Primer

Heterochromatin: silence is golden

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The large genomes of higher eukaryotes suggest a need for stable packaging, particularly as most of the DNA does not code for proteins, and much consists of repetitious sequences, including remnants of invading retrotransposons, transposable elements and the like. Cytological studies first demonstrated that much of the repetitous DNA is packaged in a condensed form referred to as heterochromatin, and indicated that such packaging limits transcription. During the last few years, remarkable progress has been made in identifying the biochemical characteristics of heterochromatin, suggesting mechanisms by which heterochromatin formation is targeted and maintained.

Biochemical marks of heterochromatin

An important characteristic of heterochromatin is that this mode of packaging is epigenetically inherited; i.e. the packaging state is generally maintained after replication and mitosis, independent of the underlying DNA sequence. This implies a biochemical mark and a cellular machinery that can recognize and maintain the mark locally.

The DNA of eukaryotic genomes is packaged in nucleosomes, with around 167 base pairs (bp) of DNA wrapped in two left-handed turns around a core of eight histones (an $[H3+H4]_2$ tetramer and two dimers of [H2A+H2B]). Histone H1 binds to the DNA where the DNA enters and exits from association with the core. 'Linker' DNA of around 10–50 bp extends to the next histone core. The carboxy-terminal two thirds of the core histones establish the very stable interactions that create the octamer and bind DNA to its surface, whereas the amino-terminal tails are available for interaction with other chromosomal components. The tails are substrates for a number of enzymes that modify specific amino acids of specific histones. While general patterns had been noted, the significance of these histone modifications was first recognized with the demonstration that a particular histone acetyltransferase was the product of a gene previously identified as an activator of gene expression. Heterochromatin is characterized by histone hypoacetylation (in all eukaryotes) and by methylation of histone H3 on lysine 9 in higher eukaryotes, but not in some single-celled eukaryotes such as *Saccharomyces*. Histone H3 methylated at lysine 9 (H3-mK9) is bound by Heterochromatin Protein 1 (HP1), a highly conserved protein that is directly associated with pericentric heterochromatin.

'Constitutive heterochromatin', which is commonly found around centromeres and telomeres, displays the same condensed packaging in all somatic cell types of an organism (Table 1). 'Facultative heterochromatin' refers to regions of the chromosome that appear densely packaged and have lost gene expression, in which the decision to package the DNA represents a selective, cell-specific event that is clonally inherited (e.g., the inactive X chromosome in mammalian cells). The typical condensed appearance of heterochromatin is reflected in a general loss of accessibility to a variety of reagents, particularly nucleases, accompanied by a loss of accessibility at gene-specific 5' hypersensitive sites (HS sites), and packaging in a more regular nucleosome array. Juxtaposition of genes normally found in euchromatin with heterochromatic domains, either by rearrangement or transposition, generally results in silencing of the gene in some of the cells in which the gene is normally expressed, giving rise to a variegating phenotype, termed position effect variegation (PEV). This effect is attributed to 'spreading' of the heterochromatic form of packaging. The observation that heterochromatic packaging can lead to gene silencing supports the suggestion that this mode of chromatin assembly emerged as a defense to minimize the activity of invading retrotransposons and transposable elements.

In contrast to histone hypoacetylation and H3-K9 methylation, 5-methylcytosine (5mC), a third mark of silent chromatin in most eukaryotes, is a modification of the DNA itself. Postreplicative methylation of cytosine is carried out by a diverse group of DNA methyltransferases. Some of these are capable of de novo methylation, whereas others primarily maintain the pattern of methylation by acting preferentially on hemimethylated sites after replication. CpG is the most common site of methylation, but CpNpG sites and asymmetric sites are also used. Why this mark is prominently used in some eukaryotic genomes, but not in others remains a puzzle.

What is remarkable is the number of interactions between the three major biochemical marks — histone hypoacetylation, H3-K9 methylation and DNA methylation — during heterochromatin formation. Each of the epigenetic marks can be directly propagated and each can influence the acquisition of the other two. For example, genetic analysis shows that in *Neurospora crassa* DNA methylation is completely dependent on H3-K9 methylation, and a similar but less complete relationship has been found in *Arabidopsis*. Conversely, loss of targeted DNA methylation can lead to H3-mK9 depletion. A mechanism for this is suggested by the observation that the 5-methylcytosine binding protein MeCP2 can recruit both histone deacetylase activity and H3-K9 methyltransferase activity. One's general impression is that of an internally redundant

Table 1. Characteristics of different chromatin forms.

