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RITS- connecting transcription, RNAi and heterochromatin assembly in Fission Yeast

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Abstract

In recent years a bevy of evidence has been unearthed indicating that 'silent' heterochromatin is not as transcriptionally inert as once thought. In the unicellular yeast *Schizosaccharomyces pombe*, processing of transcripts derived from centromeric repeats into homologous small interfering RNA (siRNA) is essential for the formation of centromeric heterochromatin. Deletion of genes required for siRNA biogenesis revealed that core components of the canonical RNAi pathway are essential for centromeric heterochromatin assembly as well as for centromere function. Subsequent purification of the RITS (RNA-induced initiation of transcriptional gene silencing) complex provided the critical link between siRNAs and heterochromatin assembly, with RITS acting as a physical bridge between non-coding RNA scaffolds and chromatin. Here, we review current understanding of how RITS promotes heterochromatin formation and how it participates in transcription coupled silencing.

TRANSCRIPTION COUPLED SILENCING

Eukaryotic chromosomes consist of cytologically distinct chromatin domains, with euchromatin having an open chromatin structure, and heterochromatin defined as more heavily condensed regions of chromatin. Heterochromatic domains tend to be less transcriptionally active than euchromatin, and often replicate later and are localized at the nuclear periphery. Usually, heterochromatic regions are gene-poor, and genes that become translocated within these domains are transcriptionally silenced.

An intriguing paradox is that studies in several organisms have revealed a widespread requirement for transcription of sequences that are destined to become heterochromatic (reviewed in¹⁻⁴). A classic example of such transcription coupled silencing occurs during X chromosome inactivation in female mammals. At the onset of X chromosome inactivation, the long non-coding RNA Xist is transcriptionally upregulated on one X chromosome, and then spreads in *cis* to coat and silence that chromosome^{5,6}. Perhaps the most extreme example of transcription coupled silencing occurs during programmed DNA elimination in ciliates such as *Tetrahymena*, where transcription of sequences in the micronucleus promotes heterochromatin formation and subsequent removal of homologous sequences within the developing macronucleus (reviewed in⁷). Silencing can involve recruitment of chromatin modifying complexes to particular loci by long non coding RNAs, or short RNAs

that are generated by the RNAi pathway can recruit chromatin modifiers or endonuclease activity to homologous sequences. Transcription coupled silencing can act in *cis* to silence linked sequences, in *trans* on another chromosome, or even influence chromatin in a different nucleus.

In this review we will focus on the current knowledge of one particular type of transcription coupled silencing- that of the assembly of heterochromatin in fission yeast- which relies on the RNAi pathway, and in particular on a complex of proteins called RITS or RNA- induced initiation of transcriptional gene silencing complex.

HETEROCHROMATIN

Heterochromatin and euchromatin are made up of the same basic unit of chromatin, the nucleosome, comprising an octamer of 4 core histones which are wrapped by 147 bp of DNA⁸. Histones are highly conserved basic proteins, and their N terminal tails extend out from the surface of the nucleosome. Differences in heterochromatin and euchromatin can be linked to post-translational modification of histone tails that can change the degree of compaction of the chromatin and “mark” them to provide binding surfaces for additional chromatin proteins⁹. Several different types of post-translational modification can occur, and at multiple positions on the histone tails, leading to the idea of a complex regulatory “histone code”¹⁰. In general, histone acetylation correlates with transcriptional activity in euchromatic regions, whereas histone methylation can correlate with active or inactive states¹¹. Heterochromatin is typified by the presence of methylation on histone H3 lysine (K) 9, histone H3K27 and on histone H4K20, and a paucity of H3K4 methylation. This contrasts with enrichment for histone H3K4 methylation in transcriptionally active euchromatic domains.

A hallmark of heterochromatin in most eukaryotes is the presence of methylation on histone H3 lysine 9 (H3K9me)¹². This mark correlates with the assembly of heterochromatin, and serves as an acceptor for binding of chromodomain proteins, such as Heterochromatin Protein 1 (HP1)^{13,14}. In metazoans, several families of histone methyltransferases can specifically methylate H3K9. Trimethylation of H3K9 (H3K9me3) is catalyzed by the Suv39 family of enzymes in pericentric heterochromatin, while G9a family members catalyze H3K9me1 and me2 in regions of euchromatin that are subject to silencing^{15,16}. Until recently, H3K9me was thought to be restricted to heterochromatin, but genome wide chromatin immunoprecipitation (ChIP) analyses have revealed H3K9me2, me3 and HP1 protein in regions of actively transcribed euchromatin in metazoans, correlating with transcriptional elongation by RNA polymerase II¹⁷⁻¹⁹.

HETEROCHROMATIN IN FISSION YEAST

The *S. pombe* genome possesses three major regions of constitutive heterochromatin, which assembles at the pericentromeres, telomeres, and mating-type locus²⁰. These regions share some DNA sequence similarity, notably the repetitive *dg* and *dh* elements which are particularly enriched in pericentromeric regions (see Figure 1)²¹⁻²³. Assembly of these domains into heterochromatin is critical for maintenance of genomic stability, and chromatin of these domains is methylated on histone H3K9²⁴⁻²⁶. Fission yeast lack the enzymatic machinery responsible for H3K27 methylation and lack DNA methylation²⁸. Additionally, H4K20 methylation, although present in fission yeast, does not correlate with centromeric heterochromatin or gene silencing, but instead with DNA damage²⁷.

