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Transcription and RNAi in the formation of heterochromatin

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Abstract

Preface—Our title appears to be an oxymoron – surely “silenced” chromatin should not be transcribed! But there have been frequent reports of low level transcription in heterochromatic domains, and in *Drosophila* several hundred genes are found within heterochromatic regions¹. Most striking, recent investigations implicate RNAi (in the broadest sense) in targeting and maintaining heterochromatin, and RNAi-based mechanisms are inherently dependent on transcription. While this might involve *trans*-acting sources of the critical small RNAs, in some cases transcription of the domain to be silenced appears to be required at least initially, a seeming contradiction.

At the first level of organization, the chromatin fiber is composed of generic nucleosome arrays, built using a small number of histones. However, great variety is achieved by a complex system of accessory proteins which modify, bind and reorganize histone complexes to produce different functional domains within the eukaryotic nucleus. Heterochromatin and euchromatin domains reflect different patterns of histone modification and are associated with different modes of nucleosome packaging²; presumably this is reflected in differences in higher order packaging^{3, 4} and nuclear organization (see Fraser and Bickmore, this issue).

Heterochromatin was initially defined as that portion of the genome that retains deep staining with DNA-specific dyes as the cell returns to interphase from metaphase. Subsequent investigation suggested a constellation of properties for heterochromatin (see Box 1). A link between heterochromatin formation and gene silencing has been inferred from the loss of most gene activity on the inactive X chromosome (the visibly condensed Barr body) in female mammals, and the loss of gene expression (correlated with condensed packaging) in position effect variegation (PEV) in *Drosophila* and other organisms. PEV results from the juxtaposition of a normally euchromatic gene with heterochromatin through rearrangement or transposition; the resulting variegating phenotype indicates that the gene has been silenced in a fraction of the cells in which it is normally active². Reporters displaying PEV have a more regular nucleosome array, and (perhaps as a consequence) suffer a loss of the nuclease hypersensitive sites, presumed nucleosome-free regions generally associated with regulatory sequences of active or readily induced genes^{3, 5}. Loss of hypersensitive sites is dependent on Heterochromatin Protein 1 (HP1)⁶. Studies in *Schizosaccharomyces pombe* (*S. pombe*) have shown that HP1 family proteins mediate recruitment/spreading of chromatin modifying factors, such as a histone deacetylase (HDAC)- and Snf2 ATPase-containing complex, SHREC. Such complexes presumably facilitate the nucleosome modification and positioning needed to organize the higher-order chromatin structures essential for heterochromatin functions, including transcriptional silencing, suppression of recombination, maintenance of long-range chromatin interactions, and successful chromosome segregation^{4, 7}.

An important characteristic of heterochromatin is the ability of this form of packaging to spread, as evidenced by the occurrence of PEV, and further demonstrated in *S. pombe*⁸ (see below). Once established, heterochromatin structures can be stably maintained through mitosis, and even meiosis, as exemplified by the clonal nature of the calico cat, where coat color reflects

the choice of X chromosome for inactivation. The general properties of euchromatin, the gene-rich portions of the genome, are antagonistic to those of heterochromatin (see Box 1), although one anticipates much more variation in the histone modification state and disposition of the nucleosome array, depending on the transcription status of a given gene (see Berger, this issue). Indeed, increased expression of a variegating gene (embedded in heterochromatin) in *Drosophila* has been reported in response to increased amounts of a positive (GAL4) transcription factor, suggesting constant competition in establishing these alternative states⁹. In this review we will focus on recent findings on how heterochromatin formation is targeted and maintained in specific regions of the genome, examining in particular the potential role of transcription related to the RNAi system. We will draw primarily on results in fungi and animals; very interesting results in plants are reported by Henderson and Jacobson (this issue).

Heterochromatin assembly: *Drosophila* as a model system

A key tool in investigating heterochromatin has been the ability to screen for suppressors of PEV, second site mutations that lead to loss of silencing at a variegating locus. About 15 such loci have been characterized in *Drosophila melanogaster*, and many more candidates have been identified¹⁰. The *Su(var)* genes typically code for either proteins that participate directly in the structure of heterochromatin, or enzymes that control changes in histone modification. To a first approximation, a transition between euchromatin and heterochromatin (as might occur in PEV) can be viewed as a series of reactions where the histone modifications and proteins associated with the active state are removed, and the histone modifications and proteins associated with the inactive state are added (Figure 1). A sequential set of reactions is required: e.g., lysine 9 of histone H3 (H3K9) cannot be methylated until it is de-acetylated; binding of SUV4-20 occurs through interaction with HP1 and requires SU(VAR)3-9 activity¹¹. This no doubt contributes to the relative stability of the alternative packaging states. While the heterochromatic state can be inherited through mitotic and even meiotic cell divisions, a given site will switch from a repressed to an active chromatin state and vice versa at a low frequency. Of course, PEV cannot be scored as such in single cell organisms such as yeast, but this switching can be observed in the phenotype of sectors of a growing colony (Figure 2).

A small group of proteins are considered likely structural components of pericentric heterochromatin in flies because of the observed dosage response: while one copy of the encoding gene results in loss of silencing, three copies results in increased silencing, presumably as a consequence of mass action¹². This set of proteins includes HP1¹³, the initial chromo domain protein; HP2, a large protein with no conserved structural motifs¹⁴; SU(VAR)3-7, a zinc finger protein¹⁵; and SU(VAR)3-9, an H3K9-specific histone methyl transferase (HMT)¹⁶. However, no well-defined complex of heterochromatin proteins has been isolated (despite interactions of HP1 with all of these proteins, and others¹⁷), suggesting that an organized assembly exists only on the chromatin fiber. The small HP1 protein (206 amino acids in *D. melanogaster*) has two conserved domains, an N-terminal chromo domain and a C-terminal chromo shadow domain (CSD), separated by a short hinge (Figure 3). HP1 dimerizes through the CSD, forming a peptide-binding surface. HP1 interacts stably with SU(VAR)3-9 via its chromo shadow and hinge domains, and with the H3 N-terminal tail marked by di- or tri-methylated K9 (K9me2/3) through its chromo domain². By interacting with both the modified histone and the modifying enzyme, HP1 can provide a foundation for a self-assembly and spreading mechanism, anticipated from observations of PEV (Figure 3) (see ref¹⁸ for review of possible spreading mechanisms). This core assembly appears to be conserved in animals and fungi^{19, 20} (see Table 1). We note that in many organisms there are multiple homologues of HP1, and multiple H3K9 HMT's, suggesting the possibility of alternative assemblies¹⁹. However, in flies only HP1a (referred to simply as HP1 in this review) appears to be generally associated with known heterochromatic domains, and the ability of the other

homologues to mimic HP1 in establishing heterochromatic packaging remains to be established.

