RNAi and Heterochromatin Assembly

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SUMMARY

The involvement of RNA interference (RNAi) in heterochromatin formation has become clear largely through studies in the fission yeast *Schizosaccharomyces pombe* and plants like *Arabidopsis thaliana*. This article discusses how heterochromatic small interfering RNAs are produced and how the RNAi machinery participates in the formation and function of heterochromatin.

Outline

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OVERVIEW

The intersection between RNA interference (RNAi) and heterochromatin formation has brought together two areas of gene regulation that had previously been thought to operate by different, perhaps even unrelated, mechanisms. Heterochromatin was originally defined nearly 80 years ago using cytological staining methods as those chromosome regions that retain a condensed appearance throughout the cell cycle. Early investigators studying the relationship between chromosome structure and gene expression noticed that certain chromosome rearrangements resulted in the spreading of heterochromatin into adjacent genes, which then became silent. But, the seemingly stochastic patterns of spreading gave rise to genetically identical populations of cells that had different phenotypes. This phenomenon, initially described in *Drosophila* as position-effect variegation, provides a striking example of epigenetic regulation. The term RNAi was first used to describe gene silencing that resulted from the introduction of homologous antisense or double-stranded RNA (dsRNA) into the nematode *Caenorhabditis elegans*. But, it was soon recognized that a related mechanism involving RNA accounted for posttranscriptional transgene silencing (PTGS) described earlier in petunia and tobacco. In contrast, heterochromatin was widely believed to operate directly at the chromatin level to cause transcriptional repression by a mechanism referred to as transcriptional gene silencing (TGS). This article focuses on the relationship between the RNAi pathway and the formation of epigenetically heritable heterochromatin at specific chromosome regions. It draws on recent examples that show this relationship in the fission yeast *Schizosaccharomyces pombe* and the mustard plant *Arabidopsis thaliana*.

The fission yeast nuclear genome is composed of three chromosomes that range in size from 3.5 to 5.7 Mb. Each chromosome contains large blocks of repetitive DNA, particularly at centromeres, which are packaged into heterochromatin. The mating-type loci (which control cell type) and subtelomeric DNA regions also contain repetitive sequences that are packaged into heterochromatin. We now know that the assembly of DNA into heterochromatin plays both regulatory and structural roles. In the case of the mating-type loci

in yeast, regulation of gene transcription by heterochromatin is important for cell-type identity. In the case of telomeres and centromeres, heterochromatin plays a structural role that is important for proper chromosome segregation during cell division. Moreover, repetitive DNA sequences and transposable elements account for a large fraction, in some cases more than half, of the genomes of many eukaryotic cells. Heterochromatin and associated mechanisms play a critical role in regulating the activity of repeated sequences, thus maintaining genome stability.

Recent studies have uncovered a surprising requirement for components of the RNAi pathway in the process of heterochromatin formation in fission yeast and have provided insight into how these two pathways can work together at the chromatin level. Briefly, small interfering RNA (siRNA) molecules and their Argonaute-binding proteins assemble into the RNA-induced transcriptional silencing (RITS) complex and direct epigenetic chromatin modifications and heterochromatin formation at complementary chromosome regions. RITS uses siRNA-dependent base pairing to guide association with nascent RNA sequences at the target locus destined to be silenced, an association that is stabilized by direct binding to methylated histone H3 at lysine (K)9 (H3K9me). The presence of these two activities in RITS (i.e., siRNA base-pairing and association with chromatin via methylated H3K9) triggers heterochromatin formation in concert with well-known heterochromatin-associated factors, and RNA polymerase II (Pol II) directly linking RNA silencing to heterochromatin modification and silencing.

In *A. thaliana* and many other eukaryotes, repeat sequences such as retroelements and other transposons are targeted for inactivation at the chromatin level by mechanisms that couple small RNA-mediated targeting with histone H3K9, but also DNA methylation. Although the existence of a RITS complex is not always clear, components of the RNAi and related pathways are required for the initiation and maintenance of these repressive methylation events, along with Pol II and related polymerases. In this article, we will discuss how heterochromatic siRNAs are produced, and how they mediate DNA and/or chromatin modifications in fission yeast and *A. thaliana*.

1 OVERVIEW OF THE RNAi PATHWAY

Although the term RNA interference (RNAi) was originally used to describe silencing that is mediated by exogenous double-stranded RNA (dsRNA) in *Caenorhabditis elegans* (Fire et al. 1998), it now broadly refers to gene silencing that is triggered by some kind of small RNA in association with a member of the Argonaute family of proteins (Fig. 1). In most cases, the small RNA is produced from dsRNA. However, new RNA-silencing pathways have recently been uncovered wherein the small RNA is produced from long single-stranded RNA (ssRNA) precursors. Despite the diversity of small RNA biogenesis pathways (briefly discussed below), the downstream steps use similar effector proteins and mechanisms; in all cases described so far, Argonaute-associated small RNAs target either messenger RNAs (mRNAs) posttranscriptionally (i.e., posttranscriptional transgene silencing, PTGS) or chromatin regions (i.e., transcriptional gene silencing, TGS) to effect silencing. Therefore, before introducing the components of the RNAi machinery specific to TGS—the topic of this article—the source of small RNAs that harness the RNAi machinery into action will be discussed.

1.1 Small RNA Biogenesis

Two classes of small RNA, small interfering RNA (siRNA) and microRNA (miRNA), are processed from longer dsRNA precursors. dsRNA may originate from the bidirectional transcription of repetitive DNA elements or transcription of RNA molecules that can base-pair internally to form dsRNA segments (see Fig. 2A,B, respectively). For example, transcription through inverted repeat regions

produces RNA molecules that fold back on themselves to produce hairpin structures. dsRNAs are then cleaved by Dicer, an RNase III class ribonuclease, to generate siRNAs, or processed into miRNAs through a series of related steps (Bartel 2004; Filipowicz et al. 2005). The miRNA biogenesis pathway is distinct because miRNAs are produced from the introns of endogenous coding genes or from endogenous noncoding transcripts. The Dicer products are complementary duplexes, 21–24 nt in size, which have a characteristic 2-nt overhang at each 3' end of the duplex (Hamilton and Baulcombe 1999; Zamore et al. 2000; Bernstein et al. 2001; Elbashir et al. 2001; Hannon 2002; Zamore 2002; Bartel 2004; Baulcombe, 2004). These duplexes are unwound into single-stranded siRNA (or miRNA) to act as guides through base-pairing interactions with complementary target sequences. miRNAs and siRNAs are therefore specificity factors and play a central role in all RNAi-mediated silencing mechanisms.

Although dsRNAs can form by the annealing of forward and reverse RNAs that result from bidirectional transcription or are present in hairpin structures, in some cells RNAi requires an additional enzyme to make dsRNA. This is the RNA-dependent RNA polymerase (RdRP) found in some viruses, many fungi, all plants, and *C. elegans* (Dalmay et al. 2000; Sijen et al. 2001). It is directed by siRNAs to generate more dsRNA, which can then be processed into additional siRNA by Dicer (Fig. 2C). The primary function of RdRP is thus thought to be in amplification of the RNAi response. Indeed, it seems to be involved in a process adapted for producing a better host defense response to the introduction of exogenous dsRNA. This idea is strengthened by the fact that RdRPs are not involved in the miRNA-

Figure 1. Overview of small RNA-silencing pathways. Silencing requires the biogenesis of a small RNA from either long ssRNA or dsRNA. The resulting small RNA is loaded onto effector complexes that contain a member of the Argonaute/Piwi (AGO) family of proteins, which bind to the small RNA (~22 to 28 nt in size) via their conserved middle (MID) and PIWI-Argonaute-Zwille (PAZ) domains. RNAi-mediated silencing occurs via multiple mechanisms. In the nucleus, RNAi promotes DNA and chromatin modification to induce heterochromatin formation and TGS. In addition, it cotranscriptionally degrades RNAs that are transcribed in heterochromatic domains by a process called cotranscriptional gene silencing (CTGS). In the cytoplasm, RNAi mediates the degradation of target mRNAs or their translational repression (PTGS).