H3 K9 methylation is catalyzed by a single methyltransferase, Clr4. Clr4 is a homolog of Suv39, and is responsible for mono, di and tri-methylation of H3K9 in fission yeast^{12,29}. Clr4 is a component of a multi-subunit complex called Clr-C consisting of additionally

Rik1, Pcu4 (Cul4), Raf1 (Cmc1, Dos1, Clr8) and Raf2 (Cmc2, Dos2, Clr7)^{25,30-34}. These proteins, together with a ring box protein, Rbx1, are components of a cullin 4-dependent E3 ubiquitin ligase^{30,32}. Although each component is necessary for heterochromatin assembly, their exact role in directing E3 ligase activity and promoting Clr4 function is presently unclear. However, catalytic activity of Pcu4, the cullin 4 protein that acts as a scaffold for assembly of ubiquitin ligases, is necessary for assembly of heterochromatin³². Rik1 is likely a nucleic acid binding protein since it shares homology with the UV DNA damage binding protein, and with CPSF-A, a subunit of the polyA polymerase complex³⁵. Rik1 may help target the complex to chromatin, since it is localized to sites of heterochromatin even in mutant backgrounds such as *pcu4*, in which Clr4 localization is lost^{32,36}. All components of Clr-C are required for heterochromatin assembly, and mutants deficient for these proteins completely lack H3K9me on centromeric repeats and at other normally heterochromatic loci. The target of Clr-C's ubiquitin ligase activity is not known, although Clr-C can ubiquitinate histone H2B *in vitro*³⁰. In addition to its H3K9 target, Clr4 may methylate other proteins, since yeast bearing histone H3 mutants that block K9 methylation exhibit less severe phenotypes than *clr4* mutants that are deficient in methyltransferase activity³⁷.

The H3K9me mark in fission yeast is bound by the chromodomain-containing proteins Swi6 and Chp2 (homologs of HP1), Chp1, and even by the Clr4 enzyme itself^{14,24,36,38,39}. The latter provides an example of an amplification switch where the protein that “reads” a chromatin mark can then “write” the mark on adjacent nucleosomes. Assembly of Swi6-containing heterochromatin is critical for centromere function in fission yeast and prevents the formation of aneuploid cells⁴⁰. Swi6 physically recruits cohesin to the outer repeats of the centromere^{41,42} which holds the sister chromatids together until satisfaction of the spindle assembly checkpoints and anaphase onset⁴³. Recruitment of cohesin appears to be the major role of Swi6 at centromeres, since artificially tethering cohesin to centromeres bypasses the requirement for Swi6 in centromere function during mitosis⁴⁴.

A clear understanding of how the Clr-C complex is recruited to chromatin is key to deciphering the process of heterochromatin assembly. Detailed analyses of the requirements for assembly of heterochromatin have revealed that different mechanisms can contribute to Clr-C recruitment at distinct loci. For example, genetic screens have identified proteins that specifically affect the silencing of an individual heterochromatic locus through specific DNA sequences present at a given site^{22,39,45-54}. At centromeres, there is a high concentration of repetitive *dg* and *dh* sequences, which play a major role in centromeric heterochromatin assembly through the RNAi pathway (see below). Similar sequences are present at lower copy number at the mating type region and telomeres, but at these loci, heterochromatin is unaffected by loss of RNAi components since alternative DNA sequences can recruit Clr-C independently of RNAi^{23,25,50,51,53,54} (see Figure 1). Thus centromeric heterochromatin appears to be particularly sensitive to the loss of the conserved components of the cellular RNA interference (RNAi) pathway. This leads to loss of silencing and decreased enrichment of H3K9me2 and Swi6 on the repetitive sequences surrounding fission yeast centromeres, causing aberrant chromosome segregation due to centromere dysfunction^{55,56}.

THE RNAi PATHWAY IN FISSION YEAST

RNAi is well recognized as a process by which post transcriptional gene silencing (PTGS) can occur within the cytoplasm via the action of small RNAs derived from double stranded RNA (dsRNA). In several organisms, including *Tetrahymena*, *Drosophila* and plants, there is evidence that RNAi can also influence gene expression at the level of chromatin (transcriptional gene silencing)(reviewed in⁵⁷). Several conserved proteins are instrumental for the RNAi pathway, and in fission yeast, these components are encoded by single genes.

This has contributed to the development of fission yeast as an excellent model system in which to study RNAi.

Heterochromatic loci are transcriptionally active and regulated by RNAi

RNAi relies on the processing of dsRNA into short interfering RNAs (siRNAs) that are then bound by Argonaute family proteins. The finding that RNAi is critical for centromeric heterochromatin assembly led to the discovery that regions of heterochromatin are not in fact silent and refractory to access of RNA polymerases, but are transcribed at low levels as a pre-requisite for heterochromatin assembly^{56,58,59}. Thus, transcripts derived from centromeric repeats can serve as template for RNAi-mediated production of centromeric siRNAs^{26,60}. Initially, this notion seemed inconsistent with the finding that in general, only low levels of RNA polymerase associate with heterochromatic loci⁶¹. More recently, however, resolution of these differences came with the realization that heterochromatin assembly is cell cycle regulated.