The role of RNAi: *S. pombe* as a model system

Genetic and biochemical studies using *S. pombe* as a model system have provided important insights into mechanisms of heterochromatin assembly. Many factors involved in heterochromatin formation in *Drosophila* and mammals are conserved in *S. pombe*^{19, 20}. In particular, the evolutionarily conserved Clr4 protein – the fission yeast homolog of *Drosophila* SU(VAR)3-9 – present in a Cullin 4-containing E3 ubiquitin ligase complex, has been shown to specifically methylate H3K9^{21–26}. Methylated H3K9 serves as a binding site for recruitment of chromodomain proteins, including Chp1, Chp2 and Swi6 (the latter a homologue of *Drosophila* HP1) to heterochromatic loci^{22, 27–29}. Mapping of heterochromatin factors, including H3 methylated at K9 and Swi6, has identified extended chromosomal domains coated with heterochromatin complexes at centromeres, telomeres and the mating type locus³⁰. Interestingly, all three heterochromatic regions share a common feature – each of these domains contains *dg/dh* repeat elements that are preferential targets of heterochromatin formation^{8, 31–33}. Recent investigations into mechanisms by which these repeat elements might trigger heterochromatin formation have led to the surprising discovery that the RNA interference (RNAi) system is involved in the nucleation and assembly of heterochromatin^{8, 33}.

RNAi was first described as a posttranscriptional silencing mechanism in which double-stranded RNA (dsRNA) triggers destruction of cognate RNAs³⁴. Subsequent studies have implicated RNAi-related mechanisms in diverse cellular functions. In *S. pombe*, mutations in factors involved in RNAi such as Dicer (Dcr1, an enzyme that cuts double-stranded RNA), Argonaute (Ago1, a PAZ/PIWI domain protein, capable of binding small RNAs), and Rdp1 (an RNA-dependent RNA polymerase) result in defects in heterochromatin assembly, as shown by loss of silencing at reporter loci^{8, 33}. An “RNAi-induced initiation of transcriptional silencing” (RITS) complex, containing both a chromatin-associated protein and an RNAi-associated protein, has been identified³⁵. RITS contains Chp1 (a chromo domain protein), Ago1, and a protein of unknown function, Tas3. In addition, RITS also contains siRNAs (small interfering RNAs) derived from the *dg/dh* repeats present at the different heterochromatic loci^{30, 35}. Genome mapping analyses have shown that components of RITS and Rdp1 are distributed throughout heterochromatic domains in a pattern almost identical to the H3K9 methylation and Swi6 distributions³⁰. Stable binding of RITS to chromatin depends at least in part on the binding of the Chp1 chromodomain to methylated H3K9³⁶. Deletion of Clr4, as well as a mutation in the Chp1 chromodomain, results in delocalization of RITS from heterochromatic loci. Interestingly, one sees concurrent defects in the processing of repeat transcripts into siRNAs^{30, 36}, suggesting that siRNAs are produced within a heterochromatin environment. RITS also recruits an RNA-directed RNA polymerase complex (RDRC) containing Rdp1; this RdRP activity is essential for siRNA production and heterochromatin assembly^{37, 38}. The generation of siRNAs also requires an RNaseH-like RNA cleavage activity (Slicer) known to be associated with Argonaute family proteins, such as Ago1, found in RITS; mutations in conserved Ago1 residues that abolish this activity severely affect processing of repeat transcripts, and result in defects in heterochromatin assembly^{39, 40}. The slicer function of Ago1 has been suggested to be important for spreading of heterochromatin³⁹. It is also possible that siRNAs generated by Ago1-mediated processing of transcripts have a direct structural role in the assembly of higher-order structures, that in addition to mediating silencing facilitates local heterochromatin spreading. These mechanisms, however, cannot by themselves account for spreading of heterochromatin across large domains, as this requires HP1 proteins to serve as a platform for recruiting chromatin-modifying activities involved in heterochromatin assembly^{2, 8}.

These findings suggest that RNAi-mediated heterochromatin assembly in *S. pombe* might occur via a self-reinforcing loop mechanism^{36, 37}. In this model, siRNAs (possibly generated elsewhere) and/or DNA binding proteins mediate initial targeting of heterochromatin factors, leading to establishment of H3K9 methylation marks. The presence of methylated H3K9 and associated silencing factors in turn allows stable binding of RITS across heterochromatin domains (Figure 4). RITS presumably serves as a core for binding other RNAi factors, such as RDRC, essential for processing any transcripts of the *dg/dh* repeats. The siRNA-guided cleavage of nascent repeat transcripts by Ago1 (a component of RITS) is believed to be an important step in producing additional siRNAs. It is possible that cleaved transcripts serve as preferential targets for Rdp1, which generates double stranded RNAs, necessary for generation of short siRNAs by Dicer. siRNAs produced in *cis* can feed back to target additional heterochromatin complexes, but might also have additional functions (see below).