Figure 2. Pathways for the biogenesis of primary small RNAs that mediate silencing. (A) Bidirectional transcription has been observed at the *Schizosaccharomyces pombe* centromeric repeats and the *cenH* region of the silent mating-type locus and may provide a dsRNA substrate for the Dicer ribonuclease. (B) Transcription through inverted repeats found in many plant and animal cells can potentially produce dsRNA. (C) Transcription of aberrant RNAs that may lack proper processing signals may trigger dsRNA synthesis by RNA-dependent RNA polymerases (RdRPs). (D) Transcription from several driver loci gives rise to Piwi-associated small RNAs (piRNAs) that silence dispersed transposons. Piwi proteins together with other ribonucleases, which are not fully defined (represented by the gray dotted line), mediate primary piRNA generation.

silencing pathways (Sijen et al. 2001). Interestingly, insects (including *Drosophila*) and vertebrates (including mammals) lack recognizable RdRP-like sequences in their genome, but the possibility that other polymerases perform dsRNA synthesis in these organisms cannot be ruled out.

In the metazoan germline, a class of small RNAs called piRNA are generated from long single-stranded primary RNA transcripts, and function to silence transposons (Fig. 2d) (Aravin et al. 2007). piRNAs are produced from several long RNA polymerase II (Pol II) transcripts and have extensive sequence complementarity with dispersed transposons. Rather than RdRP or Dicer enzymes, the generation and amplification of piRNA transcripts from piRNA-producing loci involves association with the Piwi clade of Argonaute proteins and relies on the slicer activities of these proteins (Aubergine and AGO3 proteins in *Drosophila*; see Table 1). In *S. pombe*, Argonaute, Dicer, and RdRP are all required for silencing (Volpe et al. 2002) and for the biogenesis of siRNA from noncoding transcripts in centromeric repeats (Motamedi et al. 2004; Verdel et al. 2004). Dicer-independent small RNAs, called primal RNAs or priRNAs, have also been described and originate from single-stranded centromeric repeats as well as nearly the entire transcriptome (Halic and Moazed 2010). priRNAs have been proposed to initiate RdRP-dependent siRNA amplification by targeting long noncoding centromeric transcripts. The pathways that generate priRNAs are

not fully understood. However, both siRNAs and priRNAs are trimmed to their mature size by a conserved 3–5′ exoribonuclease called Triman, which is required for efficient heterochromatin establishment (Marasovic et al. 2013).

1.2 RNA-Silencing Pathways

To date, two related complexes have been identified that incorporate siRNA: RISC and RITS. In the RNA-induced silencing complex (RISC), siRNAs or miRNAs recognize target mRNAs and initiate PTGS via their degradation by endonucleolytic cleavage within the mRNA region that is base-paired to the siRNA (Hannon 2002; Bartel 2004). The RNase H domain of the Argonaute/PIWI family protein (a subunit of RISC) performs this initial mRNA cleavage event (Song et al. 2004). In the nuclear RITS complex (RNA-induced transcriptional silencing; similar to the RISC complex), siRNAs and other protein components target the complex to chromosome regions for chromatin modification and silencing (i.e., TGS [Verdel et al. 2004; Bühler et al. 2006]) as well as cleavage of centromeric transcripts (Irvine et al. 2006). It is the RNAi-mediated TGS silencing pathway that is the focus of this article.

Argonaute and Dicer proteins are central components of virtually all RNA-silencing mechanisms including those involving siRNAs and miRNA, except, for example, the piRNA pathway (see Sec. 7 of Elgin and Reuter 2013).

Table 1. Conservation of RNAi and heterochromatin proteins

<i>Schizosaccharomyces pombe</i>	<i>Arabidopsis thaliana</i>	<i>Caenorhabditis elegans</i>	<i>Drosophila</i>	<i>Homo sapiens</i>
Dcr1	DCL1 to 4	Dcr-1	Dcr1 and 2	Dcr-1
Ago1	AGO1 to 10	Rde-1, Alg-1, and -2	Ago1 to 3, Piwi	Ago1 to Ago4
–	–	Prg-1 and 2, and 19 others	Aubergine/Sting	Piwi1 to Piwi4
Chp1 ^a	CMT3	–	–	–
Tas3 ^b	–	AIN-1	GW182	TNRC6
Rdp1	RDR1 to 6	Ego-1, Rrf-1 to -3	–	–
Hrr1	SGS2/SDE3 ^c	ZK1067.2	GH20028p	KIAA1404
Cid12	–	Rde-3, Trf-4 ^c	CG11265 ^c	POLS ^c
Swi6	LHP1 (TFL2)	Hpl-1, Hpl-2, F32E10.6 ^d	HP1a, b	HP1α, β, γ
Clr4	SUVH2 to 6	–	Su(var)3-9	Suv39h1 and 2
Rik1 ^e	DDB1	M18.5	Ddb1	Ddb1
Cul4	CUL4	Cul4	Cul4	Cul4
Sir2	SIR2	Sir2–1	Sir2	SirT1
Clr3	–	–	–	–
Clr6	HDA6	Hda-1	Rpd3	HDAC1
–	DDM1	–	–	–
Eri1	ERI1	Eri-1	CG6393	THEX1

^aAn obvious ortholog of the chromodomain protein Chp1 has not been identified in the other model organisms listed here, but most eukaryotic cells contain multiple chromodomain proteins. CMT3 in *Arabidopsis* is a chromodomain DNA methyltransferase, which acts in the same pathway as AGO4 and may be analogous to Chp1.

^bTas3 is a GW motif protein. Members of this conserved family are found associated with Argonaute family members.

^cCid12 belongs to a large family of conserved proteins that share sequence similarity with the classical poly(A) polymerase as well as 2′–5′-oligoadenylate enzymes.

^d*C. elegans* have about 20 SET domain proteins, but a histone H3 lysine (K) 9 methyltransferase (KMT) specific for H3K9 has not yet been identified in this organism.

^e*Schizosaccharomyces pombe* contains another Rik1-like protein, Ddb1, which is involved in DNA damage repair. Metazoans and plants appear to contain only a single Rik1-like gene called Ddb1 involved in DNA damage repair; however, it is unknown whether it also participates in heterochromatin formation.

Like siRNAs, miRNAs are 21–24 nt in size and form part of the RISC complex via binding to the Argonaute protein to target specific mRNAs. This targeting can result in mRNA cleavage via the PIWI/RNase H domain and translational repression. This may be coupled to sequestration of the mRNA to cytoplasmic RNA-processing organelles known as P bodies (processing bodies). Thus, although at least two different dsRNA-processing pathways result in the generation of siRNA or miRNA (i.e., small RNA biogenesis), these small RNAs use a similar machinery to inactivate cognate mRNAs. The miRNA pathway distinguishes itself because the originating transcripts are largely developmentally regulated and, in turn, generally target and developmentally regulate the silencing of homologous genes.

