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Regulation of heterochromatin by histone methylation and small RNAs

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Heterochromatin mediates various nuclear processes including centromere function, gene silencing and nuclear organization. Although it was discovered nearly 75 years ago, the pathways involved in heterochromatin establishment, assembly and epigenetic maintenance have been elusive. Recent reports have demonstrated that distinct and novel chromatin-associated factors, including DNA, RNA and histone modifications, are involved in each of these events. These new findings define a novel conserved mechanism of heterochromatin formation that is likely to have an impact on all eukaryotic silencing pathways.

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Abbreviations

cenH	centromere homologous repeat
dsRNA	double stranded RNA
HMT	histone methyltransferase
HP1	heterochromatin protein 1
KRAB	Krupple-associated box
mAM	mouse ATFa-associated modulator
PcG	Polycomb group
PEV	position effect variegation
RdRp	RNA-dependent RNA polymerase
RISC	RNA-induced silencing complex
RITS	RNA-induced initiator of transcriptional gene silencing
RNAi	RNA interference
siRNA	small interfering RNA

Introduction

Heterochromatin was originally defined as the fraction of the genome that remained visibly condensed during interphase [1]. More recently, it has been defined as the genomic regions that are gene-poor and are inaccessible to DNA-modifying reagents [2,3]. Because the DNA within heterochromatin is condensed, it is largely believed that these regions are transcriptionally silenced. Early studies of heterochromatin led to the discovery of a phenomenon known as position effect variegation (PEV) [4], where a euchromatic gene placed near or within heterochromatin becomes epigenetically silenced [5]. The implications of epigenetic silencing in normal developmental gene regulation, aging and cancer progression have made heterochromatin the focus of intense investigation over the past few decades, although insights into heterochromatin establishment and maintenance have been lacking.

Recent findings have reshaped the way we think about heterochromatin, especially how it is formed and epigenetically maintained. In this review, we discuss some of the most recent findings in heterochromatin research with particular emphasis on histone H3 lysine 9 (H3 Lys9) mono-, di- and trimethylation and RNAi-mediated transcriptional silencing. Additionally, we will discuss some of the important implications of these recent findings and predict what the next few years will bring.

Histone methylation and heterochromatin

Within the eukaryotic nucleus, DNA is packaged with chromosomal proteins to form chromatin. The most fundamental repeating unit of chromatin is the nucleosome, which consists of 146 bp of DNA wrapped around an octamer of histone proteins made up of two copies each of H2A, H2B, H3 and H4 [6]. These evolutionarily conserved proteins consist of a globular C-terminal domain critical to nucleosome formation and a flexible N-terminal tail that protrudes from the nucleosome. These tails are targets for a variety of post-translational modifications including acetylation, phosphorylation and methylation. Typically, these modifications are associated with specific biological processes including cell division (phosphorylation), gene regulation, and DNA repair and recombination (acetylation) [7–11]. Although it was initially described forty years ago, the biological relevance of histone methylation has only recently been revealed [12–14].

The field of histone methylation evolved quickly in the past few years with the discovery of the first mammalian histone methyltransferase (HMT), *Su(var)3-9*, which specifically methylates H3 Lys9 [15]. Previous studies had demonstrated that *Su(var)3-9* was a potent modifier of PEV in *Drosophila*, immediately suggesting a direct role for histone methylation in heterochromatin formation [16–18]. Subsequent work definitively showed that H3 Lys9 methylation was required for the establishment and epigenetic inheritance of the heterochromatic mating type loci and centromeres in fission yeast, a pathway that seemed to be conserved in all eukaryotes except for

budding yeast [19]. In the meantime, two groups reported that the chromodomain of the heterochromatin protein 1 (HP1) specifically bound to the methylated form of H3 Lys9 [20,21]. Indeed, the solution of the crystal structure of this complex confirmed these findings [22,23]. Because HP1 was previously found to be essential for constitutive heterochromatin formation, these new findings led to a new model of heterochromatin formation in eukaryotes involving a pathway mediated by histone H3 Lys9 methylation. A review of this pathway was published recently [24].