The exact mechanism by which siRNAs target histone modifications is not clear. Binding of RITS to heterochromatic domains requires *dg/dh* siRNAs as part of the complex. It has been suggested that RITS, tethered to nascent transcripts via siRNAs, might mediate recruitment of histone methyltransferases such as Clr4³⁵, or that siRNAs directly facilitate recruitment of chromatin-modifying activities, such as a Clr4 complex, to heterochromatic repeats^{8, 23}. It is certainly possible that siRNAs target heterochromatin by base pairing with nascent transcripts⁴¹; subunits of the RITS and RDRC complexes can be cross linked to noncoding centromeric repeat transcripts³⁸. However, it is not known whether this binding simply reflects the roles of these factors in processing repeat transcripts, or whether it indicates an additional function in recruiting heterochromatin proteins. Artificial tethering of RITS to nascent transcripts has recently been shown to induce local heterochromatin assembly⁴². However, this process requires Dicer, presumably for production of siRNAs. Hence besides the targeting of RITS, additional siRNA-dependent steps are necessary for stable RNAi-mediated heterochromatin nucleation. The emerging view is that RNAi machinery, tethered to specific loci, though associations with heterochromatin components, helps process transcripts generated from these loci into siRNA, thus effectively causing posttranscriptional silencing in *cis*; but in addition, the siRNAs produced in this process facilitate additional targeting of heterochromatin modifications, such as H3K9 methylation. H3K9 methylation allows HP1 family proteins such as Swi6 to localize across heterochromatin domains; these factors in turn facilitate localization of diverse cellular activities such as SHREC. The HDAC and SNF2 ATPase activities of SHREC are critical for proper positioning of nucleosomes to achieve transcriptional gene silencing⁴. But how can transcription to produce siRNAs occur within a silenced domain?

Transcription of heterochromatic repeats

The results described above argue that heterochromatic repeat elements need to be transcribed to produce the siRNAs that target heterochromatin formation- indeed, a circular process! In support, recent studies have shown that heterochromatic repeats present in the *S. pombe* genome are transcribed by RNA polymerase II^{30, 43}, and that mutations in Pol II impair RNAi-mediated heterochromatin assembly^{43, 44}. An apparent paradox arises, however, in that heterochromatin in general is believed to be relatively inaccessible to factors involved in different aspects of DNA metabolism, including the transcriptional machinery². How does Pol II gain access to sequences packaged as heterochromatin? Since heterochromatic silencing is thought to be plastic and can be overcome by increased concentration of transcription factors⁹, it can be argued that promoters driving the transcription of repeats, unlike promoters of euchromatic genes, have evolved to be somewhat impervious to heterochromatic repression. Indeed, one strand of centromeric repeats in *S. pombe* is always transcribed at a low level³³ but is silenced posttranscriptionally via RNAi-mediated processing of transcripts³³.

Transcription of repeats might be facilitated by a specialized mechanism(s) that modulates heterochromatin to provide access for factors involved in chromosomal transactions. Swi6/HP1 is believed to serve as an oscillator of heterochromatin transcription by directing recruitment of both silencing and anti-silencing factors²⁰. In addition to factors such as SHREC that repress Pol II transcription⁴, Swi6/HP1 also recruits a JmjC domain-containing protein Epe1⁴⁰, which was identified in a screen for factors that negatively regulate heterochromatic silencing⁴⁵. Epe1 facilitates Pol II transcription of repeats specifically in a heterochromatin context but does not appear to have an obligatory role in transcription *per se*; it is dispensable for transcription when heterochromatin is disrupted⁴⁰. The mechanism by which Epe1 counteracts heterochromatic silencing is not known. Since several JmjC domain proteins have been shown to catalyze histone demethylation⁴⁶, it is formally possible that Epe1 affects heterochromatin stability through removal of repressive lysine methylation marks. However, no such activity has been detected for Epe1⁴⁷. Epe1 may modulate chromatin via a yet undefined mechanism. Additional factors targeted to heterochromatic loci via Swi6/HP1 or other mechanisms are also likely important for heterochromatic transcription.

Apart from heterochromatin assembly, the transcription of repeat elements embedded within heterochromatin domains likely has other biological implications. It has been suggested that transcription of heterochromatic repeats may be necessary for continuous production of siRNAs to prime RISC-like complexes required to neutralize future invasions by similar sequences²⁰. Heterochromatin-bound RNAi factors might be components of a memory mechanism that selectively generates a reservoir of siRNAs aimed against parasitic DNA elements. It should be noted that in *S. pombe*, RNAi machinery targeted to specific elements can spread to surrounding sequences, including nearby genes, via a process dependent on H3K9 methylation and Swi6³⁶. This might also allow the RNAi machinery to exert heritable control over expression of sequences located adjacent to repeat elements. Finally, the role of RNAi in destroying viral or transposable element transcripts is conserved in other systems, including *Tetrahymena* and *Drosophila*^{48, 49}.

RNAi in silencing of repetitious sequences in metazoans

While heterochromatin, built from repetitious DNA, has become an essential part of the eukaryotic chromosome, maintaining these repetitious sequences in a stable, silent form (repressing both transposition and recombination) is clearly a challenge and a necessity. Heterochromatin packaging once initiated is self re-enforcing through multiple feedback loops⁵⁰. The RNAi machinery appears to be able to detect and respond to repetitious DNA in a variety of ways. To what extent might RNAi components be utilized in metazoans, either to initially target or to maintain silent domains, and to what extent might silencing of repetitious DNA be dependent on transcription in *cis*? The system described above is unlikely to be universally applicable, as many metazoans, including *Drosophila* and mammals, appear to lack canonical RNA-dependent RNA polymerase¹⁹.

Post-transcriptional gene silencing (PTGS) using RNAi, degrading or blocking translation of mRNA, is well established in all metazoans examined to date. The first suggestions of RNAi-based transcriptional gene silencing (TGS) in *Drosophila* came from work showing a loss of expression when multiple copies of a transgene are present⁵¹. Subsequent analysis showed suppression of PEV (a loss of silencing, monitored using tandem *mini-white* arrays and *white* transgenes in heterochromatin) by mutations in factors involved in the RNAi pathway, with decreased levels of H3K9me2^{51–53}. As in other organisms (notably plants; see review by Henderson and Jacobson, this issue), the RNAi system in *D. melanogaster* may have originated as a viral defense mechanism^{54, 55}. About one-third of the genome is considered heterochromatic, and much of that DNA is made up of remnants of transposable elements, both DNA transposons and retroviruses. *Drosophila* has five PAZ/PIWI domain proteins, thought

to bind small RNAs: PIWI, AUBERGINE (AUB), AGO-1, AGO-2, and AGO-3. PIWI is required for germ stem cell self-renewal, apparently playing a key role in silencing retrotransposons and blocking their mobilization in the germ line⁵⁶. Both PIWI and AUB are found with siRNAs of 24–29 nts derived from repetitious sequences in the germline^{49, 57}. *In vitro* PIWI exhibits RNA cleavage activity⁵⁷, and it has been suggested that germline siRNA may be produced by a unique processing mechanism, dependent on cleavage from long single strand transcripts rather than dsRNA⁴⁹. How this silencing activity might impact heterochromatin formation in somatic chromosomes (if at all) is not clear at present.