Feature	Euchromatin	Constitutive heterochromatin
Staining/packaging in interphase	Dispersed	Condensed, prominently stained (heteropycnotic)
DNA sequence	Predominantly unique; gene rich	Repetitive (satellites; derivatives of viruses, transposons, etc.); gene poor
Replication timing	Throughout S phase	Late S phase
Meiotic recombination	Normal frequency	Low frequency
Characteristic modifications	Histone hyperacetylation	Histone hypoacetylation
	Histone H3-mK4 present	Histone H3-mK9 present
	Cytosine hypomethylation	Cytosine hypermethylation
Chromatin structure	HS sites, irregular nucleosome spacing; accessible to nucleases	Loss of HS sites, regular nucleosome array; less accessible to nucleases

system, designed to maintain continuous silencing.

Assembly of heterochromatin

Recent biochemical and genetic experiments in a wide range of organisms have led to a model of heterochromatin assembly based on the pattern of histone modification (Figure 1). An early event is histone deacetylation by multiple histone deacetylases (HDACs) that are specific for different core histone sites. The deacetylases appear to act in concert with a histone methyltransferase (HMT) that specifically methylates H3-K9. Histone H3-mK9 is then a target for binding by HP1.

HP1 specifically binds H3-mK9 through its amino-terminal chromo domain and interacts with the specific HMT required for this modification through its carboxy-terminal chromo shadow domain. By both recognizing the heterochromatin mark and interacting with the enzyme that makes the mark, HP1 presumably can promote epigenetic inheritance and the spread of heterochromatic packaging. Genetic studies in fission yeast (*Schizosaccharomyces pombe*) and *Drosophila* have shown that the spreading of heterochromatic packaging, observed upon loss of a normal boundary (Figure 1), is dependent on the specific HMT and on the presence of HP1. Both HP1 and the HMT display dosage-dependent effects on PEV, such that an increased dose of either results in an increased percentage of cells showing the silent state, whereas a decreased dose of either or a point mutation that weakens H3-mK9 binding to HP1 leads to a decrease in the percentage of cells showing the silent state.. HP1 interacts with several other chromosomal proteins, many of which are genetically characterized by mutations that suppress PEV. In addition, HP1 can form homodimers and thus might contribute to the regular chromatin structure observed in heterochromatic domains.

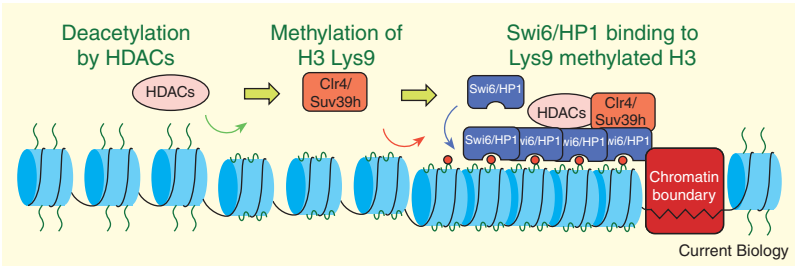

Figure 1. A stepwise model for epigenetic control of heterochromatin assembly in fission yeast (*S. pombe*).
Deacetylation by the histone deacetylases (HDACs) Clr6, Clr3, Sir2, and perhaps others, allows methylation of histone H3 lysine 9 by the Clr4/Rik1 complex. Swi6 (the *S. pombe* homolog of HP1) binds specifically to H3-mK9 to continue heterochromatin assembly. Such progression might be stopped by a boundary, potentially a site that recruits histone acetylases. (Figure adapted from Grewal and Elgin, Figure 5.)

While HP1 is commonly present in eukaryotes, it is not universal. In particular, it is not present in the yeast *S. cerevisiae*. Here the SIR complex plays a key role in establishing and recognizing a pattern of histone modification (hypoacetylation, particularly of lysine 16 of histone H4), thus providing a similar means for the spreading of heterochromatic packaging. In this instance, heterochromatin formation is targeted to the telomeres and to the silent mating type loci by specific protein–protein interactions. How HP1 is targeted to specific domains remains to be resolved.

RNA-directed targeting of heterochromatin

Several lines of evidence suggest a role for repetitive DNA elements, such as transposons and satellite repeats, in targeting heterochromatin formation in higher eukaryotes. These repeat sequences comprise a major portion of any complex genome and are preferentially associated with heterochromatin. Recent evidence supports the theory that cellular mechanisms have evolved to sense repeated elements and neutralize them. A common emerging theme is the idea that, in addition to DNA binding factors, non-coding RNAs play a key role in targeting and propagating chromatin modifications. For example, in plants the production of small

RNAs from transcripts of viral-derived genes or from transgenes producing self-complementary transcripts can lead to chromatin modifications at homologous sequences, with concomitant silencing. In mammals, the Xist RNA is essential for initiation and spreading of histone H3 methylation and heterochromatin assembly during X-chromosome inactivation.