As mentioned above (Overview and this section), nuclear small RNA-silencing mechanisms are widely conserved and play central roles in the regulation of gene expression and genome stability (through stable heterochromatin formation). The nuclear and cytoplasmic pathways are not separate and share common components. A striking example of the intersection of these pathways is represented by the recent finding that classical RNAi in *C. elegans*, first described as a purely PTGS mechanism, is coupled to histone H3K9 methylation and TGS that can be

inherited in subsequent generations (Guang et al. 2010; Gu et al. 2012). Similarly, piRNAs, which associate with Piwi proteins to degrade transposon RNAs in the cytoplasm, can also act in the nucleus to promote histone H3K9 and DNA methylation. Finally, recent evidence in *C. elegans* indicates that small RNAs and Argonaute proteins form a sophisticated self–nonself recognition mechanism (Ashe et al. 2012; Lee et al. 2012; Shirayama et al. 2012). Small RNAs originating from foreign DNA elements load onto a specific Piwi protein (PRG-1), which, in turn, silences their transcription. This loading appears to involve a general scanning mechanism so that small RNAs from all transcripts are represented in PRG-1. However, the silencing of self-transcripts is prevented by the action of another Argonaute protein (CSR-1), which serves as a repository of small RNAs from all self-transcripts and prevents their silencing by PGR-1. This illustrates how RNA-silencing mechanisms can distinguish transcription of foreign DNA elements from self-transcription. Related mechanisms in the *Drosophila* germline can also detect transposons via the piRNA pathway resulting in hybrid dysgenesis (Aravin et al. 2007; Brennecke et al. 2007; discussed in Elgin and Reuter 2013). RNA silencing clearly plays a central role in defense against transposons and RNA viruses at both the PTGS and TGS

levels (Plasterk 2002; Li and Ding 2005; Ghildiyal and Zamore 2009).

2 EARLY EVIDENCE IMPLICATING RNA AS AN INTERMEDIATE IN TGS

Before discussing the better-understood examples of RNAi-based chromatin modifications in fission yeast and *Arabidopsis*, we will briefly discuss early experiments that suggested a role for RNA in mediating chromatin and DNA modifications. The earliest evidence for the role of an RNA intermediate in TGS came from studies of plant viroids. The potato spindle tuber viroid (PSTV) consists of a 359-nt RNA genome and replicates via an RNA–RNA pathway. The artificial introduction of PSTV transgenes into the tobacco genome resulted in DNA methylation (Wassenegger et al. 1994), but only in plants that supported viroid RNA replication. Thus, these experiments suggested the involvement of an RNA intermediate that directs DNA methylation to homologous sequences (Wassenegger et al. 1994). Further evidence in *Arabidopsis* came from the production of aberrant transcripts, which somehow resulted in DNA methylation of promoter regions homologous to the promoter of the aberrant transcript, thus causing TGS (Mette et al. 1999). Importantly, the silencing of viral genomes in plants leads to the production of small RNAs that are 22 nt in size, which were the first examples of small RNA (Hamilton and Baulcombe 1999). These observations as well as homology-dependent silencing of transgenes, which was first discovered in petunia and tobacco (discussed in Pikaard and Mittelsten Scheid 2014), are now widely recognized as some of the earliest examples of silencing by RNA (Napoli et al. 1990). Classical silencing phenomena in maize, such as paramutation and transposon control, are early examples of transcriptional silencing that depend on RNAi (Slotkin and Martienssen 2007; Chandler 2010).

Further evidence for a link between RNAi and TGS comes from studies of repeat-induced gene silencing in *Drosophila*. The introduction of multiple tandem copies of a transgene results in the silencing of both the transgene and the endogenous copies (Pal-Bhadra et al. 1999). This silencing requires the chromodomain protein Polycomb, which is also involved in the packaging of homeotic regulatory genes into heterochromatin-like structures (Francis and Kingston 2001). In addition, this repeat-induced gene silencing requires Piwi, which associates with piRNAs in the nucleus (Pal-Bhadra et al. 2002; Aravin et al. 2007). However, Piwi and the piRNA amplification machinery appear to be exclusively localized in the germline and it remains to be determined how they can affect silencing of reporter genes in somatic cells in the above studies. In

Tetrahymena, another Piwi protein family member Twil is required for small RNA accumulation and the massive DNA elimination that is observed in the somatic macronucleus of the protozoa (detailed in Chalker et al. 2013). These and more recent results discussed in Section 4 suggest that the RNAi pathway is involved in the assembly of repressive chromatin structures in the fly germline and possibly somatic cells.

Other repeat-induced silencing mechanisms have been described in filamentous fungi (see Aramayo and Selker 2013), including repeat-induced point mutation (RIP) in *Neurospora crassa* and methylation-induced premeiotically (MIP) in *Ascobolus immersus*, that do not appear to involve an RNA intermediate because they occur independently of the transcriptional state of the locus (Galagan and Selker 2004). Instead, RIP and MIP involve paired loci, in which, for example, two out of three gene copies are silenced, suggesting some kind of DNA–DNA interaction mechanism involving homologous loci for the induction of silencing. Conversely, meiotic silencing of unpaired DNA (MSUD), which occurs in *Neurospora*, requires the RNAi pathway (Shiu et al. 2001) and may have parallels in other organisms including *C. elegans* (Maine et al. 2005).

3 RNAi AND HETEROCHROMATIN ASSEMBLY IN *S. POMBE*

S. pombe chromosomes contain extensive heterochromatic regions that are associated with underlying repetitive DNA elements at the centromeres and the silent mating-type loci (*mat2/3*; Grewal 2000; Pidoux and Allshire 2004). The DNA sequence structure of fission yeast centromeres contains a unique central core region (*cnt*) that is flanked by two types of repeats called the “innermost” (*imr*) and “outermost” (*otr*) repeats (Fig. 3). The *otr* region itself is composed of *dh* and *dg* repeats.

Heterochromatin formation in *S. pombe* involves the concerted action of a number of *trans*-acting factors. These include histone deacetylases (HDACs), the histone H3 lysine (K) 9 methyltransferase (HKMT or KMT) called Clr4, and three chromodomain proteins that bind specifically to dimethylated (me2) or trimethylated (me3) histone H3K9 called Swi6, Chp2 (both HP1 homologs), and Chp1. It has been proposed that after their initial recruitment, Swi6 and Clr4 contribute to the spreading of H3K9 methylation and heterochromatin formation through sequential cycles of Clr4-catalyzed H3K9 methylation coupled to chromodomain-mediated spreading into adjacent nucleosomes (Grewal and Moazed 2003).

Fission yeast contains a single gene for each of the RNAi proteins: Dicer, Argonaute, and RdRP (*dcr1*⁺, *ago1*⁺, and *rdp1*⁺, respectively). Mutations in the components of the

Figure 3. Organization of heterochromatic chromosome regions in *S. pombe* and *A. thaliana*. (A) The centromere of *S. pombe* chromosome 1 is shown as an example (top line), seen in the context of the whole chromosome below. The centromere core (orange) consists of the unique central core (*cnt1*) region flanked by innermost (*imrL* and *imrR*) and outermost (*otrL* and *otrR*) repeats. The pericentric *otr* region (green) is transcribed in both directions, giving rise to forward (blue) and reverse (red) transcripts. *A. thaliana* centromeres illustrated below are composed of 180-bp repeats (orange) interspersed with retrotransposable elements (yellow). Forward transcripts initiating within the long terminal repeat (LTR) of the retroelement and reverse transcripts initiating within the 180-bp repeats are indicated. (B) The region between the *mat2* and *mat3* genes contains a domain that is homologous to the centromeric repeats (*cenH*) and is also bidirectionally transcribed. Atf1 and Pcr1 are DNA-binding proteins that act in parallel with RNAi in mating-type silencing.