Detailed studies have revealed that transcription of centromeric repeats is mediated by RNA polymerase II (RNA pol II) and occurs during S phase following the early replication of the centromeric domains^{58,59,61-63}. Transcriptional competence during this period suggests that heterochromatin normally blocks the passage of RNA pol II, and that transcription occurs when heterochromatic marks are diluted by the incorporation of “new” nucleosomes onto the newly replicated DNA strand. Consistent with this, the heterochromatin protein Swi6, which precludes centromeric transcription⁵⁶, is removed from chromosomes during mitosis and does not reassociate until G2^{61,63}. Swi6's affinity for H3K9me is reduced during mitosis by the H3S10 phosphorylation activity of the Aurora kinase, which causes loss of binding of chromodomain proteins to H3K9me^{64,65}.

Production of centromeric siRNAs correlates with centromeric transcription during S phase of the cell cycle. These siRNAs are thought to contribute to the recruitment of Clr-C to centromeric repeats during late S phase and G2^{61,63}. Thus in fission yeast, there is a cell cycle regulated component to heterochromatin formation, with transcription from centromeric repeats in S phase priming siRNA-dependent recruitment of Clr-C to reassemble H3K9me2 chromatin following its dilution by replication. It is possible that RNAi largely operates during S phase of the cell cycle, but whether heterochromatin maintenance in G2 also relies on RNAi is not known.

In mammals, there is also evidence for cell cycle regulated transcription of centromeric sequences, and that these transcripts are suppressed by the RNAi pathway^{66,67}. However, although enforced overexpression of such transcripts causes centromere dysfunction⁶⁸, there is little evidence supporting that RNAi is directly required for heterochromatin accumulation at mammalian centromeres. In contrast, accumulation of H3K9 methylation and HP1 at centromeric repetitive elements depends on several RNAi proteins in *Drosophila* including the Argonaute proteins PIWI, Aubergine and Ago2 as well as the RNA helicase Spindle-E^{69,70}. PIWI, in addition to binding small RNAs of heterochromatic origin⁷¹, physically interacts with HP1⁷².

RNAi proteins in fission yeast

Fission yeast express an RNA-dependent RNA polymerase, Rdp1. These polymerases have been shown to be important for the amplification of the RNAi response in fission yeast, worms and plants, but have not been identified in flies or mammals⁷³. Rdp1 can act in a primer independent fashion to generate dsRNA from single stranded centromeric transcripts to provide template for Dicer (Dcr1) function⁷⁴ (see Figure 2).

Dcr1 is an RNase III endonuclease which cleaves dsRNA to form siRNAs. Fission yeast Dcr1, unlike that of metazoans, has an unusual PAZ domain, lacking a conserved helix. The PAZ domain normally anchors the position of the duplex RNA relative to the active site of the enzyme⁷⁵. The variant PAZ domain in fission yeast may contribute to the size variability of fission yeast siRNAs compared with other organisms⁷⁶. Once generated, double stranded small RNA products of Dcr1 cleavage are loaded into Argonaute. In humans this loading is accomplished by the TRBP RNA binding protein which recruits Ago2 to Dicer⁷⁷. In fission yeast, ds siRNAs are loaded into the Argonaute siRNA Chaperone Complex (ARC) comprised of Argonaute (Ago1), and Argonaute-binding proteins 1 and 2 (Arb1/2)⁷⁸.

Argonaute is a key component of RNAi. Small RNAs bind to Argonaute proteins and act as guides to target Argonaute to homologous sequences. Targeting of Argonaute to perfectly homologous mRNAs can lead to sequence specific destruction of that RNA. Argonaute proteins are typified by the presence of PAZ and PIWI domains separated by the MID domain. The 3' end of the guide small RNA is bound by the PAZ domain⁷⁹⁻⁸¹ and its 5' phosphate is bound by the MID domain⁸². Some Argonaute proteins have endonucleolytic or "slicer" activity (including mammalian Ago2 and fission yeast Ago1), and in these proteins, the PIWI domain is responsible for the endonucleolytic cleavage of RNA homologous to the guide small RNA^{78,83,84}. In the fission yeast ARC complex, Arb1 suppresses the endonucleolytic activity of Ago1, thus ARC contains primarily ds siRNAs⁷⁸. Activation of Ago1 and cleavage of the passenger siRNA strand is thought to occur after passage of Ago1 from ARC to another complex called RITS⁷⁸, although the details of this hand-off are not yet clear.

Interestingly, deletion of any of these RNAi proteins causes accumulation of transcripts from the outer repeats of the centromere, reduced centromeric H3K9me and Swi6 localization and chromosome missegregation^{55,56}.

TWO KEY RNAi EFFECTOR COMPLEXES: RITS AND RDRC

RNAi pathway mutants show similar phenotypes to mutants lacking the H3K9me- binding protein, Chp1. These mutants exhibit greatly reduced Clr-C activity at centromeres, but retain heterochromatin at the mating-type locus and at telomeres^{25,39,50,56,85-87}. The similarity in phenotypes suggested that there would be a link between Chp1 and RNAi, and that link was made by biochemical purification of the RITS (RNA induced initiation of transcriptional silencing) complex - isolated by purification of Chp1. In addition to Chp1, the RITS complex consists of Ago1, a poorly characterized protein Tas3, and siRNAs derived from centromeric repeat sequences⁸⁸.