A major advance came from studies demonstrating that the RNA interference (RNAi) pathway, in addition to its role in targeted destruction of mRNAs, is involved in initiating heterochromatin formation and silencing at repeated sequences. Factors involved in RNAi include the RNase III-like enzyme Dicer, which cleaves double-stranded transcripts into small interfering RNAs (siRNAs). siRNAs provide specificity for the targeting of the RNA-induced silencing complex (RISC), which degrades homologous mRNA sequences. RISC contains members of the PAZ/Piwi family, such as Argonaute. RNA-dependent RNA polymerases (RdRP) that presumably use siRNA primers and mRNA templates to synthesize double-stranded RNAs (dsRNAs) have been shown to be important for RNAi in several systems. Deletions of Argonaute (*ago1*), Dicer (*dcr1*) or RdRP (*rdp1*) in *S. pombe*, which unlike higher eukaryotes contains single copies of these genes, cause defects in heterochromatin assembly. Specifically, RNAi is required for the establishment of heterochromatin-specific histone modifications, such as H3-mK9, and for the targeting of the HP1 homolog Swi6 to the centromeres and the silent mating-type region of *S. pombe*. It has been demonstrated that centromere repeats, which are believed to be remnants of transposable elements, are transcribed at low levels, producing double-stranded transcripts. siRNAs generated by RNAi-mediated processing of these double-stranded transcripts appear to provide the specificity for targeting heterochromatin complexes to the corresponding genomic locations. siRNAs corresponding to centromere repeats have been isolated from several organisms. The centromere-homologous sequence (*cenH*) found at the *mat* locus of *S. pombe* is an RNAi-dependent heterochromatin nucleation center that controls silencing throughout a 20-kilobase domain that includes the developmentally important mating-type genes (Figure 2).

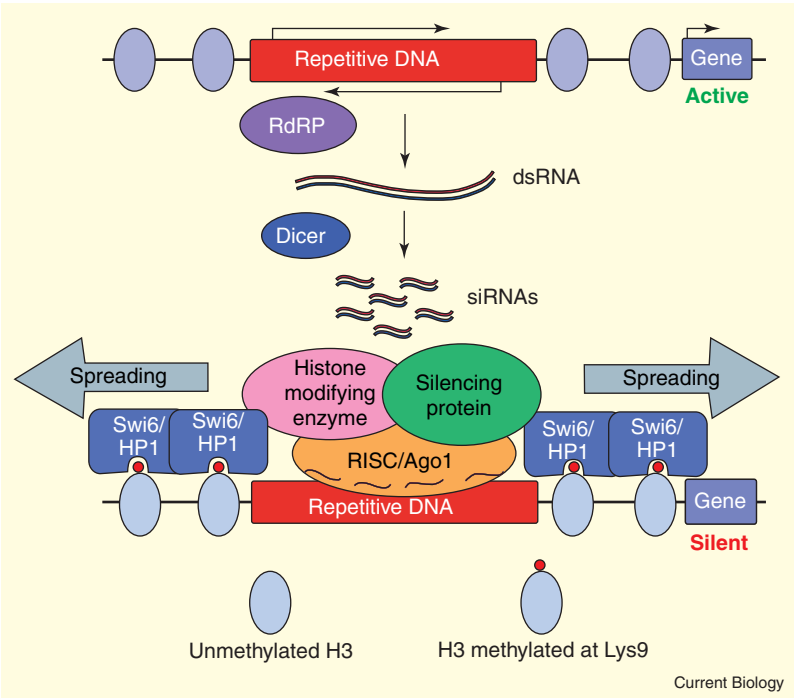

Figure 2. A stepwise model for targeting of heterochromatin formation by an RNAi mechanism.
Double-stranded RNA (dsRNA) is generated from a repetitive sequence, either as a consequence of RNA-dependent RNA polymerase activity (RdRP), or because of transcription from internal and external promoters with opposite orientation. The dsRNA is processed by Dicer to generate short interfering RNAs (siRNAs). The siRNAs are utilized to localize histone modifying complexes; heterochromatic packaging spreads due to the combined activity of the H3-K9 histone methyltransferase and associated Swi6/HP1.