RNAi pathway surprisingly result in a loss of centromeric heterochromatin, and accumulation of noncoding forward and reverse transcripts from bidirectional promoters within each *dg* and *dh* repeat (Volpe et al. 2002). Deleting any of these genes also results in the loss of histone H3K9 methylation, and mutants display defects in chromosome segregation, which are generally associated with defects in heterochromatin assembly (Volpe et al. 2002, 2003). Moreover, sequencing of a fission yeast, small RNA library identified ~22-nt RNAs that mapped exclusively to centromeric repeat regions and ribosomal DNA repeats, suggesting that *cen* RNAs can produce dsRNAs that are processed into siRNAs (Reinhart and Bartel 2002). Thus, it was first suggested that the RNAi pathway could recruit Swi6 and Ctr4 to chromatin to initiate and/or maintain heterochromatin formation at centromeric and ribosomal DNA repeat loci (Hall et al. 2002; Volpe et al. 2002).

Interestingly, both TGS and PTGS mechanisms appear to contribute to the down-regulation of *cen* RNAs. The forward-strand transcript is primarily silenced at the transcriptional level, as shown in RNAi mutants (Volpe et al. 2002). The reverse-strand *cen* transcripts, however, are not affected by Swi6 mutants (Volpe et al. 2002), and silencing of this *cen*-reverse transcript occurs primarily at the post-transcriptional level. Moreover, the TRAMP complex, containing the Cid14 poly(A) polymerase among other proteins, is required for efficient silencing of *dg*, *dh*, and centromeric transgenes (Bühler et al. 2007). TRAMP targets RNAs for degradation by a 3–5′ exonuclease complex called the Exosome (Houseley et al. 2006). Thus, efficient silencing of heterochromatic RNAs requires RNAi-dependent and -independent degradation mechanisms, which appear to act on the chromosome in *cis* and are thus referred to as *cis*-PTGS or -CTGS (co-TGS) mechanisms (Fig. 4A,B). The Swi6 protein, which binds to methylated H3K9, plays a major role in linking heterochromatic transcription with RNA degradation by promoting the interaction of heterochromatic transcripts with the RNAi and exosome pathways (see Fig. 4, dotted lines) (Motamedi et al. 2008; Reyes-Turcu et al. 2011; Hayashi et al. 2012; Keller et al. 2012; Rougemaille et al. 2012).

RNAi also plays a role in silencing the mating-type locus (*mat2/3*) but acts redundantly with other mechanisms (Hall et al. 2002). *mat2/3* is interrupted by a region of DNA that is highly homologous to centromeric repeats (called *cenH*, for *cenHomology*; Fig. 3). Like the *cen* repeats, the *cenH* region is divergently transcribed to produce forward and reverse RNA (Noma et al. 2004). These *cenH* transcripts accumulate to high levels in RNAi mutants in combination with mutations in Pcr1 and Atf1, site-specific DNA-binding proteins that can recruit the heterochromatin machinery independently of RNAi (Jia et al. 2004).

3.1 Small RNAs Initiate Heterochromatin Assembly in Association with an RNAi Effector Complex

The discovery that the RNAi pathway is involved in heterochromatin formation in fission yeast and TGS in other systems raised the question of how it could directly regulate chromatin structure. Purification of Chp1, a chromodomain protein that is a structural component of heterochromatin, led to the identification of the RITS complex (Verdel et al. 2004). RITS contains the fission yeast Ago1 protein and the Tas3 GW domain protein, in addition to Chp1. It also contains centromeric siRNAs, which are produced by the Dicer ribonuclease. Importantly, RITS associates with centromeric repeat regions in an siRNA-dependent fashion. RITS has therefore been proposed to use centromeric siRNAs to target specific chromosome regions for inactivation, and this provides a direct link between RNAi and heterochromatin assembly (Fig. 4A). The fission yeast Ago1 is present in a second complex called the Argonaute chaperone (ARC) complex, which may function in the specific delivery of duplex siRNAs to Ago1 before it is exchanged into the RITS complex (Fig. 4A) (Buker et al. 2007).

RITS uses siRNAs for target recognition, but unlike RISC, which mediates PTGS via mRNA inactivation, it associates with chromatin and initiates heterochromatin formation. How can siRNAs target specific chromosome regions? Two possible mechanisms have been proposed. In the first model, siRNAs bound to Ago1 in the RITS complex somehow base-pairs with an unwound DNA double helix. In the second model, RITS-associated siRNAs base-pair with noncoding RNA transcripts at the target locus (Fig. 4A). As discussed below (Sec. 3.3), the available evidence strongly supports the second model.

According to either model, the association of RITS with chromatin via siRNA results in the recruitment of the Ctr4 KMT, with subsequent methylation of histone H3K9. However, Ctr4 is also required for the association of RITS with chromatin, suggesting that it provides methylated H3K9 to which the RITS complex can bind, thereby stabilizing its association with chromatin (Fig. 4A). The chromodomain of Chp1 was already known to bind specifically to methylated H3K9 residues (Partridge et al. 2002), and mutations in Ctr4 or the chromodomain of Chp1 that are involved in this interaction result in a loss of RITS binding to chromatin (Partridge et al. 2002; Noma et al. 2004). Moreover, RITS can also bind to chromatin domains that are coated with methylated H3K9 through the chromodomain of Chp1 at the *mat2/3* and telomeric regions in the absence of siRNAs (Noma et al. 2004; Petrie et al. 2005). In summary, the RITS complex shows affinity to chromatin via Chp1 binding to methylated H3K9 and through base-pairing of siRNA with either DNA or RNA transcripts. This

Figure 4. RNAi-mediated cotranscriptional assembly of heterochromatin in *S. pombe*. Transcription of pericentric repeats gives rise to long noncoding RNAs that are processed into primary small RNAs by Dicer-dependent and -independent pathways. (A) A small RNA loaded onto the RITS complex targets the nascent noncoding RNA by base-pairing interactions. This leads to the recruitment of the RDRC (RNA-directed RNA polymerase complex) and conversion of the targeted RNA into dsRNA, which is diced into siRNAs by Dicer. The resulting duplex siRNA is loaded onto the Argonaute chaperone (ARC) complex and converted into single-stranded siRNA after cleavage and release of the passenger strand in the RITS complex. The mature RITS complex containing single-stranded siRNA can now target additional noncoding RNAs completing a positive feedback loop. The RITS complex also recruits the CLRC H3K9 methyltransferase complex to chromatin via interactions with the Rif1 subunit of CLRC and Stc1, an adaptor protein. (B) H3K9 methylation stabilizes the association of RITS with chromatin and also provides binding sites for HP1 proteins (Swi6 and Chp2). Swi6 facilitates the recruitment of RDRC and degradation by the exosome (C), whereas Chp2 recruits the SHREC complex containing the Clr3 HDAC promotes TGS by mechanisms that remain to be defined (D). In addition to TGS, efficient silencing requires cotranscriptional RNA degradation (CTGS) by RNAi-dependent (A, dicing and slicing) and RNAi-independent (C, TRAMP/exosome degradation) mechanisms. Dicer-independent priRNAs contribute to low levels of H3K9 methylation (E) and may trigger siRNA amplification (A). The 3' ends of priRNAs and siRNAs are trimmed by the Triman exonuclease (A,E). Black tapered arrows indicate enzymatic activity.

dual mode of RITS recruitment may provide an explanation for the epigenetic inheritance of heterochromatin by promoting the preferential establishment of heterochromatin at regions that inherit H3K9 methylation during the replication of chromatin (Moazed 2011).

