RNA Interference and Heterochromatin Assembly

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SUMMARY

In most eukaryotes, histone and DNA modifications are responsible for the silencing of genes integrated in heterochromatic sequences, as well as the silencing of pericentromeric repeats and transposable elements themselves. But the mechanisms that guide these modifications to heterochromatin during the cell cycle have been elusive. RNA interference takes advantage of heterochromatic transcription to process small RNAs and recruit enzymes required for both histone and DNA modifications, and is one such mechanism that has been identified. The processes are best understood in fission yeast and plants, but recent work in mammalian cells, especially in the germline, suggests these mechanisms may be highly conserved.

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1 INTRODUCTION

Heterochromatin is defined as chromosomal material that remains condensed during interphase, when euchromatin unravels co-incident with gene expression. Heterochromatin is faithfully inherited from one cell cycle to the next, but (at least in some cases) can be reset in each sexual generation, and so fulfills many of the criteria for epigenetic inheritance (Djupedal and Ekwall 2009). Nonetheless the function of heterochromatin is still incompletely understood. One important function is the ability to silence genes that are closely linked to heterochromatin—leading to the concept of “spreading” of silencing from one chromosomal location to the next. This type of heterochromatic silencing is known as “position effect variegation” (PEV) (Fig. 1), in which variegation refers to the mosaic pattern of silencing visualized by clones of cells in the developing eye of *Drosophila* (Huisinga and Elgin 2009). Since its discovery in the 1930s, more than 100 loci have been identified that either enhance or suppress PEV, which is also acutely sensitive to the position of the gene on a chromosome, to dosage, and to environmental factors such as temperature and nutrition (Ebert et al. 2006).

Importantly, silencing of transposons shares many of the same properties as PEV, and was recognized as a related phenomenon when it was first discovered in maize in the 1950s (Lippman and Martienssen 2004). Since the advent of genome sequencing, it is clear that transposons make up a substantial portion of heterochromatic sequences in most eukaryotes, and heterochromatic silencing has become a paradigm for epigenetic mechanisms of gene

regulation. However, although silencing clearly has a role in regulating transposable elements, the biological function of PEV remains unclear. In addition to its role in silencing, heterochromatin also has profound effects on chromosome organization, especially at centromeres in which it plays a major role in establishing the kinetochore (Durand-Dubief and Ekwall 2008).

RNA is an attractive intermediate in heterochromatic silencing and spreading, as base-pairing with target sequences might account for sequence specificity. Furthermore, RNA interference has been established as a potent silencing mechanism, albeit at the posttranscriptional level. Silencing is accomplished by the Argonaute family of RNase H-related endonucleases, which use 20–30nt small RNA to guide cleavage or translational inhibition of target mRNA (see Joshua-Tor and Hannon 2010). In this article we review the role of RNA interference in establishment and maintenance of heterochromatic transcriptional silencing, focusing on studies in the fission yeast *Schizosaccharomyces pombe*.

2 HETEROCHROMATIN AND HETEROCHROMATIC MODIFICATIONS

Heterochromatin is distinguished by the covalent modification of histones and of DNA. Histone modifications specific to heterochromatin are more highly conserved and are found in organisms that lack DNA methylation, such as fission yeast and *Caenorhabditis elegans*, as well as in most animals and plants, in which DNA methylation is widespread. 5-methyl cytosine at symmetric CpG dinucleotides, on the other hand, is found in all plant and vertebrate genomes, but is lacking from some invertebrate and fungal genomes (Feng et al. 2010). CpG methylation is maintained by the methyltransferase Dnmt1, and guided during DNA replication by the hemimethylation binding protein Uhf1, and their orthologs MET1 and VIM1 in plants, respectively. In plants and filamentous fungi heterochromatic sequences are also enriched in CpNpG as well as asymmetric (CpHpH) methylation, which are targeted by alternative DNA methyltransferases (Henderson and Jacobsen 2007). However, DNA methylation in these contexts is much rarer in animal genomes, in which CG methylation is distributed more widely (Popp et al. 2010).

