatin serves as a recruiting platform for factors involved in different chromosomal processes [11]. Both posttranscriptional and transcriptional silencing activities rely on heterochromatin for their stable localization across silenced chromosomal domains (Figure 1).

As mentioned above, heterochromatin allows preferential recruitment of RITS and Rdp1 to centromeric repeats [14,23,25,26]. H3K9me and Swi6 also allow these factors to spread *in cis* and operate across large domains, thus providing a mechanism to degrade transcripts produced by repeats and other loci [23]. Indeed, transcripts derived from marker genes artificially inserted within heterochromatic domains are processed by the RNAi machinery [54] (M. Zofall and S. Grewal, unpublished data). Therefore, heterochromatin-mediated

tethering of the RNAi machinery across domains broadens its target range to include sequences that are otherwise incapable of eliciting RNAi response [11].

In addition to post-transcriptional gene silencing in cis (cis-PTGS), heterochromatin causes transcriptional gene silencing (TGS). HP1 proteins Chp2 and Swi6 bound to H3K9me provide recruiting platform for the chromatin-modifying activities involved in TGS [20] (Figure 1). Chp2 and Swi6 target SHREC containing a class II HDAC Clr3 and an Snf2 family ATPase Mit1 [19,20,41,42]. Swi6 also associates with another complex containing class I HDAC Clr6 [9,42]. Whereas Clr3 targets histone H3 lysine 14, Clr6 displays broad substrate specificity for lysine residues on both histone H3 and H4 tails [55]. Chp2 and Swi6, and their associated HDACs have overlapping functions in limiting Pol II occupancy across heterochromatic domains [42]. Deacetylation of histones, which is a hallmark of heterochromatin in most eukaryotes, may prevent chromatin remodeling. It might also facilitate the positioning of nucleosomes required for higher-order chromatin. To this end, activities associated with Clr3 and Mit1 subunits of SHREC influence nucleosome positions that correlate with TGS defects [19].

Heterochromatic silencing can be regulated in response to changes in environmental or developmental signals. Modifications of HP1 proteins may alter their affinities for silencing factors as in the case of SHREC association with Swi6 [56]. A surprising finding however is that in addition to HDACs, Swi6 recruits a JmjC domain-containing anti-silencing factor Epe1 [38,39]. Epe1 promotes Pol II transcription in a heterochromatin-specific context of centromeric repeats [38], and several other genomic loci [39]. Mutations in JmjC domain impair Epe1 function but its exact function is not known. Therefore, HP1 can recruit opposing activities, the balancing of which determines the expression state of target loci (Figure 1). A recent study reported that transcription of piRNA precursors in *Drosophila* requires an HP1 homolog Rhino [57]. It is possible that Rhino also recruits Epe1-like anti-silencing factor.

Heterochromatin-independent targeting of HDAC activities

RNAi and heterochromatin while dramatically affecting the silencing of centromeric repeats have only minor impact on retrotransposons and their remnants dispersed across *S. pombe* genome [58]. As retrotransposons also pose threat to genome stability, additional mechanisms must exist to silence these elements. An emerging theme is that a “toolkit” of repressors are targeted to different classes of repeats by distinct recruitment mechanisms (Figure 2A). Similar to HP1 targeting HDACs to heterochromatic repeats, CENP-Bs derived from transposases of DNA transposons localize at and recruit SHREC and Clr6 to silence retrotransposons and their remnants [59]. Also, as in the case of heterochromatin, CENP-Bs promote genome stability by blocking recombination at their target repeats, and by preventing retrotransposon mobilization [59]. CENP-Bs bind to repeat elements in higher eukaryotes and may represent an ancient transposon surveillance mechanism.

CENP-Bs and other site-specific recruitment mechanisms also target HDACs to heterochromatic loci. Genetic studies have identified three major sites of HDAC recruitment at the silent mating-type region (Figure 2B). Two of these correspond to silencers located adjacent to *mat2* and *mat3* loci [60,61], including an element bound by ATF/CREB proteins (Atf1 and Pcr1) that associate with HDACs [17,18,20]. CENP-Bs localize to the third site to target HDAC activities [59]. In addition, Swi6 and Chp2 target SHREC and Clr6 across the entire heterochromatic domain. Therefore, multiple overlapping mechanisms recruit at least two distinct HDAC activities with partially redundant functions to enforce transcriptional and recombinational suppression. Similar mechanisms operate at telomeres where a telomere binding protein Ccq1 and Taz1 (a homolog of mammalian TRF1/2) acts in a

parallel pathway to heterochromatin to target HDAC activities [16,19]. The redundancy in silencing activities and their recruitment mechanisms underscore the importance of higher-order chromatin organization at these loci.