This model also appears to apply to another region of constitutive heterochromatin: telomeres. A new study demonstrates that decreased H3 Lys9 methylation results in abnormally lengthened telomeres [25]. Concomitant with the loss of H3 Lys9 methylation was a decrease in localization of chromodomain-containing Cbx proteins, homologues of HP1. However, recent findings suggest that this model may only be valid for constitutive heterochromatin. For example, many labs have demonstrated that H3 Lys9 methylation is an early event associated with facultative heterochromatin formation in the mammalian inactivated X chromosome [26–28]. To date, there is no definitive evidence that HP1 localizes to the inactive X. These observations suggest that alternative pathways are responsible for facultative heterochromatin formation, possibly mediated by other H3 Lys9 methyl-binding proteins. The identity of these proteins and the basis of their selectivity for specific genomic regions, such as the inactive X, remains elusive. In addition, as Suv39h1 and Suv39h2 are predominantly localized to pericentric (constitutive) heterochromatin [15,29], the H3 Lys9 methylation of facultative heterochromatin is most likely to be mediated by different H3 Lys9 HMTs such as G9a, ESET/SETDB1 and/or EuHMTase1 [30–32].

To date, there is an overwhelming amount of data demonstrating that a histone-methylation-mediated heterochromatin pathway is essential for a number of biological processes in mammalian systems, including genomic stability and development. Using knock-out mouse models, it was found that mice lacking *Suv39h1/Suv39h2* not only had decreased levels of H3 Lys9 methylation but also displayed massive chromosomal mis-segregation [33]. Similarly, mice lacking *G9a*, another H3 Lys9 HMT, also had decreased levels of H3 Lys9 methylation, but presented a strikingly different phenotype: early embryonic lethality attributed to severe differentiation defects [34••]. These disparate findings raise several questions. How can different enzymes that impinge on the same target be associated with such different biological roles? Why do mammalian cells have so many different H3 Lys9 HMTs? Shouldn't the lack of one of the enzymes be compensated for by the other H3 Lys9 HMTs? The answers to these questions are just beginning to be elucidated.

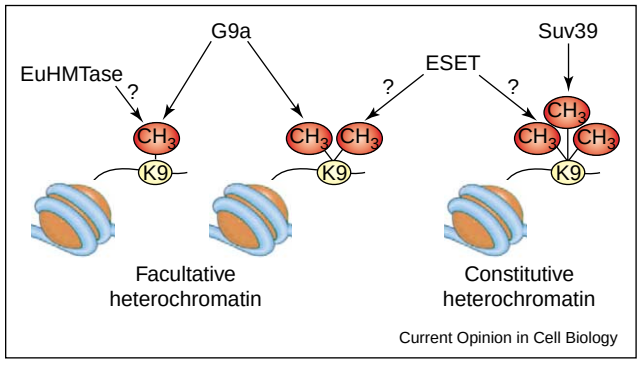
Three degrees of methylation

One possible answer to these questions is that specific HMTs differentially methylate H3 Lys9 to a certain degree. The ε-amino group of lysine can accept up to three methyl groups and hence can be mono-, di- or trimethylated [13]. Different mass spectrometric techniques have identified several human histone lysine residues, including H3 Lys9, that are mono-, di- or trimethylated *in vivo* [35–37]. Although the biological significance of these differences was unknown, recent reports indicate that these different degrees of methylation are correlated with different degrees of gene regulation. For example, the conversion of dimethyl to trimethyl histone H3 lysine 4 at gene promoters by the budding yeast HMT, Set1, was exclusively associated with actively transcribed genes [38]. Similarly, could increased methylation of H3 Lys9 be associated with an increased repressive state? A recent report demonstrates that recombinant ESET, a murine HMT, dimethylates H3 Lys9 *in vitro* [31]. Interestingly, when ESET is complexed with its endogenous partner, a protein known as mAM (mouse ATFa-associated modulator), ESET facilitates the conversion of dimethyl to trimethyl H3 Lys9 [39••]. In chromatin-templated assays it was found that H3 Lys9 dimethylation by ESET alone repressed transcription; however, transcription was significantly repressed by H3 Lys9 trimethylation by the ESET/mAM complex.