Histone modifications include methylation of histone H3 lysine-9 (H3K9), methylation of H3K27 (in multicellular animals and plants) and H3 arginine-2, as well as demethylation of H3K4, deubiquitination of H2BK123 and deacetylation of several lysine residues in histones H3 and H4 (Garcia et al. 2007). Importantly, these modifications occur in readily accessible, unstructured histone “tails” that extrude from the nucleosome, and enzymes such as SET domain methyltransferases can modify nucleosomes

Figure 1. Position effect variegation in *Drosophila* and fission yeast. In *Drosophila*, the white gene *w[m4h]* is silenced by heterochromatin, causing white sectors on an otherwise red eye. Silencing is enhanced in males. In fission yeast, loss of *ade6* silencing when integrated near heterochromatic sequences gives rise to white sectors on red colonies.

in situ, without having to (completely) unravel individual nucleosomes. Additional covalent modifications are found in internal domains, which presumably can only be efficiently targeted to histone monomers and dimers. Methylation of lysine residues by SET domain methyltransferases can result in mono- (me1), di- (me2), or tri- (me3) methylated derivatives, and different derivatives are associated with heterochromatin in different species. Demethylation is accomplished sequentially by Jumonji C (JmJC)-domain oxidoreductases (from me3 to me2 to me1) and Lysine Specific Demethylase (LSD)-like amine oxidases (from me2 to me1) (Mosammaparast and Shi 2010). Deacetylation is accomplished by type I and type II deacetylases as well as type III, or “sirtuin” deacetylases that use an NADH cofactor (Shahbazian and Grunstein 2007). Importantly, many of these enzymes can also modify nonhistone proteins, such as the DNA methyltransferase Dnmt1, and so impact chromatin modification indirectly as well (Nicholson and Chen 2009).

Modifications of histone tails are specifically recognized by “effector” and “adaptor” proteins that recognize intact modified nucleosomes (Taverna et al. 2007). Adaptor domains include chromodomains, PHD domains, and WD repeat domains (all of which bind methylated lysines), bromodomains and BAH domains (which bind acetylated lysines) and 14-3-3 domains (which bind phosphorylated serines). Recognition can be reduced by nearby modifications, such as S10 phosphorylation (which prevents K9me2/3 recognition by chromodomains) or R2 methylation (which prevents K4me3 recognition by PHD domains), or enhanced by modification of other histone tails or even of the underlying DNA sequence itself (Cheng and Blumenthal 2010). Effector domains include a variety of enzymatic activities such as ATP-dependent chromatin remodeling and further histone and DNA modification. These observations have led to the idea that a combination (or code) of chromatin modifications is required to dictate any given readout (Jenuwein and Allis 2001).

Many of the enzymes that modify histones, as well as proteins that recognize them, were first discovered in genetic screens for mutants that either enhance or suppress position effect variegation in *Drosophila*, and related silencing phenomena in yeast, other animals and plants. For example SuVar (suppressor of variegation) 3–9, responsible for H3K9 methylation, was first discovered in *Drosophila*, but homologs were subsequently found in almost every other eukaryotic genome (Ebert et al. 2006).

3 RNA INTERFERENCE

Double-stranded RNA (dsRNA) can trigger posttranscriptional silencing of cognate genes through sequence specific

recognition of endogenous transcripts (see Joshua-Tor and Hannon 2010). The RNase III enzyme, Dicer, specifically cleaves dsRNA into small interfering RNAs (siRNA) 21–24nt in length. These siRNAs can act as guides to target effector complexes, such as the RNA-induced silencing complex (RISC), to endogenous transcripts in a sequence-specific manner resulting in cleavage and subsequent degradation or in translational inhibition. Several components of RISC have been isolated. One of these components, the small RNA binding protein Argonaute, is a key effector and can cleave endogenous messages via its endonuclease domain, which resembles RNaseH. RNA dependent RNA polymerase (RdRP) is thought to be involved in amplification of small interfering RNAs (siRNAs) in some organisms by using endogenous messages as templates to generate additional dsRNA substrates.

RNAi is found in almost all eukaryotes though interesting exceptions exist. The budding yeast *S. cerevisiae* appears to have lost the RNAi machinery (Aravind et al. 2000), although recent studies of other budding yeast species reveal the presence of Argonaute as well as noncanonical Dicer genes and suggest their involvement in transposon regulation (Drinnenberg et al. 2009). Similarly, whereas the function of RdRP has been well studied in plants, worms, and fission yeast, RdRP genes in insects and mammals have been elusive. Recent studies, however, had revealed RdRP activity in *Drosophila* and in humans suggesting that the activity itself is conserved (Lipardi and Paterson 2009; Maida et al. 2009).