The next step in the assembly of fully silenced heterochromatic domains involves the recruitment of HP1 proteins, Swi6 and Chp2, which associate the methylated H3K9. Swi6 appears to function mainly by stabilizing the RNAi and exosome complexes on the chromosome and thereby promotes efficient *cis*-RNA degradation (Fig.

4A,B) (Motamedi et al. 2008). Specifically, Swi6 interacts with an accessory factor called Ers1, which, in turn, interacts with the RDRC complex to promote dsRNA synthesis and RNA degradation (Hayashi et al. 2012; Rougemaille et al. 2012). In addition, Swi6 has nonspecific affinity for RNA and may help retain heterochromatin-transcribed RNAs on the chromosome until they are degraded by the exosome (Keller et al. 2012). Chp2, on the other hand, plays a critical role in recruitment of the SHREC histone deacetylase complex (Motamedi et al. 2008; Fischer et al. 2009). Deacetylation of histone H3 lysine 14 (H3K14) by the Clr3

subunit of SHREC is critical for shutting down transcription from the repeats. RNAi-mediated silencing acts in parallel to Ctr3 to release Pol II from the centromeric repeats during S phase, preventing it from clashing with replicating DNA polymerase, which would lead to stalling of replication forks in centromeric repeats (Li et al. 2011; Zaratiegui et al. 2011). The consequent requirement for DNA repair in RNAi mutants has been proposed to underlie spreading of heterochromatic modifications along with the replication fork, as described below.

Recent evidence strongly suggests that RITS and siRNAs can initiate de novo heterochromatin assembly. Bühler et al. used a site-specific RNA-binding protein to artificially tether the RITS complex to the RNA transcript of the normally active *ura4⁺* gene (Bühler et al. 2006). Remarkably, this tethering results in the generation of *ura4⁺* siRNAs and silencing of the *ura4⁺* gene in a manner that requires both RNAi and heterochromatin components. In addition, this system allowed a direct evaluation of the ability of newly generated siRNAs to initiate H3K9 methylation and Swi6 binding, which are molecular markers for heterochromatin formation. Interestingly, the newly generated *ura4⁺* siRNAs were found to be largely restricted to the locus from which they were generated (*cis*-restricted). However, when the gene encoding Eri1, a conserved siRNA ribonuclease, is deleted, *ura4⁺* siRNAs are able to act in *trans* to silence a second copy of the *ura4⁺* gene, which is inserted on a different chromosome in the same cell. This experiment shows that tethering of RNAi to a nascent transcript mediates heterochromatin formation. In addition, it indicates that siRNAs can act as specificity factors that direct RITS and heterochromatin assembly to a previously active region of the genome.

The ability of siRNAs to initiate silencing in *S. pombe* has also been examined using a different method that relies on the expression of a hairpin RNA to produce siRNAs homologous to *ura4⁺* or a green fluorescent protein transgene (Sigova et al. 2004; Iida et al. 2008; Simmer et al. 2010). Hairpin siRNAs can promote silencing at the PTGS, or both PTGS and TGS levels, depending on the locus, suggesting that properties of the targeted locus affect the ability of siRNAs to induce heterochromatin formation. For the *ura4⁺* locus, both preexisting H3K9 methylation and antisense transcription contribute to siRNA-mediated silencing (Iida et al. 2008). Thus, although siRNAs can initiate ectopic heterochromatin formation, their ability to do so is strongly affected by properties of the targeted locus, which have not yet been fully defined.

3.2 dsRNA Synthesis and siRNA Generation

Bidirectional transcription of centromeric DNA repeats could, in principle, provide the initial source of dsRNA

in fission yeast (Volpe et al. 2002). dsRNA resulting from the annealing of forward and reverse transcripts could then be a substrate for the Dicer ribonuclease. However, RNA-directed RNA polymerase (Rdp1) and its associated cofactors, as well as the Ctr4 KMT, are also required for siRNA production by Dicer (Hong et al. 2005; Li et al. 2005; Bühler et al. 2006). These observations indicate that the generation of heterochromatic siRNAs by Dicer is coupled to chromatin and Rdp1-dependent events (Fig. 4A). Moreover, recent high-throughput sequencing experiments, which are more sensitive than northern blots used in earlier experiments, have detected a class of Dicer-independent small RNAs in fission yeast, termed primal RNAs, or piRNAs (Halic and Moazed 2010). These small RNAs have the same size and 5' nucleotide preference as Dicer-produced siRNAs and, not surprisingly, contribute to low levels of Dicer-independent H3K9 methylation at the pericentromeric repeats.

The Rdp1 enzyme resides in a multiprotein complex that also contains Hrr1, an RNA helicase, and Cid12, a member of the β family of DNA polymerases that includes poly(A) polymerase enzymes (Motamedi et al. 2004). This complex has been termed RDRC and all of its subunits are required for heterochromatin formation at centromeric DNA regions (Motamedi et al. 2004). As expected from the presence of Rdp1, RDRC has RNA-directed RNA polymerase activity *in vitro*, and mutations that abolish this activity also abolish RNAi-dependent silencing *in vivo* (Motamedi et al. 2004; Sugiyama et al. 2005). The *in vitro* RNA synthesis activity of RDRC does not require an siRNA primer (Motamedi et al. 2004). Therefore, *in vivo*, the RITS siRNA may just provide the specificity for recruiting RDRC to specific RNA templates. Consistent with this hypothesis, subunits of the RDRC complex are required for siRNA amplification, and RITS complexes purified from cells that lack any subunit of the RDRC complex contain only trace levels of small RNA (Motamedi et al. 2004; Li et al. 2005; Sugiyama et al. 2005; Bühler et al. 2006; Halic and Moazed 2010).

The presence of Cid12 in the RDRC complex is intriguing and raises the possibility that another polymerase activity participates in chromosome-associated RNA silencing. As some members of this family have poly(A) polymerase activity, one possibility is that adenylation may be important for either Rdp1-dependent dsRNA synthesis or further processing of Rdp1-produced dsRNA. The fission yeast Cid12 has monoadenylation activity and can adenylate template RNAs that are targeted by Rdp1, suggesting that it functions upstream of dsRNA synthesis (Halic and Moazed 2010). Interestingly, Cid12-like proteins are conserved throughout eukaryotes and appear to target small RNAs for exosome-mediated degradation (Table 1; Fig. 4A,B). In *C. elegans*, mutations in *rde-3*, a

member of this family, results in defective RNAi (Chen et al. 2005), corroborating a conserved role for these enzymes in the RNAi pathway.

There is evidence for dsRNA synthesis and processing associated with the generation of heterochromatic siRNAs occurring on the chromosome at sites of transcription of noncoding centromeric RNAs (Fig. 4). Evidence includes, first, that Rdp1 can be cross-linked to centromeric DNA repeats (Volpe et al. 2002; Sugiyama et al. 2005), and to the forward and reverse RNA transcripts that originate from *cen* regions (Motamedi et al. 2004). Cross-linking to centromeric RNAs requires Dicer and Clr4, and is therefore siRNA- and chromatin-dependent. Second, siRNA generation requires chromatin components, including Clr4, Swi6, and the HDAC Sir2 (Hong et al. 2005; Li et al. 2005; Bühler et al. 2006). Finally, the association of RDRC with RITS is dependent on siRNAs as well as Clr4 suggesting that it occurs on chromatin (Motamedi et al. 2004), although Clr4 also has nonhistone H3 targets that are critical for efficient siRNA generation (Gerace et al. 2010). Thus, the generation of dsRNA and heterochromatic siRNAs may involve the recruitment of RDRC to chromatin-associated nascent pre-mRNA transcripts (Martienssen et al. 2005; Verdel and Moazed 2005; Moazed 2011). The fact that transcription and siRNA generation are likely to occur simultaneously reinforces the difference between RNA-silencing mechanisms that mediate chromatin modifications and PTGS. However, this distinction is unlikely to be absolute. For example, in *C. elegans*, similar to *S. pombe*, mutations in several chromatin components result in defects in RNAi and transposon-induced RNA silencing (Sijen and Plasterk 2003; Grishok et al. 2005; Kim et al. 2005; see Table 1), raising the possibility that in some cases dsRNA synthesis and processing may occur on the chromosome regardless of whether silencing occurs at the TGS or PTGS level.