The involvement of RNAi in heterochromatin formation suggested that heterologous repeat sequences capable of generating dsRNA might also serve as heterochromatin nucleation centers. A recent study confirmed this prediction by showing that retrotransposable element long terminal repeats (LTRs) recruit heterochromatin complexes in an RNAi-dependent manner in *S. pombe*. Moreover, the generation of double-stranded transcripts by artificial hairpin expression leads to silencing and heterochromatin formation at genomic sequences homologous to the dsRNA. Similar to the *cenH*-mediated heterochromatin formation at the *mat* locus, RNAi-based heterochromatin at LTRs spreads into neighboring sequences in a Swi6/HP1-dependent manner, thus silencing nearby genes.

The role of RNAi in epigenetic modification of the genome is likely to be conserved among diverse species. Factors involved in the RNAi pathway are found in fungi, plants and animals. For example, Argonaute proteins have been shown to be required for gene silencing in *Drosophila*, for targeting of H3 K9 methylation to epigenetically silenced loci in *Arabidopsis* and for programmed DNA elimination in *Tetrahymena*. In all cases, generation of siRNAs accompanies chromatin modification, suggesting that RNAs might provide specificity for targeting the chromatin-modifying activities. However,

some organisms, such as *S. cerevisiae,* lack components of the RNAi system as well as Swi6/HP1 and the specific H3-K9 methyltransferase.

Mechanism for siRNA-mediated initiation of heterochromatin formation

The precise mechanism(s) by which siRNAs target heterochromatin formation to specific genomic locations remain(s) to be delineated. The specificity suggests that the mechanism for targeting involves the pairing of homologous DNA–RNA or RNA–RNA sequences. We propose that a RISC-like heterochromatin-targeting complex containing Argonaute protein binds siRNAs and promotes their pairing to either nascent transcripts or homologous DNA sequences at the target locus (Figure 2). The multiple Argonaute proteins found in higher eukaryotes might contribute to distinct RISC complexes that perform specialized functions, such as mRNA degradation or heterochromatin targeting. Certain chromo domain proteins have also been suggested to link siRNAs to heterochromatin. The chromo domain has been shown to bind RNA in some cases, but the specific involvement of these chromatin-associated factors in targeting RNAi-based silencing remains to be demonstrated.

Based on recent studies in *S. pombe*, the assembly of a heterochromatic domain can be divided into two steps. In the initial nucleation step, Dicer produces siRNAs from the double-stranded transcripts generated from the repeated sequences. siRNAs join a RISC-like targeting complex that is capable of recruiting histone-modifying enzymes and guide it to homologous sequences. Although it remains to be shown whether the RNA dependent RNA polymerase activity of RdRP *per se* is important for silencing in *S. pombe*, its essential function in heterochromatin formation suggests a role in the synthesis of dsRNA. In other instances, dsRNA might be transcribed from promoters within the repetitious element and from a flanking promoter on the opposite strand. siRNA-mediated recruitment of histone-modifying enzymes at the repeated sequences is proposed to nucleate heterochromatin formation by establishing a specific histone modification pattern for binding of Swi6/HP1. In the second, spreading step, which is not dependent on RNAi, chromatin-bound Swi6/HP1 directly recruits histone-modifying enzymes, such as H3-K9 histone methyltransferase, which modify adjacent nucleosomes and create additional Swi6/HP1 binding sites. This allows Swi6/HP1 and heterochromatin-specific histone modifications to spread sequentially in *cis*, leading to epigenetic silencing of repetitious sequences and nearby genes. Unlike in *S. pombe*, RNAi-mediated silencing in *Arabidopsis* fails to spread and is mainly restricted to sequences homologous to the dsRNA trigger. This difference might be explained by the observation that RNA-based silencing in *Arabidopsis* is primarily dependent upon DNA methylation; the role of HP1, a factor essential for spreading in *S. pombe* and *Drosophila* has not yet been resolved.

Concluding remarks

Epigenetically heritable heterochromatin domains control a variety of chromosomal functions. In addition to stable repression of large pericentric and telomeric domains, heterochromatin plays an essential role in the maintenance of genomic integrity through its role in sister chromatin cohesion and chromosome segregation. Moreover, heterochromatic structure is known to prohibit deleterious recombination between repeated DNA sequences. Finally, the discovery of RNAi being required for targeted heterochromatin assembly has had a significant impact on our current thinking about the mechanisms of higher-order chromatin assembly and genome regulation. Future investigations of RNAi-based chromatin remodeling should lead to an increased understanding of how cells modify their epigenetic landscape in response to distinct developmental signals and under diverse environmental conditions.

Further reading

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