Many RNAi mechanisms depend on dsRNA, and aside from exogenous sources (such as viruses and transgenes), dsRNA can also be generated endogenously. One mechanism involves bidirectional transcription followed by annealing of complementary transcripts, although the large amount of antisense transcription observed in eukaryotic genomes raises questions regarding specificity. Another method involves dsRNA synthesis via RdRP activity on single stranded RNA templates. Exogenously generated siRNA, or “primary siRNAs” directing amplification of endogenous “secondary siRNAs” via activity of RdRP has been shown in plants and worms, and occurs in fission yeast (Halic and Moazed 2010).

In higher eukaryotes endogenously expressed single stranded stem-loop RNAs are recognized and processed by Dicer resulting in small RNA molecules known as micro RNAs (miRNAs). miRNAs are incorporated into Argonaute, which then targets RISC to endogenous transcripts. This targeting results in posttranscriptional silencing via inhibition of translation or, in some cases, cleavage of the associated message (Aravin and Hannon 2008). The versatility of miRNAs in regulation of gene expression is vast, and some miRNAs have also been shown to result in

transcriptional activation as well as repression (Kim et al. 2008). Yet another class of small RNAs, PIWI interacting RNAs (piRNAs), require neither Dicer nor RdRP for biogenesis and associate with a subset of argonaute homologs called PIWI proteins. These piRNAs have been implicated in germ line development, transposon regulation, and genome organization (Thomson and Lin 2009). The biogenesis and function of miRNA and piRNA are described by Joshua-Tor and Hannon (2010).

4 HETEROCHROMATIC RNA INTERFERENCE AT FISSION YEAST CENTROMERES

S. pombe has proven to be a powerful system for studying the endogenous function of RNAi. One reason is that the fission yeast genome contains only a single homolog each of Dicer, Argonaute, and RdRP thus eliminating much of the redundancy exhibited in other organisms (Aravind et al. 2000; Carmell et al. 2002). One startling discovery from early studies in fission yeast was the dramatic loss of reporter gene silencing near centromeres in the absence of RNAi, which was accompanied by the accumulation of transcripts derived from heterochromatic repeats (Volpe et al. 2002). These observations suggested that heterochromatic silencing was mediated, at least in part, by RNA interference.

Heterochromatin in *S. pombe* exists at centromeres, telomeres, and the silent mating-type locus (Cam et al. 2005). Proper heterochromatin assembly at centromeres and telomeres is required for chromosome stability whereas heterochromatin within regions of the mating-type locus is required for proper cell fate determination. A major

function of heterochromatin at centromeres appears to be the recruitment of cohesin, which is required for proper chromosome segregation. This recruitment is mediated by the *S. pombe* HP1 homolog Swi6, which can bind both H3K9me2 and cohesin (Bernard et al. 2001). The centromeres of the three fission yeast chromosomes (Fig. 2) are organized in a similar manner in that each is composed of a central core region (cnt) enriched with the histone variant Cenp-A and the site of kinetochore assembly (Durand-Dubief and Ekwall 2008). The central core is then flanked by large inverted repeats referred to as the innermost repeats (imr). The central and innermost regions are further flanked by tandem alternating copies of dg and dh centromeric repeats (Takahashi et al. 1991); it is from these the outermost repeats (otr) that most centromere transcription originates (Volpe et al. 2002).