3.3 RNA–RNA versus RNA–DNA Recognition Models

As we discussed earlier, RITS and other nuclear Argonaute complexes are targeted to specific chromosomal regions by siRNA-mediated base-pairing interactions. Studies in *S. pombe* have provided support for base-pairing interactions between siRNAs and nascent RNA transcripts, giving rise to the general consensus that nuclear Argonaute complexes associate with chromosomes via siRNA-mediated base-pairing with nascent transcripts. The observation that tethering components of the RNAi machinery to the RNA transcript of a gene can initiate RNAi and heterochromatin-dependent gene silencing that is *cis*-restricted clearly shows that the process can initiate via initial interactions

with nascent RNA transcripts (Bühler et al. 2006). Importantly, *cis*-restriction rules out the possibility that the initial events of dsRNA synthesis and siRNA generation occur on mature transcripts in which mRNA products from different alleles cannot be distinguished. Furthermore, a direct prediction of the RNA–RNA interaction model is that transcription at the target locus should be required for RNAi-mediated heterochromatin assembly. Although the requirement for transcription has not been directly tested, mutations in two different subunits of RNA Pol II, denoted Rpb2 and Rpb7, have specific defects in siRNA generation and heterochromatin assembly, but not on general transcription (Djupedal et al. 2005; Kato et al. 2005). This is reminiscent of Rbp1 mutants, which have defects in certain active histone modifications (i.e., H3K4 methylation and H2B ubiquitination), coupling it to the process of transcriptional elongation (Hampsey and Reinberg 2003). The Rbp1 paradigm provides a precedent for the hypothesis that RNAi-mediated H3K9 methylation and heterochromatin formation could be coupled to transcriptional elongation via the association of RNAi complexes with RNA Pol II. A notable development is the demonstration that transcription of the centromeric repeat regions and siRNA generation occur largely within a restricted window during S phase of the cell cycle (Chen et al. 2008; Kloc et al. 2008). Thus, both nascent transcripts and high levels of siRNA are present during the process of chromosome duplication when heterochromatin needs to be reestablished, ensuring heterochromatin maintenance (Fig. 4).

The RNA–RNA targeting model is also supported by the observation that components of both the RITS and RDRC complexes can be localized to noncoding centromeric RNAs using *in vivo* cross-linking experiments (Motamedi et al. 2004). This localization is siRNA-dependent, which suggests that it involves base-pairing interactions with the noncoding RNA. In addition, it requires the Clr4 KMT, suggesting that it is coupled to binding of RITS to methylated H3K9 and occurs on chromatin. Nonetheless, the possibility that siRNAs can also recognize DNA directly through base-pairing interactions cannot be ruled out. For example, in plants, siRNAs that are complementary to promoter regions that are (presumably) not transcribed still direct DNA methylation, another modification that takes place during heterochromatin formation within these regions (see Pikaard and Mittelsten Scheid 2014).

3.4 How Does RNAi Recruit Chromatin-Modifying Enzymes?

The recruitment of Clr4 is a key step in initiating histone H3K9 methylation and heterochromatin assembly.

However, because RITS association with chromatin and Clr4-catalyzed histone H3K9 methylation are codependent processes, it has been difficult to determine the event that provides the initial trigger for RNAi-dependent heterochromatin assembly. Furthermore, low levels of H3K9 methylation are present at pericentromeric repeats in RNAi mutant cells, suggesting that other mechanisms also contribute to Clr4 recruitment. Nonetheless, as we discussed above, *de novo* generation of siRNAs, either by tethering RITS to RNA or from transcription of a long hairpin RNA, promotes silencing of a previously active copy of the *ura4⁺* gene, and that is coupled to H3K9 methylation and the recruitment of RITS and Swi6 to chromatin (Bühler et al. 2006; Iida et al. 2008; Simmer et al. 2010). These observations clearly suggest that siRNAs are capable of recruiting Clr4. However, the sensitivity of this capability to properties at the targeted locus suggests that unidentified factors may work together with an siRNA-programmed RITS to initiate H3K9 methylation.

Clr4 is a component of a multiprotein complex called CLRC, which contains the heterochromatin protein, Rik1, a Cullin E3 ubiquitin ligase, Cul4, and two variously named associated proteins, Raf1/Clr7 and Raf2/Clr8, which encodes a β propeller protein (Hong et al. 2005; Horn et al. 2005; Jia et al. 2005; Li et al. 2005). Consistent with a direct role for RNAi in recruitment of Clr4, subunits of the RITS and RDRC complex physically associate with the Clr4 complex (Bayne et al. 2010; Gerace et al. 2010). Interestingly, the efficient interaction of RITS/RDRC with Clr4 requires an adaptor protein called Stc1 and is diminished in cells that harbor a catalytically dead Dicer enzyme, and therefore lack siRNAs (Bayne et al. 2010; Gerace et al. 2010). These observations suggest that the interaction of these complexes may be stabilized by accessory proteins and siRNA-mediated mechanisms. In this regard, the Rik1 subunit of CLRC is a member of a large family of β propeller WD repeat proteins that have been implicated in RNA or DNA binding. Members of this protein family include the cleavage polyadenylation specificity factor A (CPSF-A) involved in pre-mRNA splicing, and the DNA damage binding 1 (Ddb1) protein involved in binding UV-damaged DNA. CPSF-A is of particular interest because Rik1 shares sequence similarity with its putative RNA-binding domain involved in the recognition of mRNA polyadenylation sequences (Barabino et al. 2000). The Ddb1 protein, like Rik1, is a component of a Cul4 E3 ubiquitin ligase complex and is involved in the recognition and repair of UV-damaged DNA (Higa et al. 2003; Zhong et al. 2003). An exciting possibility is that Rik1 acts in a fashion that is similar to CPSF-A and Ddb1, binding to an RNAi-generated product during heterochromatin assembly. Once recruited, the Rik1 complex is associated with the leading strand DNA

polymerase, providing a mechanism for spreading of histone modification along with the replication fork. Because the repeats are transcribed during S phase, fork progression requires Pol II release by a mechanism that requires RNAi (Li et al. 2011; Zaratiegui et al. 2011). Failure to release Pol II invokes homologous recombination (HR) repair.

Studies in fission yeast have made enormous contributions to our understanding of RNAi-mediated heterochromatin formation, but important questions remain unanswered. Although progress has been made in defining the Dicer-dependent and -independent pathways of small RNA generation, the determinants that distinguish centromeric noncoding RNAs from other genomic transcripts as sources of abundant siRNAs as well as chromosome-bound scaffolds for siRNA-mediated heterochromatin formation remain to be determined. Furthermore, much remains to be learned about how RNAi contributes to the maintenance of genomic stability through the coordination of transcription, replication, and DNA repair within centromeric DNA repeats.