RITS is a key player in the assembly of heterochromatin. It localizes to all sites of heterochromatin and is dependent on each of its protein subunits and on the RNAi pathway for its centromeric localization^{86,88}. In addition, RITS depends on Clr-C for its recruitment to chromatin⁸⁸ (see Figure 3). Most intriguingly, centromeric recruitment of RITS is required for much of the Clr-C activity at centromeric sites^{25,39,88}. Thus there has been intense interest in deciphering how RITS is recruited to centromeres, and how RITS facilitates Clr-C function to generate robust centromeric heterochromatin.

A second RNAi effector complex was identified from purification of factors associated with the RNA-Dependent RNA Polymerase Rdp1. Rdp1 was found in complex with a putative RNA helicase (Hrr1) and polyA polymerase (Cid12), which together make up the RNA Dependent RNA Polymerase Complex (RDRC)⁷⁴. This purification also identified a physical association between RDRC and RITS which depends on Clr4 as well as Dcr1, suggesting RITS and RDRC interact in the context of heterochromatin and through nascent transcripts⁷⁴.

Subsequent experiments have suggested that RDRC is also physically coupled to the C terminus of Dcr1⁸⁹. However, these C terminal sequences confer nuclear shuttling ability to Dcr1, and truncation mutants in Dcr1 that were presumed to cause loss of interaction with RDRC may actually cause cytoplasmic restriction of Dcr1⁹⁰. *In vitro* experiments have revealed that Dcr1 promotes RDRC activity, but that Dcr1 function is not accentuated by addition of RDRC. *In vivo*, however, physical coupling of Dcr1 and RDRC enhances the production or stabilization of siRNAs likely through more efficient targeting of template dsRNA to Dcr1^{89,91}. Together these findings have led to the model that the RNAi machinery primarily acts in *cis*, with RITS tethered to centromeric chromatin. Stably localized RITS/RDRC complexes process nascent transcripts, generating siRNAs for further recruitment of Clr-C, H3K9me deposition and recruitment of heterochromatin assembly factors. These complexes act in a self-reinforcing positive amplification loop to yield robust heterochromatin^{92,93}.

Before entering a detailed discussion of RITS's contributions to heterochromatin assembly, it will be useful to first mention that heterochromatin formation can be broken down into several different components, which although not strictly separable, provide a useful framework for thinking about the process. **Heterochromatin initiation** is the process of establishing *de novo* heterochromatin. This is followed by **heterochromatin assembly** or re-assembly, which is the process by which robust heterochromatin forms at an initiation site, and re-assembles every cell cycle following its dilution by DNA replication. A third component is the **spreading** of heterochromatin from its sites of nucleation, wherein neighboring DNA sequences are silenced. RITS and the RNAi pathway are important players in both the assembly and spreading of heterochromatin, and may also contribute to the initiation of heterochromatin. Below we will outline the role of RITS in heterochromatin assembly.

HOW IS RITS RECRUITED TO CHROMATIN?

Prior to the purification of RITS, study of RNAi in several organisms had revealed the remarkable ability of small RNAs to target the RNA-induced silencing complex (RISC) to homologous mRNA for subsequent degradation^{94,95}. The discovery that Ago1 associates with siRNAs of centromeric origin provided a logical means for directing RITS to heterochromatic loci^{60,88}, either through association with single stranded DNAs produced during replication, or through binding to nascent transcripts derived from the centromeric sequences. Supporting the idea of siRNA-mediated targeting, Chp1 and Tas3 association with centromeric repeats is lost in cells defective for siRNA production⁸⁸. Evidence that transcription itself is necessary to drive heterochromatin formation supported the idea that nascent transcripts may in fact be the platform to which RITS is recruited^{74, 96}. This led to the development of the “nascent transcript model” (Figure 2) which has been widely accepted as the route by which heterochromatin assembles and is even initiated in fission yeast^{88,97-99}.

In an updated version of this model, centromeric repeats are transcribed by RNA pol II during S phase of the cell cycle. These single stranded transcripts serve as template for the RNA-dependent RNA polymerase activity of Rdp1 to generate dsRNA, which then yields siRNAs through the activity of Dcr1. These siRNAs are incorporated into Ago1, and following cleavage of the passenger strand, the guide siRNA targets RITS to homologous centromeric transcripts. Once recruited, RITS facilitates recruitment of Clr-C, which methylates H3K9 in centromeric chromatin, providing an additional binding surface for RITS via the Chp1 chromodomain, and for assembly of heterochromatin. Critically, this model relies on the chromosomal tethering of the RNAi effector complexes, and the generation of siRNAs and recruitment of H3K9 methyltransferase activities in a mutually

dependent fashion that contributes to both *cis*-restriction of silencing and amplification of the response.