Heterochromatic RNAs are expressed bidirectionally from both the top (forward transcripts) and the bottom (reverse transcripts) DNA strands in *rdp1*[−] (RNA-dependent RNA polymerase), *dcr1*[−] (Dicer), and *ago1*[−] (Argonaute) mutant cells. Analysis of nascent RNA by nuclear run-on revealed reverse strand centromeric transcripts are synthesized but rapidly degraded in both wild type and *swi6*[−] mutant cells, suggesting that RNAi is involved in the posttranscriptional degradation of reverse strand centromeric transcripts. In addition, nascent forward strand transcripts were not detected by nuclear run-on in wild type. These transcripts do accumulate in *swi6*[−] mutants, however, suggesting the promoter driving forward transcription is transcriptionally silenced in wild type cells and this silencing is dependent on Swi6. Furthermore, accumulation of forward strand centromeric transcripts

Figure 2. Centromeric heterochromatin in the fission yeast *S. pombe*. The three centromeres of *S. pombe* each include a central region (green rectangles) flanked by large inverted innermost repeats (red rectangles). These are flanked by tandem copies of outermost elements (blue arrows, orange arrows, white rectangles and grey rectangles) that are composed of dg and dh repeats. Centromeres also contain clusters of tRNA genes (yellow rectangles) at the boundaries between heterochromatin and euchromatin. Reporter genes integrated into centromere 1 (*ade6* or *ura4*) are silenced by RNAi-mediated heterochromatic silencing.

in RNAi mutants suggests transcriptional regulation of the forward promoter is also regulated by RNAi (Volpe et al. 2002). Therefore, both transcriptional and posttranscriptional mechanisms are involved in silencing of centromeric repeats in *S. pombe*.

Heterochromatic regions such as the *S. pombe* centromeric repeats are normally enriched in the chromodomain-containing Swi6 protein and H3K9me2 whereas sites of active transcription are associated with H3K4me2 (Cam et al. 2005). However, in RNAi mutants Swi6 and H3K9me2 enrichment is replaced by H3K4me2 (Volpe et al. 2002). This led to a model in which transcripts derived from heterochromatic centromeric repeat sequences are processed by the RNAi machinery resulting in sequence specific targeting of histone modifications to centromeres. This was further supported by the identification of small interfering RNAs (siRNAs) corresponding to *S. pombe* centromeric transcripts and the discovery that at least one component of the RNAi machinery, RdRP, is enriched at centromeric repeat sequences (Reinhart and Bartel 2002; Volpe et al. 2002). Insights into effector complexes involved in RNAi mediated heterochromatin assembly mechanisms would soon follow (summarized in Fig. 3).

Biochemical purification of the fission yeast chromodomain-containing protein, Chp1, identified an RNAi effector complex consisting of Chp1, Ago1, and a novel protein, Tas3 as well as heterochromatic siRNAs (Verdel et al. 2004). This complex, the RNA-induced initiation of transcriptional silencing complex (RITS), is associated with heterochromatin (Verdel et al. 2004; Cam et al. 2005). This enrichment appears to be mediated by base

pairing of RITS associated siRNAs with heterochromatic nascent transcripts, although it has also been shown that binding of RITS to heterochromatic regions requires H3K9me2, which is recognized by the Chp1 chromodomain (Schalch et al. 2009). RITS can then recruit several other protein complexes to heterochromatin.

One protein complex recruited by RITS is the RNA-directed RNA polymerase complex (RDRC), which consists of Rdp1, the RNA helicase Hrr1, and the oligoadenylate polymerase Cid12. This complex acts on single-stranded RNA templates to generate dsRNA independently of siRNA primers (Motamedi et al. 2004). RITS association with RDRC is dependent on heterochromatin as well as siRNAs (Motamedi et al. 2004). The endonuclease activity of Ago1 is used first in a siRNA processing step whereby one strand of the siRNA duplex is "sliced" to yield single stranded siRNA. This siRNA processing step occurs in a complex called ARC, which acts to chaperone siRNA duplexes generated by Dicer to the RITS complex (Buker et al. 2007). Once in the RITS complex, the slicer activity of Ago1 on heterochromatin transcripts has a further function in generating 3' ends to act as substrates for RdRP (Irvine et al. 2006; Halic and Moazed 2010).