These *in vitro* findings are consistent with recent *in vivo* findings. Two reports using methyl-specific antibodies demonstrated that H3 Lys9 trimethylation, mediated by the Suv39h1 and Suv39h2 HMTs, was enriched almost exclusively within pericentric heterochromatin of mouse cells [40••,41••] (Figure 1). H3 Lys9 trimethylation was enriched within major satellite repeats of wild type cells but significantly decreased in *Suv39h1/Suv39h2* null cells [41••]. Interestingly, with this decrease, there was a concomitant increase in H3 Lys9 monomethylation. These findings suggest that H3 Lys9 must be monomethylated, either by deposition of an H3 Lys9 monomethylated histone or by an unidentified HMT, prior to conversion to the trimethylated form by Suv39h1/Suv39h2. Consistent with this, *in vitro* analysis demonstrates that monomethyl H3 Lys9 is the preferred substrate for both Suv39h1 and Suv39h2 [40••].

In contrast to the localization of H3 Lys9 trimethylation to constitutive heterochromatin, H3 Lys9 mono- and dimethylation were found to be enriched within transcriptionally silent regions in the chromosomal arms [40••,41••] (Figure 1). These findings suggest that these modifications are associated with facultative heterochromatin, although this has yet to be proven experimentally. The G9a HMT was found to be responsible for the vast majority of H3 Lys9 dimethylation and most monomethylation in mouse embryonic stem cells. Consistent with its proposed role in regulatory gene silencing, lack of *G9a*

Figure 1



Degree of histone H3 lysine 9 (K9) methylation defines specific heterochromatic regions. The different known HMTs are depicted above. Recent reports demonstrate that the Suv39h1 and Suv39h2 HMTs specifically trimethylate H3 K9 at constitutive heterochromatin [40••,41••]. The G9a HMT is a dominant H3 K9 dimethylase and minor monomethylase within the chromosomal arms, most likely at facultative heterochromatin. The remaining H3 K9 monomethylation may be attributed to EuHMTase1, an HMT with significant homology to G9a [32]. Although it is known that ESET is an H3 K9 HMT *in vitro*, it is unclear if it is a di- or trimethylase *in vivo* [39••].

resulted in a global increase in histone modifications associated with transcription [34••]. Importantly, the severe phenotypes observed in the *G9a* knockout mice imply that H3 Lys9 mono- and dimethylation play a causative role in the establishment and maintenance of developmentally regulated gene silencing [34••]. Much like the discovery of HP1α, which binds to trimethyl H3 Lys9 at pericentric heterochromatin, it is likely that factors binding to mono- and dimethyl H3 Lys9 will be discovered and their role in silencing and heterochromatin formation within the chromosomal arms will be elucidated in the years ahead. Two possible candidates are the HP1 β and γisoforms, which are both dispersed throughout the nucleus, much like H3 Lys9 mono- and dimethylation [42]. At present, these factors, if they exist, remain a mystery.

Targeted for silence: transposons and repeats

In addition to understanding the role of histone-modifying activities and the factors that recognize differentially modified histone tails, it is important to comprehend the mechanisms that define specific chromosomal domains as sites of heterochromatin assembly. In higher eukaryotes, a significant amount of genomic DNA is assembled into heterochromatic structures. Small blocks of silent chromatin structures are interspersed throughout the chromosomes and are essential for the maintenance of heritable transcriptional states during development. The major targets of heterochromatin formation, however, are DNA repetitive elements such as transposons and the satellite repeats associated with centromeres and telomeres [43]. It is largely believed that cellular defense mechanisms have evolved in higher eukaryotes to neutralize the invasion of these transposable elements and viruses by forming repressive heterochromatin structures.