The impacts of mutations in *ago-2* are clearly seen in the early *Drosophila* embryo as defects in chromosome condensation, nuclear kinesis, and spindle assembly, all potentially correlated with defects in the formation of centric heterochromatin⁵⁸. Similar defects are observed on failure of heterochromatin formation in *S. pombe* and other systems^{59–61}. In flies with mutations in the genes for SU(VAR)3-9 (one of three known H3K9 methyltransferases), HP1, or Dicer 2, cells display disorganized nucleoli as well as disorganized centric heterochromatin. Under these circumstances, there is a substantial increase in extrachromosomal repetitious DNA in mutant tissues⁶². Similarly, mutations in the RNAi machinery in *S. pombe* have also been found to result in defects in maintaining chromosome integrity, including high rates of recombination at rDNA loci³⁰. Thus while repetitious DNA now contributes to essential chromosome structures, it is critical to maintain that DNA specifically in a heterochromatic form, and genetic analysis indicates that the RNAi system plays a role. In the absence of any recognizable RdRP activity, this might be accomplished by targeting heterochromatin formation to specific sites either by DNA-protein interactions or by an RNAi-based recognition system, followed by spreading of the heterochromatin modifications and structure. As in *S. pombe*, spreading of heterochromatin in *D. melanogaster* (as monitored by PEV) is dependent on HP1 and on SU(VAR)3-9.

Targeting heterochromatin formation

While much of our discussion focuses on RNAi-based mechanisms, it is important to note that heterochromatin proteins can be recruited to specific sites ('silencers') by DNA binding factors. For example, in addition to the RNAi-mediated targeting of heterochromatin to a *dg/dh*-like repeat element located in the silent mating-type region of *S. pombe*, the DNA binding proteins Atf1/Pcr1 (belonging to the ATF/CREB family) have been shown to cooperate with components of SHREC to independently nucleate heterochromatin assembly in this domain^{7, 63, 64}. Similarly, redundant mechanisms of heterochromatin nucleation also operate at telomeres in *S. pombe*, where the TRF family DNA binding protein Taz1, in conjunction with Ccq1, acts in parallel to the RNAi machinery to nucleate heterochromatin^{4, 32}. Regardless of nucleation mechanism, however, heterochromatin targeted to specific sites is able to spread and provides a sequence-independent platform for recruitment of appropriate cellular activities (such as SHREC, the RNAi machinery and cohesin) across large domains²⁰.

In several cases documented in mammalian cells, HP1 can be targeted to specific promoters by interaction with DNA binding complexes, and apparently contributes to silencing at these loci (see ref.⁶⁵ for an example). However, in these cases a different HMT appears to be responsible for the accompanying H3K9 methylation, and spreading is generally not observed. This suggests that the interactions of HP1 with both the modified histone (H3K9me3) and the modifying enzyme [usually SU(VAR)3-9] are critical for heterochromatin spreading (Fig. 3). Repetitious DNA is a hallmark of heterochromatin. In the case of satellite DNA (simple sequence tandem repeats), one might suggest that a specific DNA binding protein could recognize that particular DNA sequence to trigger heterochromatin assembly. In *Drosophila* the D1 protein, which has a mutant *Su(var)* phenotype, binds AT-rich satellite III preferentially^{66, 67}. Similarly, the heterochromatin-associated protein DDP1 binds to a

conserved dodeca-satellite sequence; however, this protein, with 15 tandemly organized KH domains, binds strongly to single-stranded nucleic acids with this sequence, including RNA⁶⁸. Recent work has shown that DDP1, which also has a mutant *Su(var)* phenotype, plays a critical role in HP1 and methylated H3K9 deposition at chromocenter heterochromatin⁶⁸. Given the ability of DDP1 (and its mammalian homologues, the vigilins) to bind RNA, it is possible that RNA mediates this interaction. Aside from the blocks of satellite DNA, repetitious sequences in *Drosophila* (primarily remnants of transposable elements and DNA transposons), are very diverse. Consequently, a recognition process based on RNA (rather than specific protein binding) appears most parsimonious, and this suggestion has been supported by studies on the heterochromatic *D. melanogaster* fourth chromosome.

The small fourth chromosome of *D. melanogaster* is considered entirely heterochromatic by the criteria cited above (see Box), but has 88 genes in the distal 1.2 Mb. Mapping with a *white* reporter transgene has shown interspersed heterochromatic domains (inducing a variegating phenotype) and euchromatic domains (allowing expression giving a full red eye). Detailed examination of the region around the *Hcf* gene identified the *1360* element, remnants of a DNA transposon, as a potential site for heterochromatin initiation; reporters lying within 10 kb of a *1360* element showed a variegating phenotype, while those further away showed a red eye⁶⁹. A direct test, using a *P* transposon carrying one copy of *1360* upstream of a *white* reporter, demonstrated that the *1360* element contributes to silencing, as silencing of the reporter is largely lost when the adjacent *1360* is deleted. However, stable heterochromatin (resulting in a variegating phenotype) is only observed when that *P* element is located in a region close to the centromere, indicating a requirement for a high local repeat density and/or proximity to the pericentric heterochromatin, where HP1 is most abundant. Genetic analysis indicates that this silencing is dependent not only on HP1 and SU(VAR)3-9, but also on RNAi components, notably AUB⁵³. Whether or not transcription occurs at this *1360* element is unknown. Small RNAs have been recovered from *1360* and from about 40 other *Drosophila* transposable elements⁷⁰. *1360* is probably not the only TE targeted for heterochromatin formation. However, it appears unlikely that all transposable element remnants are targets, given the mapping results obtained on chromosome four with the *white* reporter⁶⁹. The critical characteristics of targets are as yet unknown, but could include the presence of start sites for transcription⁵³. Many *1360* remnants have a sequence known to function as a promoter in the multi-copy *Su[Ste]* locus, where they generate inverse transcripts used in suppression of the multi-copy *Stellate* gene⁷¹. These results suggest that remnants of transposable elements could be targeted for silencing by a mechanism using a small RNA, and that transcription of some such elements might be involved.