Tethering of RITS suffices to induce *de novo* heterochromatin assembly

Whether targeting of RITS via RNA to a chromosomal locus could suffice to induce *de novo* transcriptional silencing was rigorously tested by tethering RITS to nascent transcripts. Strains were constructed that expressed a Tas3- λ N fusion protein, conferring high-affinity recognition of five BoxB RNA motifs that were inserted into the 3'UTR of the normally euchromatic endogenous *ura4⁺* gene⁹⁶. Targeting RITS to *ura4-5BoxB* pre-mRNA was able to initiate silencing of the transgene. Importantly, the silencing was both heterochromatin and RNAi-dependent, enriching the locus with H3K9me2 and accompanied by production of siRNAs corresponding to the transgene, indicating that RITS can act as the initial signal in *de novo* heterochromatin formation. Heterochromatin assembly required transcription of the *ura4-5BoxB* hybrid, as removal of the upstream promoter prevented RITS recruitment to the locus. Thus, silencing was initiated by recognition of the nascent transcript rather than DNA sequence. In these experiments, silencing occurred only in *cis*, as siRNA generated from the *ura4-5BoxB* hybrid could not silence a second *ura4⁺* gene inserted elsewhere in the genome unless in an *eri1⁺*, a suppressor of RNAi¹⁰⁰, deficient background. RITS-mediated RNAi is thus *cis*-restricted, requiring stable association at chromatin to silence transcription⁹⁶. This contrasts with cytoplasmic RNAi-mediated PTGS, which occurs independent of Tas3 and Chp1, but intriguingly shows some dependence on Clr4, suggesting that Clr4 plays a role in RNAi-mediated silencing independent of its role in H3K9 methylation⁹¹.

While these experiments demonstrate that targeting of RITS to a nascent transcript can suffice to promote *de novo* heterochromatin assembly, the relative role of siRNA-mediated or H3K9me-mediated targeting of RITS to centromeres for heterochromatin assembly was unclear. To clarify this issue, we took the approach of mapping functional domains of RITS and then engineering mutants to remove potential targeting components from the complex. While Tas3 and Chp1 can interact independent of Ago1⁸⁶, Ago1 can interact with Tas3 in the absence of Chp1¹⁰¹, suggesting that RITS is a linear complex with Tas3 serving as a bridge between Chp1 and Ago1 (Figure 3).

Role of H3K9me2 in targeting RITS

First we addressed the role of Chp1 in the RITS complex. Chp1 interacts with the extreme N terminus of Tas3¹⁰². Cells were engineered to express a mutant Tas3 that lacks residues 10-24. This mutant Tas3 cannot associate with Chp1, but can still bind Ago1. This mutant is defective for centromeric heterochromatin assembly; it cannot associate with the centromere, and no longer produces centromeric siRNAs, similar to any RNAi defective mutant¹⁰². Thus Chp1 association with Tas3 and Ago1 is important for normal function of RITS.

Stable association of RITS with H3K9me2 is critical for centromeric heterochromatin assembly, as strains bearing a *chp1* mutant that lacks the chromodomain, or which bear point mutations that prevent association with H3K9me2, cannot maintain heterochromatin or process centromeric transcripts into siRNAs^{86,92,103}. The chromodomain of Chp1 has a high affinity for H3K9me2 *in vitro* compared to other chromodomain-containing proteins, and is several fold higher than that of Swi6 or HP1¹⁰³. The crystal structure of Chp1's chromodomain in association with an H3K9me3 peptide and comparison with a similar HP1 chromodomain structure revealed a unique salt bridge which forms between E23 of Chp1 and K4 of the histone tail peptide that is not predicted to form with Swi6^{103,104}. This

interaction seems to largely account for the difference in affinity between the two proteins for H3K9me.

Interestingly, mutants causing up to a 40-fold reduction in Chp1's affinity for H3K9me2 can still maintain heterochromatin¹⁰³. Maintenance of heterochromatin in these partial loss of affinity mutants is contingent on additional tethering to chromatin by Ago1, as mutations that partially disrupt Ago1 binding to siRNAs, that do not cause a significant disruption of heterochromatin on their own, result in a complete loss of centromeric silencing in these backgrounds¹⁰³.

While RITS recruitment to centromeres depends on the RNAi pathway, recruitment of RITS to the mating-type locus or telomeres can occur independent of RNAi⁸⁶ where, unlike centromeres, redundancy in heterochromatin assembly pathways permits the maintenance of H3K9me2 even in the absence of *rdp1*, *dcrl*, and *ago1* function^{25,86}. The RNAi-independent enrichment of H3K9me2 at these loci is then sufficient to recruit Chp1.

Role of siRNA-mediated targeting of RITS

We also sought to identify the role of Ago1 in targeting RITS to centromeres. Tas3 associates with Ago1 through a glycine and tryptophan-rich (GW) domain^{101,105}, a conserved Argonaute interaction motif¹⁰⁶. Surprisingly, dissociating Ago1 from Tas3 and Chp1 by directed point mutagenesis did not disrupt the maintenance of centromeric heterochromatin¹⁰¹. Interestingly, despite no longer being tethered to chromatin by association with Chp1, Ago1 was still able to associate with heterochromatin through homologous siRNA- transcript base pair interactions. Transient removal of H3K9me2 and siRNAs, however, revealed that this interaction was required for the re-establishment of heterochromatin, after transient depletion of H3 K9 methylation, suggesting Ago1 must be tightly coupled to H3K9me2 to initiate RNAi-dependent heterochromatin formation¹⁰¹.

In another study, RNAi was shown to initiate the transient accumulation of H3K9me3 at regions of convergent transcription from endogenous promoters¹⁰⁷. This work revealed that H3K9me3 accumulates in G1, and that Swi6 and cohesin recruitment to these sites is dependent on Dcr1 and Ago1. Transcription-induced remobilization of cohesin led to transcriptional termination in G2, prior to removal of Swi6 and cohesin in mitosis and a new round of heterochromatin formation in the next cell cycle. Although siRNAs were not originally detected corresponding to convergent gene pairs, more recent work has identified low levels of siRNAs at some sites of convergent transcription, and that Dcr1 associates transiently with some of these loci¹⁰⁸.