The specific features of transposons that are recognized by the heterochromatin formation machinery, as well as the features that distinguish them from endogenous genes, are not fully understood. Increasing evidence indicates, however, that the repetitive nature of these DNA elements is a key factor [44]. It was shown that ectopic transgenes and repetitive DNA elements, particularly when present in an inverted orientation, can induce homology-dependent silencing [45–48]. In fission yeast, the *dg* and *dh* repeats (also known as K repeats), which are associated with centromeres, telomeres and the silent mating-type region, are potent modulators of heterochromatin formation [43]. Also in fission yeast, the deletion of a DNA element (*cenH*), which shares homology to centromeric repeats, resulted in defects in heterochromatin assembly throughout a 20-kilobase chromosomal domain of the mating-type region [49]. Moreover, it was demonstrated that *cenH* is sufficient to nucleate heterochromatin assembly at an ectopic, otherwise euchromatic, site in the genome [50,51••]. Similar results were obtained with repeat sequences derived from fission yeast centromeres [52].

In contrast to the involvement of centromeric repeats in heterochromatin assembly, the transgene-induced silencing reported in plants and, to a lesser degree, in *Drosophila* has not yet been observed in fission yeast. One possible explanation for these differences is that the genome defense mechanisms against foreign elements and transposons are more robust in organisms that utilize DNA methylation to assemble silent chromatin (i.e. plants) than in systems lacking DNA methylation (i.e. fission yeast). This hypothesis may explain why DNA methylation is prominently used in organisms with a widespread transposon content in their genome, but not in organisms that contain relatively few transposable elements.

In the current models proposed to explain the detection of repetitive elements by the heterochromatin formation machinery, it has been suggested that the DNA–DNA pairing between repeated sequences could result in unique structures that are recognized by the silencing machinery [44,53,54]. Consistent with this, the silencing of homeotic genes in *Drosophila*, which requires the binding of Polycomb-group (PcG) proteins to cellular memory modules or PcG response elements (PRE), is subject to mitotic pairing defects [55] (see also review by Lund and van Lohuizen in this issue). In addition, a series of elegant experiments performed by Cavalli and colleagues showed that Fab-7, a well-defined memory module, promotes long-distance association of transgenes that depend upon PcG proteins and DNA sequence homology

[56]. A new model of homology-dependent gene silencing has recently been described that involves RNA [57]. Evidence suggests that RNA, in particular double-stranded RNA, produced from transgenes or repeats is able to induce heterochromatin assembly at homologous sequences *in trans* (see below).

DNA-based targeting of heterochromatin

Studies from plants, animals and fungi suggest that multiple mechanisms may be responsible for the initial targeting of heterochromatin complexes in different chromosomal contexts. One of these mechanisms probably involves factors that target specific DNA sequences. Certain DNA-binding factors that recognize specific DNA elements or silencers can nucleate heterochromatin, one example being the Sir-mediated silencing in *S. cerevisiae*. These DNA binding factors often possess modular structures with separable DNA binding domains and an effector domain that recruits cofactors or silencing proteins such as histone-modifying activities and structural chromatin proteins. For example, the Krupple-associated box (KRAB) domain contains a DNA-binding region and another region that recruits the KAP-1 corepressor to cause transcriptional repression. Biochemical studies indicate that KRAB/KAP-1 recruits histone deacetylase activity and H3 Lys9-specific methylation and may mediate repression by initiating the formation of heterochromatic structures [58]. This form of heterochromatin recruitment is likely to be used for gene-specific silencing during development. The localization of heterochromatin to specific promoters has also been implicated in the silencing of cell-cycle-controlled genes, directly implicating integral components of the heterochromatin machinery in the regulation of normally euchromatic genes [59,60]. It remains to be explored whether specific DNA-binding proteins can recruit heterochromatin to heterogeneous repetitive sequences, such as pericentric regions, in higher eukaryotes. This type of recruitment is likely to be analogous to that of the fission yeast CENP-B proteins that bind pericentromeric DNA and are required for H3 Lys9 methylation and heterochromatin assembly [61].