4 RNAI-MEDIATED CHROMATIN AND DNA MODIFICATIONS IN *ARABIDOPSIS*

The mechanism by which RNAi guides heterochromatic modifications in plants resembles the mechanism in fission yeast, but there are also many differences. The most important difference is that plants have methylated DNA at most heterochromatin regions, resembling vertebrates in this respect, but differing from *S. pombe*, worms, and *Drosophila* (Fig. 5). Genetic screens for mutants that relieve RNA-mediated TGS have recovered mutants in H3K9-specific KMTs and RNAi components, but they have also uncovered the requirement for DNA methyltransferases, SWI/SNF remodeling complexes, and two novel RNA polymerases in heterochromatin formation. The genes are listed in Table 1 of Pikaard and Mittelsten Scheid (2014) and the findings from these screens are described in more detail in the article, but here we will briefly compare the mechanism in fission yeast and plants.

Many of the silencing mutant screens in *Arabidopsis* have used inverted repeats introduced in *trans* to induce the silencing of endogenous or transgenic reporter genes, driven by tissue-specific promoters or promoters that responded to epigenetic signals. In each case, the promoter was targeted for silencing through a TGS pathway involving local chromatin changes at the promoter. Genes of note in the RNAi and TGS *Arabidopsis* pathways (Fig. 5) include DNA methyltransferases, particularly those related to the mammalian DNMT3 encoded by *DRM1* and *DRM2*. Also, there were components of the RNAi apparatus responsible for biogenesis and usage of 24-nt siRNA, such as *DICER-*

Figure 5. RNAi-mediated histone and DNA methylation in *Arabidopsis*. A summary of RNAi and chromatin proteins required for RNAi-mediated DNA and histone methylation in *Arabidopsis* are indicated. Synthesis of dsRNA from repeated DNA elements provides a substrate for Dicer-mediated cleavage and siRNA generation (DCL3 and other Dicers). RNA-directed RNA polymerases (RdRP, RdR2) and RNA polymerase IV (RNA Pol IV) may be directly involved in the synthesis of dsRNA or its amplification. siRNAs then load onto Argonaute proteins (e.g., AGO4), which is likely to help target cognate repeat sequences for DNA methylation (pink hexagons catalyzed by pink DNA methyltransferase enzymes) and H3K9 methylation (red hexagons via the red HKMT enzyme) in association with other factors, including chromatin remodeling proteins (pale green) and HDAC enzymes (cyan). See Pikaard and Mittelsten Scheid (2014) for more detail.

LIKE 3 (*DCL3*) and *ARGONAUTE 4* (*AGO4*). Yet, other mutants included those for the H3K9 methyltransferase genes *KYP/SUVH4*, *SUVH5* and *SUVH6*, and the chromodomain-containing DNA methyltransferase gene *CMT3*. Apart from the DNA methyltransferase genes, the parallels with fission yeast are striking, namely, that the *S. pombe* RITS complex contains both an Argonaute protein and the chromodomain protein Chp1, which depends on H3K9 methylation for its association with the chromosome. Unlike fission yeast, however, loss of heterochromatin-associated proteins that mediate TGS, like *CMT3* or loss of the H3K9me2 mark does not result in loss of siRNA in *Arabidopsis* (Lippman et al. 2003), and it is not yet clear if these

DNA/chromatin-modifying proteins form a complex with AGO4.

It turns out there is considerable redundancy in the proteins involved in *Arabidopsis* RNAi and TGS pathways, making it harder to dissect the detailed mechanisms. Furthermore, the pathway used to achieve heterochromatin formation depends on the underlying sequence—whether it is a transposon, retrotransposon, or tandem repeat—resulting in both RNAi and RNAi-independent mechanisms of silencing. Among the known players, *SUVH4*, -5, and -6, for example, all contain an SRA domain that binds methylated DNA, whereas DNA methyltransferase *CMT3* has a chromodomain and a BAH domain that

each recognize K9me2 (see Pikaard and Mittelsten Scheid 2014). We know that, in some cases, DNA methylation and H3K9me2 in heterochromatic regions act to reinforce each other, bypassing the role of RNAi in heterochromatin maintenance. Interestingly, mutants in DNA polymerase epsilon and cullin 4, components of the Ctr4-containing CLRC complex in *S. pombe*, relieve silencing of some RNAi-sensitive transgenes in *Arabidopsis*, implicating that it is a cell-cycle-restricted process in plants as well (Yin et al. 2009; del Olmo et al. 2010; Dumbliuskas et al. 2011; Pazhouhandeh et al. 2011). Furthermore, some of these mutants have elevated levels of HR repair, and Ago2 itself has been implicated in HR repair of double-strand breaks (Wei et al. 2012), reminiscent of the role of DNA replication and repair in heterochromatic silencing and spreading in *S. pombe* (Li et al. 2011; Zaratiegui et al. 2011). Thus, at least some aspects of the mechanism responsible for spreading of the Rik1/CLRC complex are conserved between fission yeast and *Arabidopsis*.

Transposons in *Arabidopsis* are a major source of 24-nt siRNA, unlike in *S. pombe*. However, *Arabidopsis* centromeric 180-bp satellite repeats, which are arranged in tens of thousands of tandem copies on either side of Athila LTR retroelements, are also transcribed and processed by RNAi as in fission yeast (Fig. 3). This processing depends on chromatin remodeling by the SWI2/SNF2 ATPase DDM1, dsRNA production from Pol IV transcripts by RDR2, and dicing by DCL3 (Fig. 5). Silencing also depends on H3K9me2 and the associated DNA methyltransferase CMT3. But silencing is more complex than in *S. pombe* as retrotransposon insertions into the repeats can silence the adjacent repeats, and this depends on other mechanisms including MET1, DDM1, and the histone deacetylase HDA6 (May et al. 2005). Interestingly, centromeres of the fission yeast *Styrax japonicus* also have multiple retrotransposon insertions, which generate siRNA-resembling plant pericentromeric regions in this respect (Rhind et al. 2011). DDM1 has an exquisite specificity for transposons and repeats, and must somehow recognize these as being different from genes, although the mechanism remains unclear. Loss of DNA methylation in *met1* and *ddm1* mutants is epigenetically inherited in crosses to wild-type plants, and only those transposons that retain siRNA can be remethylated (Teixeira et al. 2009). Thus, as in *S. pombe*, RNAi has a crucial role in the initiation of silencing in *Arabidopsis*.

As mentioned earlier, in fission yeast, subunits of RNA Pol II are required for silencing and siRNA production, supporting the idea that the RNAi and chromatin modification apparatuses are recruited to the chromosome by nascent transcripts (Fig. 4). In *Arabidopsis*, two novel RNA polymerases (Pol IV and Pol V) were recovered in some of

the screens mentioned above. It is not yet known what template is used by Pol IV, but Pol V is responsible for intergenic transcription of repeats and recruits AGO4 via GW repeats in the carboxy-terminal domain and in accessory elongation factors. Only the largest subunits are unique to Pol IV and Pol V, which use many of the same small subunits as Pol II. Additional SWI2/SNF2 chromatin remodelers, namely, CLSY2, were recovered in these screens and they may alter local chromatin structure to facilitate processivity of RNA polymerases. It is therefore likely that they facilitate transcription by Pol IV (more detail can be found in Pikaard and Mittelsten Scheid 2014). A similar role can be proposed for DDM1, although the requirement for DDM1 (also a chromatin remodeler) in silencing transposons is far more severe than that of Pol IV or the other SWI2/SNF2 proteins.