Antisense suppression by heterochromatin and RNAi factors

Recent studies have revealed a surprising function for RNAi and heterochromatin factors in suppressing antisense transcripts at euchromatic loci [62,63]. Components of Clr4 complex can be detected at euchromatic genes albeit at low levels. Deletions of Clr4 (or its interacting Rik1) and Ago1 cause upregulation of readthrough antisense RNAs at convergent genes. Heterochromatin might facilitate localization of cohesin that in turn promotes transcription termination [62]. Antisense suppression also requires a variant histone H2A.Z, which acts in a partially redundant manner with Clr4 and Ago1 [63]. When a null allele of H2A.Z is combined with *clr4Δ* or *ago1Δ*, double mutants display severe growth defects correlating with synergistic increase in readthrough antisense RNAs. Although the precise mechanism remains unclear, H2A.Z and heterochromatin/RNAi factors appear to have a role in the degradation of antisense RNAs by the nuclear exosome [63]. RNAi machinery, in particular Argonaute, appears to associate with Pol II transcriptional apparatus that may have implications for detection and elimination of aberrant transcripts. These results presumably reflect an ancient function of Argonautes in preventing accumulation of potentially deleterious RNAs capable of causing genomic instability.

Heterochromatin and genome stability

Heterochromatin-mediated repression of transcription and recombination is essential for maintaining genomic integrity [9]. Indeed, when null alleles of heterochromatin factors are combined with mutations in recombination/repair proteins, double mutants show severe growth defects [64]. HP1 proteins recruit a variety of effectors essential for genome stability. Loading of Hsk1-Dbf4 kinase essential for initiation of DNA replication at centromeres and the *mat* locus requires Swi6 [65]. In addition, HP1s target factors involved in segregation of chromosomes. Swi6 associates with Mis4 cohesin-loading complex to recruit cohesin [42], which promotes chromosome segregation and prevents chromosomal rearrangements [66]. Swi6 also recruits shugoshin (a protector of cohesin at centromeres during meiosis I) important for chromosome dynamics during cell division [67]. Further linking heterochromatin to chromosome segregation, loss of heterochromatin correlates with defective loading of centromeric-specific histone H3 variant CENP-A [68,69]. CENP-A peptides can be detected in purified Swi6 fractions [42], indicating that Swi6 participates in heterochromatin-mediated loading of CENP-A to centromeres.

Relationship of heterochromatic silencing in *S. pombe* to other systems

Heterochromatin formation in *S. pombe* shares many parallels with epigenetic silencing in other systems, although different lineages have emphasized different aspects of heterochromatin regulation. Small RNA-based mechanisms play prominent roles in chromatin modifications in plants, ciliates, *C. elegans* and *Drosophila* [70–73]. *Tetrahymena* selectively eliminates parts of its genome when it produces actively transcribed macronucleus. This process requires RNAi and small RNAs that are believed to target heterochromatin to sequences undergoing elimination [72]. In *Arabidopsis*, transcription of transposon-derived sequences by specialized RNA polymerases is essential for RNA-dependent DNA methylation (RdDM). This mechanism also involves RNAi and small RNAs, which are important for methylation of DNA and silencing of target loci [70,73,74]. DNA methylation enhances RdDM efficiency indicating that RNAi-mediated targeting of chromatin modifications involves a self-reinforcing loop, as in *S. pombe*.