RNAi-mediated nucleation of heterochromatin

In addition to the DNA-binding proteins, recent studies suggest that RNA interference (RNAi), a new and increasingly well-studied process, targets repressive chromosomal complexes to specific chromosomal loci. In this fundamentally novel process, RNA provides specificity for the precise targeting of silent chromatin complexes to particular genomic loci. RNAi is an evolutionarily conserved silencing mechanism that is triggered by double stranded RNA (dsRNA) and serves to regulate gene expression at various levels, via mechanisms including targeted degradation of mRNAs, translational repression of mRNAs and transcriptional repression [62]. This mechanism involves the generation of small interfering RNA molecules (siRNAs), ~22 nucleotides long, from longer dsRNAs by an Rnase-III-like enzyme called Dicer [62] (see also review in this issue by Murchison and Hannon). siRNAs generated by Dicer associate with a multiprotein RNAi effector complex called RISC (RNA-induced silencing complex) to provide specificity for the degradation of complementary mRNAs. One component of this complex, the Argonaute family members, contain a PAZ domain and a PIWI domain that have been suggested to possess RNA- or DNA-binding activities [63–65]. In some organisms, the RNAi response may also require an RNA-dependent RNA polymerase (RdRp) that is believed to amplify dsRNA [66].

Recent findings suggest that RNAi-mediated silencing pathways play a role in heterochromatin assembly. In *S. pombe*, Dicer (*dcr1*), RdRp (*rdp1*), and Argonaute (*ago1*) are required for heterochromatin formation [51••,67••]. Deletion of any of these genes results in a loss of H3 Lys9 methylation and Swi6/HP1 localization to centromeric repeats. Furthermore, it has been demonstrated that a DNA element homologous to centromeric repeats and the RNAi pathway cooperate to initiate heterochromatin formation at the silent mating-type region, although they are dispensable for the maintenance of the silent chromatin state [51••]. In *Arabidopsis*, both DNA methylation and histone H3 Lys9 methylation require the RNAi machinery [57,68,69••]. In *Drosophila*, the transcriptional silencing induced by alcohol dehydrogenase transgenesis requires PIWI, an Argonaute homologue, as well as Polycomb protein [70]. Furthermore, a recent study showed that mutations in PIWI, *aubergine* (encoding another Argonaute family protein) and *homeless* (encoding DEAD-motif RNA helicase) also affect heterochromatin formation at transgene arrays in *Drosophila* [71••]. These defects in heterochromatin assembly correlate with the loss of histone H3 Lys9 methylation and delocalization of HP1 proteins. In a similar process in *Tetrahymena*, programmed DNA elimination that is associated with H3 Lys9 methylation requires another member of the Argonaute family, Twi1p, and the HP1-like chromodomain-containing Pdd proteins [72••,73••,74]. Therefore, the role of RNAi in regulating heterochromatin assembly appears to be conserved among diverse species and is likely to play an important role in the epigenetic structuring of the genome.

An important feature of the RNAi-mediated heterochromatin pathway is the generation of siRNAs that are homologous to the target loci. Recent studies have identified numerous siRNAs corresponding to repetitive elements and transposons often associated with heterochromatic regions [75••,76,77••]. These repeat-associated siRNAs are likely to be derived from long dsRNAs generated by bidirectional transcription of repetitive sequences from adjacent promoters. Consistent with this,

both DNA strands of the *S. pombe* centromeric repeats are transcribed and can base-pair to form dsRNA [67••], and siRNAs 20–26 nucleotides long that match centromeric repeats have been identified [75••,78••]. The siRNAs appear to function as specificity factors for the targeting of chromatin-modifying activities to certain genomic loci. For example, a recent finding suggests that siRNAs participate in guiding the chromatin modifications for elimination of dispersed sequences in *Tetrahymena* [72••]. In addition, the synthesis of dsRNA from a hairpin can lead to the formation of silent chromatin at homologous sequences in plants and *S. pombe*, which is linked to generation of siRNAs [57,79••].