Thus, the role of RNAi-mediated heterochromatic silencing in plants is well documented, but more complicated than in *S. pombe*, largely because of DNA methylation. Parallels with fission yeast may become clearer in mutants that have lost RNAi-independent DNA methylation, such as *ddm1* (Teixeira et al. 2009) or in the germline. For example, in pollen grains, 24-nt siRNAs guide RNA-dependent DNA methylation independently of DDM1 (Calarco et al. 2012), whereas 21-nt siRNA are responsible for transposon silencing in sperm cells (Slotkin et al. 2009). Plants do not possess the piRNA pathway found in animal germlines (discussed in Secs. 1 and 5), and parallels with fission yeast may be more prominent as a result.

5 CONSERVATION OF RNAi-MEDIATED CHROMATIN MODIFICATIONS IN ANIMALS

Perhaps the most widely studied examples of epigenetic silencing are found in animals, including *Drosophila* and *C. elegans*, as well as in the mouse. The role of RNA and RNA interference in transcriptional silencing and heterochromatic modifications appears to be conserved in some model animals as well as in protists and plants. In *Drosophila*, both PIWI and the PIWI class Argonaute homolog, Aubergine (Sting), participate in epigenetic and heterochromatic silencing (Kawamura et al. 2008; Khurana et al. 2010; see also Elgin and Reuter 2013). *Gypsy* retrotransposons are the target of silencing in ovary follicle cells and female gonads by PIWI and its associated piRNAs (Sarot et al. 2004). This is mediated by heterochromatic noncoding RNAs encoded by Flamenco, which give rise to the piRNAs that target complementary transposons for silencing. Cut-and-paste DNA transposons are also affected by RNAi. For example, certain telomeric P elements can suppress P activity elsewhere in the genome when inherited through the female germline, resulting in a strongly

repressive “cytotype.” This repression is completely dependent on the PIWI homolog, aubergine, as well as the Swi6 homolog HP1, for heterochromatin protein 1 (Reiss et al. 2004). However, not all P repressive cytotypes, such as those mediated by other nontelomeric P elements, are dependent on aubergine or HP1.

Unlinked transgenes in *Drosophila* are silenced post-transcriptionally when present in many copies (Pal-Bhadra et al. 1997, 2002). Silencing is associated with large amounts of 21-nt siRNA and depends on PIWI. Transgene fusions can also silence each other transcriptionally in a manner that requires the Polycomb chromatin repressor. This silencing is not associated with increased levels of siRNA from the transgene transcript, but is (largely) dependent on PIWI. Involvement of Polycomb in this example, and HP-1 in other examples of PIWI-dependent silencing, implicates the RNAi pathway and histone methylation in the silencing process. Tandem transgene arrays also show position-effect variegation in *Drosophila*, and this variegation is strongly suppressed by mutants in HP1 as well as in piwi, aubergine, and the putative RNA helicase Spindle-E (homeless; Pal-Bhadra et al. 2004). Transgenes inserted within centric heterochromatin are also affected, and heterochromatic levels of H3K9me2 are reduced in spindle-E mutant cells. These observations support a role for both chromatin proteins and components of the RNAi pathway in gene silencing within *Drosophila* heterochromatin.

In the *Drosophila* male germline, the heterochromatic suppressor of stellate repeats (Su(ste)), located on the Y chromosome, are transcribed first on the antisense strand and then on both strands during spermatocyte development, possibly following the insertion of a nearby transposon (Aravin et al. 2001). These nuclear transcripts are required to silence sense transcripts of the closely related X-linked *Stellate* gene, whose overexpression results in defects in spermatogenesis. Although heterochromatic sequences are involved, silencing in this case appears to be posttranscriptional, is associated with 25–27-nt piRNA, and depends on both *aubergine* and *Spindle-E*.

In *C. elegans*, examples of TGS in somatic cells have been reported. This depends on the RNAi pathway genes *rde-1*, *dcr-1*, *rde-4*, and *rrf-1*, a nuclear Argonaute and its associated factors, as well as HP1 homologs and the histone modification apparatus (Grishok et al. 2005; Guang et al. 2010; Buckley et al. 2012). An example of naturally occurring RNAi-dependent heterochromatic silencing has also been described in the germline (Sijen and Plasterk 2003). During meiosis, unpaired sequences, such as the X chromosome in males, are silenced via H3K9me2, and this silencing depends on RNA-dependent RNA polymerase (Maine et al. 2005; see Strome et al. 2014), reminiscent of

meiotic silencing of unpaired DNA in *Neurospora* (MSUD; Shiu et al. 2001; see Aramayo and Selker 2013). It is possible therefore that the RNAi-dependent heterochromatin silencing pathway found in fission yeast is preserved in meiosis in higher organisms.

Finally, like *Drosophila*, mammalian cells lack genes related to RNA-dependent RNA polymerases found in plants, worms, and fungi. Nonetheless, antisense RNA has been implicated in the most widely studied epigenetic phenomena of all, imprinting and X inactivation (see Barlow and Bartolomei 2014 and Brockdorff and Turner 2014, respectively). In the case of X inactivation, a 17-kb spliced and polyadenylated noncoding RNA known as Xist is required to silence the inactive X chromosome from which it is expressed. Conversely, *Xist* itself is silenced on the active X chromosome, a process that depends in part on the antisense RNA *Tsix*. Silencing is accompanied by modification of histones associated with upstream chromatin regions, which are marked with H3K9me2 and H3K27me3 (Heard et al. 2001), and depends on the H3K27 methyltransferase Polycomb group gene *Eed*. Silencing of other imprinted loci in the mouse, including *Igf2r* and the *Dlk1-Gtl2* region, is also maintained by antisense transcripts from the paternal and maternal allele, respectively. In the case of *Dlk1-Gtl2*, this noncoding RNA is specifically processed into miRNA that targets the antisense transcript from the paternal allele, encoding a sushi (gypsy) class retrotransposon (Davis et al. 2005). Perhaps the best example of RNAi-dependent DNA methylation in mammalian imprinting is at the *Rasgrf1* locus (Watanabe et al. 2011). At this locus, a long noncoding RNA is targeted by piRNA that match an embedded retrotransposon LTR. piRNA guides DNA methylation of the differentially methylated region required for silencing, so that imprinting in spermatocytes comes under the control of RNAi (Watanabe et al. 2011). In this case, clear parallels can be drawn with heterochromatic silencing in *S. pombe*, which also depends on the cleavage and targeting activities of Ago1 (Irvine et al. 2006; Buker et al. 2007), and with RNA-dependent DNA methylation in plants, which depends on catalytic activity of AGO4 (Qi et al. 2006).

6 CONCLUDING REMARKS

The possibility that genes may be regulated by small RNA molecules was suggested more than 40 years ago (Jacob and Monod 1961). An equally important hypothetical notion was that regulatory RNAs might be related to repeats (Britten and Davidson 1969). Since the identification of the λ and lac repressors as site-specific DNA-binding proteins in *Escherichia coli* or its infecting bacteriophage λ (Gilbert and Muller-Hill 1966; Ptashne 1967), studies of gene regu-

lation have focused almost exclusively on the role of nucleic acid-binding proteins as specificity factors. The discovery of small RNA molecules as specificity agents in diverse RNA-silencing mechanisms now clearly establishes a role for RNA as a sequence-specific regulator of genes and their RNA products. Studies in fission yeast, *Arabidopsis*, and other model organisms have revealed a surprisingly direct role for small RNAs in mediating epigenetic modifications of the genome that direct gene silencing and contribute to heterochromatic domains necessary for genome stability and nuclear division. Many important mechanistic questions remain at large and future studies are likely to provide more surprises about how RNA regulates gene expression.

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