But most importantly, the catalytic activity of RITS is required for recruitment and spreading of a multifunctional histone modification complex known as the CLRC complex (Irvine et al. 2006). Along with the WD repeat protein Rik1, which resembles DDB1 (DNA damage binding protein), CLRC contains the sole *S. pombe* lysine 9 histone methyltransferase Clr4, as well as the H3K4 demethylase Lid2 and the cullin4 ubiquitin E3 ligase, and two other

Figure 3. Model for heterochromatin assembly and spreading at *S. pombe* centromeric outer repeats. Heterochromatic centromere sequences (yellow arrow) are transcribed by RNA Polymerase II. These centromere transcripts are targeted by RITS via siRNA loaded Ago1. Association of RITS with centromere heterochromatin is strengthened by binding of Chp1 to H3mK9. RITS activity can recruit both CLRC, via interactions with Stc1, and RDRC resulting in spreading of H3mK9 and amplification of siRNAs, respectively (see text for details). dsRNA generated either by bi-directional transcription from centromere promoters (black arrows) or by RDRC activity is recognized and processed by Dicer (Dcr1). The resulting centromere siRNAs are then loaded onto Ago1 first in the ARC complex and then in RITS.

proteins, Clr7 and Clr8 (Horn et al. 2005; Jia et al. 2005; Li et al. 2005; Li et al. 2008). Therefore, RITS association with heterochromatin acts both to amplify siRNAs and also to modify nucleosomal histones and thus reinforce heterochromatin in a sequence specific manner (Fig. 3). The bridging protein that allows interaction between RITS and CLRC has been identified as the LIM domain protein Stc1, which can interact with both Ago1 and Clr4 (Bayne et al. 2010). Further, Clr4 has the ability to bind H3K9me2 via its chromodomain (Zhang et al. 2008). This suggests a mechanism of heterochromatin spreading whereby recruitment of Clr4 by H3K9me2 allows modification of adjacent nucleosomes.

A key element in the formation of heterochromatic siRNAs, and therefore heterochromatin assembly, is the regulation of transcription within these domains. It seems paradoxical that transcription of heterochromatin triggers transcriptional silencing of these very same sequences. Insight into this paradox was provided by the finding that centromeric transcription is regulated in a cell cycle dependent manner (Kloc et al. 2008). Centromere repeat transcription is limited to the G1 and S phase of the cell cycle coinciding with increases in Pol II occupancy at centromeres (Chen et al. 2008). This is the time when

H3K9me2 is at its lowest, and Swi6 occupancy is reduced because of phosphorylation of H3S10, which prevents chromodomain binding (Fig. 4). Repeat transcription in early S-phase is followed by increases in siRNAs and in H3K9me2 that peaks in G2 (Kloc et al. 2008). Thus, centromere repeat expression in early S-phase occurs when pericentromeric heterochromatin is replicated and could provide a timely means for inheritance of the silent state by newly replicated chromatin (Fig. 4).

Mutations in RNA Polymerase II subunits have effects on centromere silencing, indicating that heterochromatin transcripts are Pol II derived (Djupedal et al. 2005) and that pol II plays an important role in the silencing process. For example, viable mutants in Rpb7 prevent accumulation of centromeric reverse transcripts and siRNA, whereas mutants in Rpb2 lose siRNA, but not centromere transcription, indicating that Pol II participates in both precursor (pre-siRNA) transcription and processing (Kato et al. 2005). As might be expected, factors involved in RNA processing also play a role in centromeric silencing. Mutants in several RNA splicing factors, including *prp5*, *prp8*, and *prp10* have strong defects in siRNA accumulation and centromeric reporter gene silencing (Bayne et al. 2010). Mutations in the exosome exonucleases Dis3 and Rrp6 also affect

Figure 4. Cell cycle regulation of heterochromatin transcription and assembly. Pericentromeric heterochromatin is silent through most of the cell cycle (G2) but is transcriptionally activated when Swi6 (the HP-1 homolog) is evicted from methylated histone H3 lysine-9 by phosphorylation of histone H3 serine-10. Transcription continues during G1 and S phase, when transcripts are processed by RNAi and converted into siRNA. Replication during early S phase replaces approximately half of the parental nucleosomes with freshly assembled nucleosomes that lack H3K9me2. The RITS complex promotes H3K9 methylation and H3K4 demethylation, by the CLRC complex, which recruits Swi6 and silences heterochromatin in the subsequent G2. Thus transient expression and siRNA production during S phase promotes epigenetic inheritance of heterochromatic modifications.

reporter gene silencing whereas mutants in the TRAMP complex oligoadenylate polymerase Cid14 prevent siRNA accumulation as well (Murakami et al. 2007; Buhler et al. 2008). Most of these mutations, however, have a much lower impact on centromere transcript accumulation and/or heterochromatin assembly relative to RNAi. One possibility is that these effects are mostly posttranscriptional and that transcriptional silencing remains largely intact. For example, pre-siRNA transcripts that are cleaved by Argonaute are stabilized in cells deficient for Rrp6 (Irvine et al. 2006).