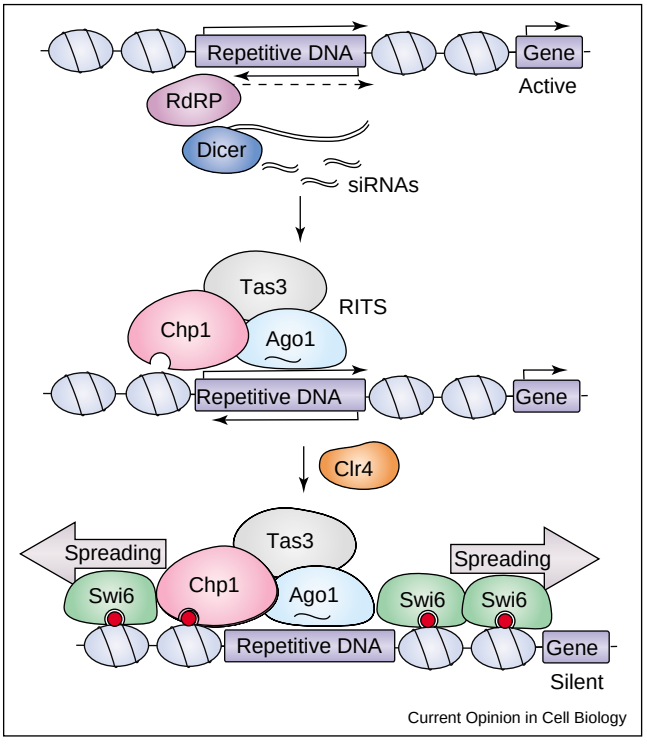
RITS: an RNAi effector complex for heterochromatin assembly

Although they were known to be involved in this pathway, it was not clear exactly how siRNAs promoted targeted assembly of heterochromatin. It had been hypothesized that a RISC-like heterochromatin-targeting complex containing the Argonaute protein binds to siRNAs and promotes their pairing to either nascent transcripts or homologous DNA sequences at the target locus [80,81]. A recent study provided the first direct evidence for the existence of an RNAi effector complex called RITS (RNA-induced initiator of transcriptional gene silencing), which links siRNAs to heterochromatin assembly in *S. pombe* [78••]. RITS contains an Argonaute family protein, Ago1, a centromere-associated chromodomain protein, Chp1, and a protein named Tas3. In addition to the three protein subunits, RITS also contains siRNAs ~22–25 nucleotides in length that were shown to originate from known targets of heterochromatin assembly, such as centromeric repeats. RITS was found to localize to heterochromatic DNA regions in an RNAi-dependent manner: in cells lacking Dicer, the protein subunits of RITS were assembled into a complex without siRNAs, but were unable to associate with chromatin. These findings suggest that siRNAs are essential for the loading of the complex onto target loci. In addition, RITS was found to play an important role in the establishment of heterochromatin-specific histone modification patterns and in the localization of Swi6/HP1. The binding of siRNAs to Ago1 is likely to mediate the targeting of RITS followed by the recruitment of histone modifying activities and the stabilization of the heterochromatin-complex via Chp1 association with H3 Lys9 methylation [78••]. These mechanisms are currently being investigated in mammalian systems.

Step-wise model for RNAi-mediated epigenetic gene silencing

Increasing evidence indicates that heterochromatin assembly is a multi-step process. Heterochromatin is believed to nucleate at specific regulatory sequences and then spreads into neighboring sequences, resulting in epigenetic gene silencing [80]. There seem to be distinct requirements for the nucleation, spreading and maintenance of heterochromatin structures [43] (Figure 2). For nucleation, dsRNAs generated from the repetitive sequences are processed into siRNAs by the Dicer ribonuclease. These dsRNAs may result from transcription from promoters within the repetitious element or from a flanking promoter on the opposite strand. The identity of the RNA polymerase involved in this process remains to be determined. In addition, RdRps have been hypothesized to use siRNAs as primers to synthesize from single stranded transcripts. The strict requirement for RdRps in the generation of siRNAs in some cases may indicate that Dicer and RdRp activities may be closely coupled. In any case, siRNAs join the RITS complex and guide the complex to homologous sequences, which probably involves pairing of siRNAs to nascent RNA transcripts or directly to DNA [80]. The RITS-mediated recruitment of histone modifying activities establishes a 'histone code' for the nucleation of heterochromatin proteins such as