Concluding remarks

Eukaryotes that tolerate high levels of repetitious sequences in their genomes generally have both the RNAi machinery and the enzymes and structural proteins necessary to generate a heterochromatin structure based on H3K9 methylation. While some features of the RNAi system (such as RdRP) and some features of the heterochromatin structure (such as DNA methylation) are used in only a subset of the metazoans, this key shift in histone modification from euchromatin to heterochromatin appears to be universal (see Table 1). The RNAi system *per se* can limit gene expression through post transcriptional gene silencing, and hence eliminate some sources of damage from invading repetitious elements, but by itself cannot generate the compact chromatin structures required to maintain chromosome integrity and mitotic chromosome function. Hence the suggestion that PTGS is sufficient to explain the silencing of repetitious elements seems quite unlikely. A model in which RNAi components play a key role in generating siRNAs involved in targeting chromatin components (H3K9 HMTs and HP1) to silence repetitious DNA remains most attractive, given the additional

understanding of a delicate balance between the need for expression and the need for silencing described above.

While an assembly of heterochromatin structure based on binding of HP1 proteins to methylated H3K9 marks provides a foundation for spreading, the molecular mechanisms by which heterochromatin exerts long-range repressive effects are not fully understood. The oligomerization of chromatin-bound Swi6/HP1 via the chromoshadow domains might mediate condensation. However, recent evidence suggests that Swi6/HP1 binding is dynamic^{72, 73}. An alternative emerging view is that HP1 proteins facilitate recruitment of other heterochromatin components responsible for silencing. Indeed, as described above, HP1 family proteins mediate preferential binding of SHREC, which includes HDAC activity⁴. Deacetylation of histones, which is a universal property of heterochromatic domains, may result in lower affinity of transcription factors for target loci or could be critical for higher-order packaging of nucleosomes, both contributing to silencing. The HP1/H3K9 methylation system may use several routes to minimize H3K9 acetylation, a key characteristic of the active state.

Evidence from different systems suggests that once triggered, a repressive chromatin structure can be sustained over many generations. In fission yeast, heterochromatin structures established by RNAi and/or DNA binding factors are inherited in *cis* for many generations in a manner dependent upon Swi6/HP1 and histone modifying activities^{8, 74}. Moreover, a recent study in *C. elegans*, an organism known to silence repetitious DNA using RNAi and chromatin factors^{75, 76}, showed that a single exposure to RNAi resulted in dominant silencing of a reporter gene in about 30% of the progeny for many generations⁷⁷. A screen for mutations that affected the maintenance of silencing identified four essential genes: *hda-4* (a histone deacetylase), *K03D10.3* (a histone acetyltransferase), *isw-1* (a homologue of the chromatin-remodeling ISW1), and *mrg-1* (a chromo-domain protein). Coupled with the observation that trichostatin A, a histone deacetylase inhibitor, relieves silencing, the results argue that maintenance of silencing is a consequence of heterochromatin formation, heritable even in the absence of the initial RNAi stimulus⁷⁷. While much remains to be learned about the mechanisms involved, it is clear that the proper interplay of the RNAi and heterochromatin systems is critical for the maintenance and function of our genomes.

Box

Properties of euchromatic and heterochromatic domains. Trying to define heterochromatin is like trying to define life itself: one can specify a cluster of important properties, but there are exceptions in every instance. For example, while centromeres are often associated with blocks of flanking heterochromatin, in *Drosophila* the inner centromere contains blocks of nucleosomes made with CENP-A, a histone H3 variant, interspersed with blocks of H3 nucleosomes showing a different modification pattern⁷⁸. The *Drosophila* telomere elements are non-LTR retrotransposons, which are transcribed⁷⁹, in contrast to the proximal TAS (telomere-associated sequences), which show more properties of heterochromatin. The characteristics given here are most consistently observed in pericentric heterochromatin, regions rich in remnants of transposable elements that flank the centromeres in the chromosomes of many eukaryotes. Note that we know very little about either the stoichiometry of HP1 or the folding of the chromatin fiber in heterochromatin; the sketch is meant to convey only HP1 association and condensation of the chromatin fiber.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Yasuhara JC, Wakimoto BT. Oxymoron no more: the expanding world of heterochromatic genes. *Trends Genet* 2006;22:330–8. [PubMed: 16690158]
2. Grewal SI, Elgin SC. Heterochromatin: new possibilities for the inheritance of structure. *Curr Opin Genet Dev* 2002;12:178–87. [PubMed: 11893491]
3. Sun FL, Cuaycong MH, Elgin SC. Long-range nucleosome ordering is associated with gene silencing in *Drosophila melanogaster* pericentric heterochromatin. *Mol Cell Biol* 2001;21:2867–79. [PubMed: 11283265]
4. Sugiyama T, et al. SHREC, an Effector Complex for Heterochromatic Transcriptional Silencing. *Cell* 2007;128:491–504. [PubMed: 17289569]
5. Wallrath LL, Elgin SC. Position effect variegation in *Drosophila* is associated with an altered chromatin structure. *Genes Dev* 1995;9:1263–77. [PubMed: 7758950]
6. Cryderman DE, Tang H, Bell C, Gilmour DS, Wallrath LL. Heterochromatic silencing of *Drosophila* heat shock genes acts at the level of promoter potentiation. *Nucleic Acids Res* 1999;27:3364–70. [PubMed: 10454645]
7. Yamada T, Fischle W, Sugiyama T, Allis CD, Grewal SI. The nucleation and maintenance of heterochromatin by a histone deacetylase in fission yeast. *Mol Cell* 2005;20:173–85. [PubMed: 16246721]
8. Hall IM, et al. Establishment and maintenance of a heterochromatin domain. *Science* 2002;297:2232–7. [PubMed: 12215653]
9. Ahmad K, Henikoff S. Modulation of a transcription factor counteracts heterochromatic gene silencing in *Drosophila*. *Cell* 2001;104:839–47. [PubMed: 11290322]
10. Elgin, SCR.; Reuter, G. Epigenetics. Allis, CD.; Jenuwein, T.; Reinberg, R., editors. Cold Spring Harbor Laboratory Press; New York: 2006. in press
11. Schotta G, et al. A silencing pathway to induce H3-K9 and H4-K20 trimethylation at constitutive heterochromatin. *Genes Dev* 2004;18:1251–62. [PubMed: 15145825]
12. Locke J, Kotarski MA, Tartof KD. Dosage-dependent modifiers of position effect variegation in *Drosophila* and a mass action model that explains their effect. *Genetics* 1988;120:181–98. [PubMed: 3146523]
13. Eissenberg JC, et al. Mutation in a heterochromatin-specific chromosomal protein is associated with suppression of position-effect variegation in *Drosophila melanogaster*. *Proc Natl Acad Sci U S A* 1990;87:9923–7. [PubMed: 2124708]
14. Shaffer CD, et al. Heterochromatin protein 2 (HP2), a partner of HP1 in *Drosophila* heterochromatin. *Proc Natl Acad Sci U S A* 2002;99:14332–7. [PubMed: 12376620]
15. Reuter G, et al. Dependence of position-effect variegation in *Drosophila* on dose of a gene encoding an unusual zinc-finger protein. *Nature* 1990;344:219–23. [PubMed: 2107402]
16. Tschiersch B, et al. The protein encoded by the *Drosophila* position-effect variegation suppressor gene *Su(var)3-9* combines domains of antagonistic regulators of homeotic gene complexes. *Embo J* 1994;13:3822–31. [PubMed: 7915232]
17. Greil F, de Wit E, Bussemaker HJ, van Steensel B. HP1 controls genomic targeting of four novel heterochromatin proteins in *Drosophila*. *Embo J* 2007;26:741–51. [PubMed: 17255947]
18. Talbert PB, Henikoff S. Spreading of silent chromatin: inaction at a distance. *Nat Rev Genet* 2006;7:793–803. [PubMed: 16983375]
19. Huisinga KL, Brower-Toland B, Elgin SC. The contradictory definitions of heterochromatin: transcription and silencing. *Chromosoma* 2006;115:110–22. [PubMed: 16506022]
20. Grewal SI, Jia S. Heterochromatin revisited. *Nat Rev Genet* 2007;8:35–46. [PubMed: 17173056]