Additional experiments expressing hairpin RNAs in fission yeast have demonstrated that heterochromatin can assemble at the hairpin-homologous euchromatic reporter sequences. In general, this silencing is weak, and requires increased gene dosage of heterochromatin proteins, physical proximity of the reporter to other sites of heterochromatin, or convergent transcription through the reporter locus for its assembly^{109,110}.

In summary then, it appears that the tight coupling of both siRNA and H3K9me binding appear to be important for recruitment of RITS for heterochromatin assembly.

MECHANISMS OF RITS/RDRC MEDIATED HETEROCHROMATIN ASSEMBLY

There are two clear mechanisms by which RITS could contribute to heterochromatin assembly. The first involves post-transcriptional, or co-transcriptional, gene silencing, where the endonuclease activity of Ago1 is actively involved in the destruction of nascent

heterochromatic transcripts to promote formation of a “silenced” locus. The second mechanism involves silencing at the level of transcriptional suppression (transcriptional gene silencing) by RITS-mediated recruitment of chromatin modifying activities to generate a heterochromatic environment that is largely refractory to access by RNA polymerases. Below, we discuss each of these mechanisms.

1. Co-transcriptional silencing by RITS

RITS possesses endonuclease activity^{78,83}. It is possible that transcription coupled silencing can be mediated by the cleavage of nascent transcripts by Ago1, and that this could contribute to heterochromatin assembly at centromeres. In this process, there is clearly also a requirement for Dcr1 to cleave dsRNAs to provide siRNAs for Ago1-mediated silencing, and for RDRC to generate dsRNA template. The physical linkage between these activities contributes to the efficiency of siRNA mediated transcriptional silencing⁸⁹.

However, it is difficult to directly test the contribution of Ago1-slicing activity to heterochromatin assembly, since catalytic mutants in Ago1 are inactive for slicing of the passenger strand of the ds siRNA, and therefore cannot bind nascent transcripts via single stranded siRNA mediated targeting⁷⁸. An alternative approach would be to test whether artificial tethering of a component of the pathway that elicits solely co- or post-transcriptional RNA processing would be sufficient to induce robust silencing. Very surprisingly, Gerace et al.³⁷ showed that tethering of Rik1 to a euchromatic transcript was sufficient to mediate co-transcriptional silencing via processing of transcripts by the RNAi pathway, but that tethered Rik1 did not promote transcriptional gene silencing, as silencing was independent of accumulation of H3K9me2 and Clr4. Thus artificially tethered Rik1 can recruit RITS to mediate slicing and turnover of transcripts via the siRNA directed endonucleolytic activity of Ago1. Under these circumstances, other components of Clr-C are either not recruited or are unable to methylate or ubiquitinate critical targets involved in heterochromatin assembly. Silencing induced by tethering of Rik1 was robust, and comparable in strength to that achieved by tethering of RITS, which mediates both transcriptional gene silencing and co-transcriptional silencing⁹⁶. Thus, co-transcriptional silencing may suffice to efficiently silence transcripts in the absence of chromatin modification.

2. Transcriptional gene silencing mediated by RITS

The proper assembly of heterochromatin at centromeres critically depends on recruitment of Clr-C by the RNAi pathway to centromeric repeats (Figure 4). This process is still not fully understood, but several studies have demonstrated physical association between RITS/RDRC and components of the Clr4-containing complex, Clr-C^{36,37,111}. Recent studies focusing on the role of Rik1 have demonstrated that it may act as a tether for the Clr-C complex. Interestingly, Rik1 interacts with the Chp1 component of the RITS complex, and its localization to centromeric transcripts depends on the enzymatic activities of Ago1 and Rdp1³⁶. Thus Rik1 (and Clr-C) may be recruited to centromeres via chromatin associated RITS/RDRC.

An additional link between Clr-C and RITS was made by the identification of Stc1, a LIM domain containing protein which interacts both with Ago1 and with Clr-C and is required for centromeric heterochromatin assembly¹¹¹. Interestingly, Stc1, like Chp1 and RNAi components, is not required for the maintenance of heterochromatin at the mating type locus, putting it into the “RNAi”-like mutant collection. However, when Stc1 is tethered to DNA, *cis*-linked euchromatic genes are transcriptionally silenced and silencing occurs independent of RNAi components, but is dependent on Clr-C¹¹¹.

Together, these experiments suggest that once RITS/RDRC has been recruited to centromeres, Clr-C is efficiently recruited to mediate accumulation of robust H3K9me2/3 for transcriptional gene silencing, and for heterochromatin assembly. This view is supported by the finding that tethering of the methyltransferase domain of Clr4 to DNA is sufficient for heterochromatin assembly even in the absence of the RNAi pathway¹¹².

THE CHICKEN AND EGG PROBLEM FOR HETEROCHROMATIN INITIATION

RITS/RDRC, Dcr1 and Clr-C activities are therefore coupled, and largely co-dependent for their physical association with centromeres. They also exhibit a shared dependence on siRNAs and Clr4 methyltransferase function for centromeric association. (Figures 2 and 4). These features have made it difficult to distinguish the ordering of events for recruitment of RITS/RDRC and Clr-C to centromeres. As described above, it seems likely that for heterochromatin assembly, RNAi and H3K9me2 cooperate in RITS recruitment.