RNAi is not the only mechanism that guides histone modification to the centromeric repeats. The histone deacetylase Clr3 is partially redundant with RNA interference, so that double mutants lose H3K9me2 completely, whereas single RNAi mutants lose H3K9me2 predominantly from embedded reporter genes but retain residual levels over centromeric repeats. In *clr3* mutants, the levels of siRNA actually increase, indicating enhanced silencing at the post-transcriptional level (Yamada et al. 2005).

5 HETEROCHROMATIC SILENCING ON CHROMOSOME ARMS

Unlike at centromeres, heterochromatin contained within the silent mating type-locus and sub-telomeric regions is maintained in *rdp1⁻*, *dcr1⁻* and *ago1⁻* mutants. Heterochromatin initiation at the mating type locus, however, seems to require RNAi (Hall et al. 2002). Once established, heterochromatin maintenance at the silent mating-type locus is dependent on the stress activated ATF/CREB (activating transcription factor/cAMP response-element binding protein) family members Atf1 and Pcr1 which can recruit Swi6 protein independently of RNAi (Jia et al. 2004). Interestingly, RNAi dependent heterochromatin assembly also occurs at sites of convergent gene transcription resulting in transient recruitment of Swi6 and binding of cohesin along chromosome arms (Gullerova and Proudfoot 2008).

Proper targeting of heterochromatin is extremely important, because misregulation of assembly can lead either to the expression or silencing of the wrong genes. Interestingly, some portions of centromeric repeat sequences integrated within euchromatic sequences are sufficient to recruit heterochromatin assembly resulting in silencing of adjacent reporter genes suggesting that these sequences act as heterochromatin nucleation elements (Ayoub et al. 2000; Partridge et al. 2002). This ectopic silencing requires factors involved in heterochromatin assembly at endogenous centromeres including RNAi (Hall et al. 2002; Volpe et al. 2003).

Whether regions of centromere homology normally nucleate heterochromatin formation in *cis* or in *trans* is

not clear. Early studies of the effects of hairpin derived siRNAs in *S. pombe* suggested that only posttranscriptional silencing of target mRNAs occurs with no obvious transcriptional silencing effects found in other organisms (Mette et al. 2000; Morris et al. 2004; Sigova et al. 2004). Further studies addressed *cis* versus *trans* silencing by tethering components of the RITS complex to RNA transcripts of a euchromatic *ura4⁺* reporter gene. Interestingly, this resulted in heterochromatin assembly at the euchromatic *ura4⁺* locus. In addition, siRNAs corresponding to *ura4* sequences were observed in these strains suggesting that *ura4* transcripts were being processed by RNAi (Buhler et al. 2006). These studies also addressed whether *ura4* siRNAs were capable of targeting heterochromatin assembly to a second *ura4* reporter in *trans*. In a wild-type background no *trans* targeting was observed suggesting that the *ura4* siRNAs could only silence in *cis*. However, in certain strain backgrounds, such as an *eri1* mutant, heterochromatin appeared to be assembled in *trans* albeit inefficiently (Buhler et al. 2006).

Eri1 is an RNA nuclease that was first identified in *C. elegans* in a screen for mutants that enhanced RNA interference on injection of exogenous siRNAs. Mutations in *Eri-1* resulted in a more robust silencing of the reporter suggesting that it acts as an inhibitor of RNAi (Kennedy et al. 2004). Consistent with this early report, Eri1 appears to play a negative role in the assembly of heterochromatin in *S. pombe* (Iida et al. 2006). Another study suggests, however, that although *C. elegans* Eri-1 may interfere with effects from exogenous siRNAs, it is actually required for post-transcriptional gene silencing triggered by endogenously expressed siRNAs (Duchaine et al. 2006). Therefore Eri1 could have a role in promoting as well as inhibiting heterochromatin assembly.