21. Rea S, et al. Regulation of chromatin structure by site-specific histone H3 methyltransferases. *Nature* 2000;406:593–9. [PubMed: 10949293]
22. Nakayama J, Rice JC, Strahl BD, Allis CD, Grewal SI. Role of histone H3 lysine 9 methylation in epigenetic control of heterochromatin assembly. *Science* 2001;292:110–3. [PubMed: 11283354]
23. Jia S, Kobayashi R, Grewal SI. Ubiquitin ligase component Cul4 associates with Clr4 histone methyltransferase to assemble heterochromatin. *Nat Cell Biol* 2005;7:1007–13. [PubMed: 16127433]
24. Hong EJE, Villen J, Gerace EL, Gygi S, Moazed D. A cullin E3 ubiquitin ligase complex associates with Rik1 and the Clr4 histone H3-K9 methyltransferase and is required for RNAi-mediated heterochromatin formation. *RNA Biology* 2005;2:106–111. [PubMed: 17114925]
25. Horn PJ, Bastie JN, Peterson CL. A Rik1-associated, cullin-dependent E3 ubiquitin ligase is essential for heterochromatin formation. *Genes Dev* 2005;19:1705–14. [PubMed: 16024659]
26. Thon G, et al. The Clr7 and Clr8 directionality factors and the Pcu4 cullin mediate heterochromatin formation in the fission yeast *Schizosaccharomyces pombe*. *Genetics* 2005;171:1583–95. [PubMed: 16157682]
27. Bannister AJ, et al. Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. *Nature* 2001;410:120–4. [PubMed: 11242054]
28. Partridge JF, Scott KS, Bannister AJ, Kouzarides T, Allshire RC. cis-acting DNA from fission yeast centromeres mediates histone H3 methylation and recruitment of silencing factors and cohesin to an ectopic site. *Curr Biol* 2002;12:1652–60. [PubMed: 12361567]
29. Sadaie M, Iida T, Urano T, Nakayama J. A chromodomain protein, Chp1, is required for the establishment of heterochromatin in fission yeast. *Embo J* 2004;23:3825–35. [PubMed: 15372076]
30. Cam HP, et al. Comprehensive analysis of heterochromatin- and RNAi-mediated epigenetic control of the fission yeast genome. *Nat Genet* 2005;37:809–19. [PubMed: 15976807]
31. Grewal SI, Klar AJ. A recombinationally repressed region between mat2 and mat3 loci shares homology to centromeric repeats and regulates directionality of mating-type switching in fission yeast. *Genetics* 1997;146:1221–38. [PubMed: 9258669]
32. Kanoh J, Sadaie M, Urano T, Ishikawa F. Telomere binding protein Taz1 establishes Swi6 heterochromatin independently of RNAi at telomeres. *Curr Biol* 2005;15:1808–19. [PubMed: 16243027]
33. Volpe TA, et al. Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. *Science* 2002;297:1833–7. [PubMed: 12193640]
34. Fire A, et al. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 1998;391:806–11. [PubMed: 9486653]
35. Verdel A, et al. RNAi-mediated targeting of heterochromatin by the RITS complex. *Science* 2004;303:672–6. [PubMed: 14704433]
36. Noma K, et al. RITS acts in cis to promote RNA interference-mediated transcriptional and post-transcriptional silencing. *Nat Genet* 2004;36:1174–80. [PubMed: 15475954]
37. Sugiyama T, Cam H, Verdel A, Moazed D, Grewal SI. RNA-dependent RNA polymerase is an essential component of a self-enforcing loop coupling heterochromatin assembly to siRNA production. *Proc Natl Acad Sci U S A* 2005;102:152–7. [PubMed: 15615848]
38. Motamedi MR, et al. Two RNAi complexes, RITS and RDRC, physically interact and localize to noncoding centromeric RNAs. *Cell* 2004;119:789–802. [PubMed: 15607976]
39. Irvine DV, et al. Argonaute slicing is required for heterochromatic silencing and spreading. *Science* 2006;313:1134–7. [PubMed: 16931764]
40. Zofall M, Grewal SI. Swi6/HP1 recruits a JmjC domain protein to facilitate transcription of heterochromatic repeats. *Mol Cell* 2006;22:681–92. [PubMed: 16762840]
41. Grewal SI, Moazed D. Heterochromatin and epigenetic control of gene expression. *Science* 2003;301:798–802. [PubMed: 12907790]
42. Buhler M, Verdel A, Moazed D. Tethering RITS to a nascent transcript initiates RNAi- and heterochromatin-dependent gene silencing. *Cell* 2006;125:873–86. [PubMed: 16751098]
43. Djupedal I, et al. RNA Pol II subunit Rpb7 promotes centromeric transcription and RNAi-directed chromatin silencing. *Genes Dev* 2005;19:2301–6. [PubMed: 16204182]