How heterochromatin initiates, though, is a thorny issue. A popular model is that RNAi also drives heterochromatin initiation. Thus nascent centromeric transcripts are thought to be converted into dsRNA by the activity of Rdp1, and this is thought to initiate the process of heterochromatin assembly. Rdp1 has, at least *in vitro*, primer independent RNA dependent RNA polymerase activity⁷⁴, and so could theoretically generate dsRNA from single stranded centromeric transcripts. Dcr1 could then generate siRNAs to recruit RITS to centromeric repeats. Following RITS recruitment, Clr-C could be efficiently recruited to centromeric repeats.

However, several observations reduce the likelihood that heterochromatin is initiated in this manner: First, low levels of H3K9me2 methylation are retained at centromeres in strains lacking a functional RNAi pathway^{25,56,113}, indicating that RNAi is not the initiating event for *de novo* Clr-C recruitment. Second, with the advent of more sensitive methods of siRNA detection, it is now clear that low levels of siRNAs are present in *rdp1* mutants^{76,114}. Thus both H3K9me and siRNA production can occur upstream of Rdp1. Third, physical association of Dcr1 and Rdp1 with chromatin does not suffice for the assembly of heterochromatin¹⁰⁸.

Alternative theories have also been proposed to account for heterochromatin initiation. Djupedal et al.⁷⁶ have proposed that single stranded transcripts from centromeric repeats form complex secondary structures including regions of dsRNA that may serve as template for Dcr1. This accounts for the authors' observation that siRNA production is not absolutely dependent on Rdp1 but does depend on Dcr1⁷⁶. In contrast, Halic and Moazed¹¹⁴ found low levels of short RNAs in Dcr1 deficient cells, and proposed that "primal" RNAs derived from random transcription can associate with Ago1 to nucleate slicing of transcripts from repetitive sequences and recruit Rdp1 for dsRNA synthesis. This study placed Ago1 as the initiating factor in heterochromatin assembly, and supported that claim by showing that mutants in Ago1 had lower levels of H3K9me accumulation than other RNAi pathway mutants. This conclusion is however not supported by other studies that demonstrate that *ago1* deficient cells retain substantial H3K9me2 on centromeric repeats^{56,113}.

Other studies suggest that RNAi is not the initiating signal for heterochromatin assembly, but that alternate mechanisms of recruitment of Clr-C lead to deposition of initially low levels of the H3K9me2 mark. This mark could then be amplified to allow full assembly of heterochromatin following recruitment of RITS and RNAi activities to centromeres (Figure 4). In studies using a crippled RITS complex, where Ago1 is dissociated from the complex by point mutation of GW sequences in Tas3, the initiation of heterochromatin shows a clear reliance on Clr-C activities, but is not disrupted by transient removal of RNAi factors^{101,113}. Mutants deficient in Clr-C completely lack H3K9me on centromeric repeats, whereas RNAi

defective cells retain sufficient H3K9me to allow reassembly of heterochromatin following reintroduction of RNAi components. These data suggest that Clr-C and not RNAi is the initiating factor for heterochromatin assembly. In addition, overexpression of Clr4 can mediate H3K9me deposition at centromeres in cells otherwise lacking H3K9me2 and either Ago1 or Dcr1¹¹³, demonstrating that RNAi is not necessary for heterochromatin initiation. Interestingly, the high affinity association of the Chp1 chromodomain for H3K9me appears critical for heterochromatin assembly. When heterochromatin is artificially disrupted by removal of *clr4*⁺ (and hence H3K9me2/3) from strains bearing reduced affinity Chp1 chromodomain mutants, and heterochromatin reassembly is assessed following reintroduction of *clr4*⁺, these reduced affinity Chp1 mutants cannot establish heterochromatin, in spite of the synthesis of wild type levels of siRNAs in these strains¹⁰³.

How then might Clr-C be recruited to centromeres independently of RNAi? Clr-C recruitment may be linked to transcription of centromeric repeats. Several different mutants in RNA pol II components have been identified in fission yeast that specifically affect heterochromatin assembly without having widespread transcriptional defects^{58,59}. It is possible that H3K9 methylation, like H3K4 and H3K36 methylation, may be linked to passage of the RNA polymerase complex¹¹⁵. Alternatively, Clr-C recruitment may rely on sequence specific DNA binding factors, similar to the role of Atf1/Pcr1 at the mating type locus^{51,52}.

HOW DOES RITS/RDRC FACILITATE SPREADING OF HETEROCHROMATIN?

RNAi can contribute to the spreading of heterochromatin through the action of RNA dependent RNA polymerases¹¹⁶. In the case of silencing of endogenous genes mediated via expression of hairpin RNAs, RdRPs can synthesize RNA that extends beyond the priming siRNA sequence, 5 prime to the initiating trigger to yield secondary siRNAs. If these siRNAs facilitate silencing, they could facilitate spreading of silencing. Recently, such secondary siRNAs have been detected in fission yeast, but whether they can facilitate the spreading of silencing is not yet known¹¹⁰.