RNAi factors, such as PIWI proteins, are required for posttranscriptional and transcriptional silencing of repeat sequences in *Drosophila* [70]. Again, interaction between Pol II and RNAi factors is important for H3K9me and heterochromatic silencing [75]. PIWI interacts with HP1, and PIWI-HP1 may have functions analogous to RITS [76]. Loss of heterochromatin in *Drosophila* causes increased rate of recombination between repeats [77]. HP1 facilitates proper positioning of nucleosomes [5], presumably in part by recruiting SHREC-like activities, to inhibit transcription and recombination. In *Neurospora*, H3K9me-HP1 platform is utilized to target DNA methylation machinery to silence repeat elements [78]. Highlighting the importance of heterochromatin platform in various chromosomal functions, H3K9me-HP1 associates with a large number of proteins in mammals [79]. Several studies suggest the involvement of long and small RNAs in histone and DNA modifications [6]. Small RNA-associated PIWI proteins have roles in the control of transposons, and their loss correlates with defects in DNA methylation [80]. Therefore mechanisms similar to in *S. pombe* may contribute to epigenetic regulation in higher eukaryotes.

The discovery of the transcription apparatus and ncRNAs facilitating heterochromatin formation has redefined our views on the assembly of specialized chromatin domains. Heterochromatin assembled at repetitive elements suppresses accumulation of potentially deleterious RNAs but it has also evolved into a powerful regulatory mechanism for protecting genomic integrity and control of various chromosomal functions. Further investigations into the mechanisms of heterochromatin assembly, in particular the role of transcriptional machinery in this process, will shed light on the epigenetic control of eukaryotic genomes.

Abbreviations

ChIP	Chromatin Immunoprecipitation
HP1	Heterochromatin Protein 1
H3K9me	Histone H3 modified by methylation on lysine 9
HDAC	Histone Deacetylase
ncRNA	non-coding RNA
RdDM	RNA-dependent DNA methylation
SHREC	Snf2/HDAC-containing Repressor Complex

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Figure 1. Heterochromatin-mediated transcriptional silencing and degradation of repeat RNAs RNAi factors RITS, Rdp1-containing RNA-dependent RNA polymerase complex (RDRC) and Dicer (Dcr1) that process centromeric transcripts into siRNAs are required for heterochromatin assembly. Heightened Pol II transcription of repeats during S-phase stimulates recruitment of heterochromatin factors including Clr4 complex subunit Rik1 and RITS subunit Ago1, as well as H3K36 methylation (blue lollipop) by Set2 implicated in heterochromatic silencing and recruitment of HDAC Clr6 [28,31]. siRNA-bound Ago1 is believed to specify RITS targeting to nascent repeat transcripts. siRNAs also facilitate recruitment of Clr4 to methylate H3K9. H3K9me (red lollipop) recruits HP1 proteins (Chp2 and Swi6) but also establishes feedback loop by stabilizing chromatin association of RITS (via Chp1 chromodomain) and Clr4 (via Clr4 chromodomain). Chromatin-bound RITS, RDRC and Dicer process heterochromatic transcripts into siRNAs to mediate posttranscriptional silencing in cis (cis-PTGS). Clr4 bound to H3K9me could modify adjacent nucleosomes, creating additional binding sites for chromodomain protein. This process, along with oligomerization of Swi6, is believed to facilitate heterochromatin spreading. Chp2 and Swi6 provide platform for targeting HDAC transcriptional gene silencing (TGS) factors SHREC and Clr6. Swi6 also recruits an anti-silencing factor Epe1 (see text for details).

Figure 2. Targeting a “toolkit” of repressors to different chromosomal addresses

(A) Heterochromatin-dependent and –independent targeting of silencing activities to repeat loci. Chp2 and Swi6 recruit HDACs across domains containing *dg* and *dh* repeats targeted by RNAi. However, RNAi and heterochromatin are dispensable for silencing Tf2 retrotransposons and their remnants (such as LTRs). CENP-Bs target SHREC and Clr6 to silence full-length Tf2 elements and solo LTRs often associated with gene promoters. (B) Redundancy in silencing mechanisms at the mating-type locus. A 20-kb heterochromatic domain containing donor mating-type cassettes *mat2* and *mat3* is flanked by boundary elements that prevent heterochromatin spreading. *cenH* sharing homology to *dg* and *dh* repeats serves as an RNAi-dependent heterochromatin nucleation center. In addition to HP1 proteins targeting HDACs across heterochromatic domain, silencer elements (REII, REIII and CENP-B silencer) recruit SHREC and Clr6 via DNA binding proteins (such as CENP-Bs Abp1 and Cbh1, and ATF/CREB family proteins Atf1 and Pcr1) bound to these sites.