44. Kato H, et al. RNA polymerase II is required for RNAi-dependent heterochromatin assembly. *Science* 2005;309:467–9. [PubMed: 15947136]
45. Ayoub N, et al. A novel jmjC domain protein modulates heterochromatinization in fission yeast. *Mol Cell Biol* 2003;23:4356–70. [PubMed: 12773576]
46. Klose RJ, Kallin EM, Zhang Y. JmjC-domain-containing proteins and histone demethylation. *Nat Rev Genet* 2006;7:715–27. [PubMed: 16983801]
47. Tsukada Y, et al. Histone demethylation by a family of JmjC domain-containing proteins. *Nature* 2006;439:811–6. [PubMed: 16362057]
48. Mochizuki K, Gorovsky MA. Small RNAs in genome rearrangement in *Tetrahymena*. *Curr Opin Genet Dev* 2004;14:181–7. [PubMed: 15196465]
49. Vagin VV, et al. A distinct small RNA pathway silences selfish genetic elements in the germline. *Science* 2006;313:320–4. [PubMed: 16809489]
50. Richards EJ, Elgin SC. Epigenetic codes for heterochromatin formation and silencing: rounding up the usual suspects. *Cell* 2002;108:489–500. [PubMed: 11909520]
51. Pal-Bhadra M, Bhadra U, Birchler JA. RNAi related mechanisms affect both transcriptional and posttranscriptional transgene silencing in *Drosophila*. *Mol Cell* 2002;9:315–27. [PubMed: 11864605]
52. Pal-Bhadra M, et al. Heterochromatic silencing and HP1 localization in *Drosophila* are dependent on the RNAi machinery. *Science* 2004;303:669–72. [PubMed: 14752161]
53. Haynes KA, Caudy AA, Collins L, Elgin SC. Element 1360 and RNAi Components Contribute to HP1-Dependent Silencing of a Pericentric Reporter. *Curr Biol* 2006;16:2222–7. [PubMed: 17113386]
54. van Rij RP, et al. The RNA silencing endonuclease Argonaute 2 mediates specific antiviral immunity in *Drosophila melanogaster*. *Genes Dev* 2006;20:2985–95. [PubMed: 17079687]
55. Wang XH, et al. RNA interference directs innate immunity against viruses in adult *Drosophila*. *Science* 2006;312:452–4. [PubMed: 16556799]
56. Kalmykova AI, Klenov MS, Gvozdev VA. Argonaute protein PIWI controls mobilization of retrotransposons in the *Drosophila* male germline. *Nucleic Acids Res* 2005;33:2052–9. [PubMed: 15817569]
57. Saito K, et al. Specific association of Piwi with rasiRNAs derived from retrotransposon and heterochromatic regions in the *Drosophila* genome. *Genes Dev* 2006;20:2214–22. [PubMed: 16882972]
58. Deshpande G, Calhoun G, Schedl P. *Drosophila* argonaute-2 is required early in embryogenesis for the assembly of centric/centromeric heterochromatin, nuclear division, nuclear migration, and germ-cell formation. *Genes Dev* 2005;19:1680–5. [PubMed: 16024657]
59. Allshire RC, Nimmo ER, Ekwall K, Javerzat JP, Cranston G. Mutations derepressing silent centromeric domains in fission yeast disrupt chromosome segregation. *Genes Dev* 1995;9:218–33. [PubMed: 7851795]
60. Fukagawa T, et al. Dicer is essential for formation of the heterochromatin structure in vertebrate cells. *Nat Cell Biol* 2004;6:784–91. [PubMed: 15247924]
61. Hall IM, Noma K, Grewal SI. RNA interference machinery regulates chromosome dynamics during mitosis and meiosis in fission yeast. *Proc Natl Acad Sci U S A* 2003;100:193–8. [PubMed: 12509501]
62. Peng JC, Karpen GH. H3K9 methylation and RNA interference regulate nucleolar organization and repeated DNA stability. *Nat Cell Biol* 2007;9:25–35. [PubMed: 17159999]
63. Jia S, Noma K, Grewal SI. RNAi-independent heterochromatin nucleation by the stress-activated ATF/CREB family proteins. *Science* 2004;304:1971–6. [PubMed: 15218150]
64. Kim HS, Choi ES, Shin JA, Jang YK, Park SD. Regulation of Swi6/HP1-dependent heterochromatin assembly by cooperation of components of the mitogen-activated protein kinase pathway and a histone deacetylase Clr6. *J Biol Chem* 2004;279:42850–9. [PubMed: 15292231]
65. Schultz D, Ayyanathan K, Negorev D, Maul G, Rauscher FR. SETDB1: a novel KAP-1-associated histone H3, lysine 9-specific methyltransferase that contributes to HP1-mediated silencing of euchromatic genes by KRAB zinc-finger proteins. *Genes Dev* 2002;16:1855–69.