Results from two recent studies indicate *trans*-targeting can in fact occur under some circumstances in fission yeast. First, it was found that siRNAs corresponding to reporter gene sequences resulted in heterochromatin targeting to a euchromatic site (Simmer et al. 2010). Interestingly, this *trans* targeting appears to be dependent on the location of the euchromatic target being near established heterochromatin. Second, Dcr1 protein is found in intracellular foci that are distinct from those containing centromere chromatin (Emmerth et al. 2010). This suggests that processing of centromere siRNAs does not occur at the site of transcription. *Trans* targeting of heterochromatin assembly has been observed in other systems (Mette et al. 2000; Morris et al. 2004) and in fission yeast portions of both the silent mating-type locus and telomeres contain regions of centromere homology (Grewal and Klar 1997; Mandell et al. 2005) suggesting these sequences can act as *trans* targets for signals such as siRNAs. Importantly, these regions

of centromere homology coincide with the centromeric regions known to be expressed and have been shown to function as heterochromatin nucleation elements suggesting that heterochromatin formation within these regions is mediated by a common mechanism (Volpe et al. 2003).

6 RNAi-DEPENDENT HETEROCHROMATIC SILENCING IN OTHER EUKARYOTES

Although the mechanisms of RNAi mediated heterochromatin assembly discovered in *S. pombe* are highly conserved, their role in higher eukaryotes is still being explored. In part this is because other transcriptional silencing mechanisms, most importantly DNA methylation, are found in mammals as well as in plants and filamentous fungi (such as *Neurospora crassa*). Furthermore, RNAi in animals appears to operate largely in the germline, rather than in cell lines and primary cells in which its impact on heterochromatin would be easier to measure. The extent to which DNA methylation in the mammalian germline is influenced by RNAi is discussed in the article by Joshua-Tor and Hannon (2010). Recently, a similar prominence for RNAi in germline silencing has been discovered in plants, and indeed it is in plants that the role of RNA in gene silencing was first described and is arguably the best understood.

When cDNA copies of plant RNA viruses are artificially integrated into the genome as transgenes, they are susceptible to DNA methylation, which is promoted by subsequent infection with viral RNA (Wassenegger et al. 1994). Viral infection can also lead to posttranscriptional silencing, if copies of chromosomal genes are included in the infectious viral genome (Ruiz et al. 1998). Similarly, when plant transgenes are integrated in the genome, they are often methylated by mechanisms that depend on RNAi especially when they carry sequences that match transposon small RNA (Chan et al. 2006).

The genes required for both posttranscriptional and transcriptional silencing have been painstakingly isolated in genetic screens, and have led to a comprehensive picture of the mechanism by which small RNA leads to transcriptional gene silencing in plants (Vaucheret et al. 2001; Baulcombe 2004; Bender 2004; Henderson and Jacobsen 2007; Matzke et al. 2009). Silent transgenes, and many transposable elements, are transcribed by a plant-specific RNA polymerase related to Pol II, known as Pol V (Wierzbicki et al. 2009). Silencing is promoted by a factor related to SPT5, first described as a protein required for transposon transcription in yeast (He et al. 2009), as well as a chromatin remodeling enzyme (DRD1) and an SMC hinge domain protein (Matzke et al. 2009). These transcripts interact with one of the ten Argonaute proteins found in plants, namely Ago4. Usually (though not always) this interaction

depends on cleavage or “slicing” of the target transcripts (Qi et al. 2006). These transcripts are processed by a combination of RNA polymerases PolIV and RDR2 (an RdRP) into dsRNA intermediates that are substrates for one or more of the four classes of Dicer enzymes found in plants (Pikaard et al. 2008). The cycle is complete when dsRNA binding proteins such as SGS3 and its homologs bind and presumably unwind the dsRNA duplexes and load one strand onto Ago4 (Ausin et al. 2009).

As in *S. pombe*, the subsequent steps that connect RNA processing with transcriptional silencing in plants are still unclear. What is clear is that RNAi leads to histone modification, via Suvar 3-9 homologs including SUVH4/KYP, and to DNA methylation, via Dnmt3 homologs DRM1 and DRM2 and the chromomethylase CMT3. In each case, the enzymes responsible for one modification are guided by the other—for example, SUVH4/KYP has a methylcytosine-binding SRA domain with a preference for methylated CpNpG sites (the product of CMT3). On the other hand CMT3 has a chromodomain that binds H3K9me2, which is the product of SUVH4 (Johnson et al. 2007). The precise details of how the 3 CMT and 3 DRM methyltransferase homologs are recruited by RNAi are still being determined, but histone modifications and SUVH enzymes may play at least an intermediate role, reminiscent of the mechanism in fission yeast (Johnson et al. 2008).