Figure 2



Mechanism for RNAi-mediated targeting of heterochromatin. dsRNA generated from repetitive sequences are processed by Dicer to generate siRNAs. siRNAs may serve as primers for RdRp to produce additional dsRNAs from single stranded transcripts. siRNAs join the RITS complex to provide specificity for localization of the complex to homologous sequences. The recruitment of histone modifying activities, such as the Clr4 H3 Lys9 HMT, by the RITS complex creates a 'histone code' for the binding of the chromodomain-containing proteins Chp1 and Swi6/HP1. The binding of Chp1 serves to stabilize RITS to the loci while the binding of Swi6/HP1 results in heterochromatic spreading as a result of the combined activity of the H3 Lys9 methylation and associated Swi6/HP1.

Swi6/HP1. In the RNAi-independent spreading step, chromatin-bound Swi6/HP1 directly recruits histone-modifying enzymes such as a H3 Lys9 HMT to methylate an adjacent nucleosomes, thus creating additional Swi6/HP1 binding sites. This process allows Swi6/HP1 and heterochromatin-specific histone modifications to spread in *cis* in a sequential manner leading to the epigenetic silencing of genes surrounding the repeated sequences. This mechanism, in which small RNAs provide the specificity for localization of chromatin proteins, is likely to be conserved in other organisms.

Conclusions

Quite recently, significant findings have led to a redefinition of heterochromatin regulation in terms of histone methylation and small RNAs. New data suggests that different degrees of H3 Lys9 methylation define specific heterochromatin domains (facultative versus constitutive) (Figure 1). It is clear that the degree of methylation is dictated by the specific HMT and where it is localized in the genome. For example, Suv39h1 and Suv39h2 localize to constitutive heterochromatin and specifically trimethylate H3 Lys9, which recruits HP1α. Although increasing data suggests that these events are associated with heterochromatin formation and silencing, recent studies suggest that factors other than histone methylation and HP1 may be important for the maintenance of the heterochromatic state. Future studies will need to be performed to verify this.

Genetic and biochemical studies in fission yeast and other experimental systems have provided new insights into the role of RNAi and siRNAs in heterochromatin assembly. Increasing evidence indicates that RNAi-mediated pathways are linked to other fundamental biological processes including cell division and chromosome segregation [82]. However, many central questions remain unanswered. For example, it remains unclear exactly how siRNAs guide the RITS complex to homologous target sequences. Future studies addressing the role of siRNAs and RNAi in higher-order chromatin assembly will provide important insights into the mechanisms responsible for protecting genomic integrity and developmental gene regulation.

Update

In recent paper, Freitag *et al*. showed that the HP1 homologue in *Neurospora crassa* was required for DNA methylation at the relics of transposons [83]. This new result extends previous studies by the Selker laboratory demonstrating that the histone H3 Lys9 trimethyltransferase DIM-5 is required for all known DNA methylation in *Neurospora crassa* [84]. Also recently, Chan *et al*. showed that factors involved in the RNAi pathway, such as RNA-dependent RNA polymerase 2 (*rdr2*), dicer-like 3 (*dcr3*), silencing defective 4 (*sde4*) and Argonaute 4 (*ago4*) are required for *de novo* DNA methylation and the establishment of silencing but are dispensable for the maintenance of preexisting silent state [85]. This situation closely resembles the role of the RNAi pathway in the establishment, but not maintenance, of the heterochromatic state at the mating-type region of fission yeast [51••].

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- • of special interest
- •• of outstanding interest

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