66. Aulner N, et al. The AT-hook protein D1 is essential for *Drosophila melanogaster* development and is implicated in position-effect variegation. *Mol Cell Biol* 2002;22:1218–32. [PubMed: 11809812]
67. Blattes R, et al. Displacement of D1, HP1 and topoisomerase II from satellite heterochromatin by a specific polyamide. *Embo J* 2006;25:2397–408. [PubMed: 16675949]
68. Huertas D, Cortes A, Casanova J, Azorin F. *Drosophila* DDP1, a multi-KH-domain protein, contributes to centromeric silencing and chromosome segregation. *Curr Biol* 2004;14:1611–20. [PubMed: 15380062]
69. Sun FL, et al. cis-Acting determinants of heterochromatin formation on *Drosophila melanogaster* chromosome four. *Mol Cell Biol* 2004;24:8210–20. [PubMed: 15340080]
70. Aravin AA, et al. The small RNA profile during *Drosophila melanogaster* development. *Dev Cell* 2003;5:337–50. [PubMed: 12919683]
71. Aravin AA, et al. Double-stranded RNA-mediated silencing of genomic tandem repeats and transposable elements in the *D. melanogaster* germline. *Curr Biol* 2001;11:1017–27. [PubMed: 11470406]
72. Cheutin T, et al. Maintenance of stable heterochromatin domains by dynamic HP1 binding. *Science* 2003;299:721–5. [PubMed: 12560555]
73. Festenstein R, et al. Modulation of heterochromatin protein 1 dynamics in primary Mammalian cells. *Science* 2003;299:719–21. [PubMed: 12560554]
74. Nakayama J, Klar AJ, Grewal SI. A chromodomain protein, Swi6, performs imprinting functions in fission yeast during mitosis and meiosis. *Cell* 2000;101:307–17. [PubMed: 10847685]
75. Grishok A, Sinskey JL, Sharp PA. Transcriptional silencing of a transgene by RNAi in the soma of *C. elegans*. *Genes Dev* 2005;19:683–96. [PubMed: 15741313]
76. Robert VJ, Sijen T, van Wolfswinkel J, Plasterk RH. Chromatin and RNAi factors protect the *C. elegans* germline against repetitive sequences. *Genes Dev* 2005;19:782–7. [PubMed: 15774721]
77. Vastenhouw NL, et al. Gene expression: long-term gene silencing by RNAi. *Nature* 2006;442:882. [PubMed: 16929289]
78. Sullivan BA, Karpen GH. Centromeric chromatin exhibits a histone modification pattern that is distinct from both euchromatin and heterochromatin. *Nat Struct Mol Biol* 2004;11:1076–83. [PubMed: 15475964]
79. George JA, Pardue ML. The promoter of the heterochromatic *Drosophila* telomeric retrotransposon, HeT-A, is active when moved into euchromatic locations. *Genetics* 2003;163:625–35. [PubMed: 12618401]

Figure 1.

Changes in histone modification implicated in the switch from a euchromatic to a heterochromatic state. Active genes are frequently marked by H3K4me (but see Berger, this issue); this mark must presumably be removed by LSD1 (not yet characterized in *Drosophila*). H3K9 is normally acetylated in euchromatin, and this mark must be removed by a histone deacetylase, typically HDAC1. Phosphorylation of H3S10 can interfere with methylation of H3K9; dephosphorylation may involve a phosphatase targeted through the carboxyl terminus of the JIL1 kinase¹⁰. These transitions set the stage for acquisition of the modifications associated with silencing, including methylation of H3K9 by SU(VAR)3-9 or another HMT, binding of HP1, and subsequent methylation of H4K20 by SUV4-20, an enzyme recruited by HP1. Other silencing marks, such as methylation of H3K27 by E(Z) (not shown), appear to be relevant in some domains, although this mark is more prominently used by the Polycomb system. Supporting data comes from genetic identification of modifiers of PEV, biochemical characterization of the activities of such modifiers, and tests of protein-protein interactions⁹. (Adapted from¹⁰)

Figure 2. Variegating phenotypes. While alternative chromatin packaging states can be inherited, they do switch at a low frequency, resulting in a variegating phenotype within a clone of cells. Picture: on the left, a fly eye; on the right a sectored colony of yeast *S. pombe*

Figure 3.

HP1 interacts with H3K9me2/3 through its chromo domain, and with SU(VAR)3-9 through its chromoshadow domain. (Adapted from ref ⁹.)

Figure 4.

Model showing RNAi-mediated heterochromatin assembly and silencing in *S. pombe*. Centromeric repeat (*dg* and *dh*) transcripts produced by RNA polymerase II are processed by the RNAi machinery, including RITS and RDRC effector complexes that interact with each other and localize across heterochromatic domains. The slicer activity of Ago1 (a component of RITS) and the RNA-dependent RNA polymerase activity of Rdp1 (a subunit of RDRC) are required for processing the repeat transcripts into siRNAs. siRNA-guided cleavage of nascent transcripts by Ago1 might make them preferential substrates for Rdp1 to generate dsRNA, which in turn is processed into siRNAs by Dicer. siRNAs are believed to mediate targeting of histone modifying activities including the Clr4 complex. This process most likely involves siRNA base pairing with nascent transcripts, but the precise mechanism remains undefined. siRNAs produced by heterochromatin-bound RNAi “factories” might also prime assembly of RISC-like complexes capable of mounting a classic RNAi response. Methylation of H3K9 by Clr4 is necessary for stable association of RITS with heterochromatic loci, apparently via binding of the Chp1 chromodomain. This methylation event also recruits Swi6, which along with other factors mediates spreading of various effectors such as SHREC. SHREC may facilitate proper positioning of nucleosomes to organize the higher-order chromatin structure essential for diverse heterochromatin functions, including transcriptional gene silencing. Swi6 also recruits an antisilencing protein, Epe1, that modulates heterochromatin to facilitate repeat transcription in addition to other functions. The system as a whole maintains a balance that results in loss of expression from reporter genes inserted within the domain.

Table 1

Factors implicated in heterochromatin formation: commonalities and differences in different systems. (Adapted from ref. 16.)

Component	Organism				
	<i>S. pombe</i>	<i>Neurospora</i>	<i>Drosophila</i>	Mouse	<i>Arabidopsis</i>
Repetitive DNA	YES	YES	YES	YES	YES
DNA methylation	NO	YES	NO*	YES	YES
H3K9 methylation	YES	YES	YES	YES	YES
HP1a	YES	YES	YES	YES	NO*
Small RNAs	YES	NO*	YES	YES	YES
RNA Pol II	YES	N/D	N/D	N/D	N/D
RNA-dependent					
RNA polymerase	YES	NO*	NO	NO	YES

YES indicates that the factor has been implicated in playing a role in heterochromatin formation in the given organism. NO indicates that the factor is not present in the organism. NO* indicates that the organism has the given component but that it does not appear to play a role in heterochromatin formation. N/D means that the organism has the component but a role for it in heterochromatin formation has not been demonstrated.