In addition to motifs directly responsible for association with target chromatin and transcripts, RITS also has the ability to self-associate. Detailed analysis of the crystal structure of Tas3 C terminal residues uncovered a compact structure consisting of seven α helices, termed the TAM (Tas3 C-terminal alpha motif) domain¹¹⁷. The TAM domain seems to be the driving force for Tas3 polymerization *in vitro*. Subsequent experiments *in vivo* supported a role for polymerization of RITS in spreading from sites of heterochromatin nucleation at centromeres. Disruption of RITS self-association by point mutagenesis of the TAM domain reduced RITS enrichment at centromeric repeats, most prominently at the inner centromeric repeat sequences¹¹⁷, where transcription is rare and perhaps an inefficient scaffold for Ago1 recruitment^{83,118}. Reduced RITS recruitment was accompanied by a marked reduction in centromeric siRNA accumulation. Interestingly, H3K9me2 was largely unaffected in these mutants, placing polymerization and, to some degree, siRNA genesis, downstream of H3K9me2 in the centromeric heterochromatin assembly pathway. RITS polymerization and *cis*-spreading of the complex is reminiscent of oligomerization of other silencing proteins most notably that of Sir3 self-association in *S. cerevisiae*¹¹⁹ and Swi6 oligomerization in fission yeast¹²⁰.

CONCLUSION

The study of heterochromatin assembly in fission yeast has provided unique insights into the complex interplay between the RNAi pathway and chromatin modifying machinery. There

is evidence that Clr-C activity and H3K9me2/3 marking can occur independently of RNAi, placing Clr-C upstream of RNAi in heterochromatin assembly, and also that RNAi can drive heterochromatin assembly. Clearly the most efficient machine for generation of heterochromatin relies on the close coupling of a functional RNAi pathway and a robust chromatin marking system, in combination with a high affinity “mark-reading” system to yield efficient heterochromatin assembly. The RITS complex provides a superb example of such a machine, and the ability to perform detailed mechanistic studies through genetic manipulation in fission yeast has allowed us to appreciate some of the beauty of this system.

Research into the assembly of transcriptionally silenced heterochromatin has revealed an intriguing dependence on the production of non coding RNA transcriptional scaffolds for nucleation and assembly of chromatin modifying complexes. Given the widespread occurrence of non coding RNA in metazoan genomes, and recent discoveries that many of these RNAs can guide localization of chromatin modifying complexes to regulate expression of key developmental regulators as well as tumor suppressors (reviewed in ¹), we look forward to an explosion of research in this fascinating area.

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Figure 1. Sites of heterochromatin assembly in fission yeast

A. Fission yeast centromeres have a common organization with a central domain consisting of central core (cnt) and inner repeat sequences (imr), which is flanked by the outer repeat elements consisting of *dg* and *dh* repeats. Centromere 1 has 2 copies of *dg* and *dh* that symmetrically flank the central domain, and larger centromeres have more copies of the repeats (upto 12 total in centromere 2).

B. At the mating type locus, heterochromatin assembles over a 20Kb region including *mat2P* and *mat3M* sequences. Within this domain, *cenH* is a 4.3 Kb region with >96% homology to *dg* and *dh* sequences. Assembly of heterochromatin on *cenH* is regulated by RNAi. Additional heterochromatin nucleation sequences exist within the *mat2P/mat3M* locus, including a 2.1 Kb region between *cenH* and *mat3*, which includes REIII. Atf1 and Pcr1 factors recruit histone deacetylase and Clr-C activity to this domain, which serves as a redundant heterochromatin nucleation site with *cenH*.

C. Sequencing of telomeres in fission yeast is incomplete, but has revealed the presence of *dg/dh* like sequences within *tln1* and *tln2* genes located in subtelomeric domains of the left arm of chromosome 1 and right arm of chromosome 2. 1.1 kb of sequence within each gene shows gapped sequences with >75% identity to centromeric sequences⁵⁴. At telomeres, heterochromatin assembly can also be specified independently of RNAi by the telomere specific factor Taz1⁵³.

Figure 2. The RNA cycle of heterochromatin assembly

Nascent centromeric transcripts expressed during S phase of the cell cycle are converted by RDRC activity to dsRNA for cleavage by Dcr1 to form siRNAs. These siRNAs pass through the ARC Argonaute chaperone complex prior to loading into RITS, where Ago1 endonuclease activity cleaves the passenger strand siRNA allowing Ago1 to target nascent centromeric transcripts for co-transcriptional gene silencing and to target Ago1 (and RITS) to centromeric transcripts to reinstate transcriptional gene silencing via recruitment of Clr-C (see Figure 4).

Figure 3. The RITS complex

RITS has a linear architecture, with Tas3 bridging between Chp1 and Ago1. Ago1 can bind siRNAs (thick red line) to mediate targeting to and destruction of nascent transcripts (red way line), and Chp1 has an N terminal chromodomain with high affinity for H3K9me chromatin (red star).

Figure 4. The H3K9me cycle

Strains lacking RNAi machinery retain low levels of H3K9me at centromeres suggesting some RNAi-independent Clr-C recruitment as seen at other sites of heterochromatin. H3K9me can be bound by the histone methyltransferase complex, Clr-C, allowing “spreading” of the H3K9me mark to adjacent nucleosomes. High affinity binding of H3K9me by Chp1 chromodomain promotes efficient recruitment of RITS/RDRC, which in turn can recruit Clr-C and amplify the H3K9me signal through an RNAi-dependent mechanism. H3K9me promotes transcriptional gene silencing. The H3K9me and RNA cycles (Figure 2) are tightly linked via RITS to promote efficient heterochromatin assembly.