Thus DNA and histone methylation are regulated via the 23-24nt siRNA pathway in plants. This pathway closely resembles the RNAi pathway in fission yeast, and as in *S. pombe*, RNAi-mediated heterochromatin formation is partially redundant with RNAi-independent mechanisms of heterochromatic modification, including (in plants) both histone deacetylation and DNA methylation (Lippman et al. 2003). The targets of heterochromatic modifications in plants include pericentromeric satellite sequences, as in fission yeast, but also many transposable elements (Hamilton et al. 2002; Lippman et al. 2003; May et al. 2005). As in *S. pombe*, RNAi can both establish as well as maintain heterochromatic modifications, unlike the methyltransferase MET1 and the chromatin remodeler DDM1, which can only maintain these modifications from one generation to the next (Teixeira et al. 2009).

In the pollen grain, down-regulation of the DDM1/MET1 pathway leaves transposons uniquely sensitive to RNAi, giving rise to a new class of 21nt heterochromatic small RNA that are mobilized into the sperm cells (Slotkin et al. 2009). Transposons are similarly sensitive to RNAi in the egg cell, indicating a similar loss of RNAi-independent silencing on the female side (Olmedo-Monfil et al. 2010). It is thought that this prevalence of transposon small RNA in germ cells may contribute to interspecific “genome

incompatibility” when they fail to recognize transposons from the other parent. Very importantly, mutants in RNAi on the male side result in meiotic failure and pollen inviability (Nonomura et al. 2007), whereas those on the female side result in misspecification of germ cells and the production of clonal, diploid eggs (Olmedo-Monfil et al. 2010). These mechanisms therefore have a profound role in plant reproduction.

In *N. crassa*, RNAi also plays a role in histone modification, gene silencing and in DNA methylation, although all three mechanisms do not always contribute to reporter gene silencing at the same time (Cogoni and Macino 1999; Tamaru and Selker 2001; Chicas et al. 2005). In meiosis, genes normally expressed at this time (such as spore color genes) are silenced if they are paired with a partially or completely deleted homolog (Kelly and Aramayo 2007). “Meiotic silencing of unpaired DNA,” also occurs in worms, and also depends on RNAi, which is required to silence the unpaired X chromosome as well as transgene arrays in the germline (Kelly and Aramayo 2007). In both *C. elegans* and *N. crassa* H3K9 methylation and silencing depend on Argonaute and RdRP, although a role for Dicer has so far proved elusive (She et al. 2009), and the origin of siRNA precursor transcripts that are presumably involved remains a mystery. In perhaps the most dramatic example of RNAi-guided histone modification, RNAi and H3K9me2 are required for elimination of intervening DNA and transposon related sequences in the protists *Tetrahymena* and *Paramecium* (Chalker 2005). Comparison of genome transcripts and small RNA via “scanning” is thought to result in modification and elimination of sequences from the new somatic macronucleus, guided by the germline micronucleus (Mochizuki et al. 2002). Clearly, RNAi-guided heterochromatin assembly has taken a prominent role in the germline of even the simplest eukaryotes.

7 CONCLUSIONS

The finding that RNA interference can guide epigenetic modification of histones and DNA raises important questions as to the origin and function of heterochromatin. Well known as a key factor in the organization of chromosomes, a developmental role for heterochromatin has been revealed by RNAi in the germlines of plants, protists, and animals. As a repository of transposon and other repeat sequences, heterochromatin is a source of sequence-specific epigenetic information—namely small RNA—that can silence transposons and modify chromosomes during meiosis and during mitosis, where small RNA provide a mechanism for epigenetic inheritance. Conversely, the programmed (or reprogrammed) loss of RNAi-mediated

heterochromatin, for example during development, reproduction or senescence, has profound effects on cell fate, especially of germ cells and their descendants. The role of RNAi in heterochromatic modification may yet have more surprises in store.

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