# RNA Interference, Silent Chromatin and Centromere Integrity

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## Introduction

*In fungal, plant and animal systems it is clear that non-coding RNAs play key roles in mediating the formation of silent chromatin. The expression of exogenous double stranded RNA homologous to a target gene can induce DNA methylation and histone H3 lysine 9 methylation and gene silencing. Double stranded RNAs are processed by the RNA interference machinery to produce short interfering siRNAs that somehow home in on homologous chromatin and induce the assembly of silent chromatin. In fission yeast endogenous non-coding transcripts are produced from centromere associated repeats and the resulting homologous siRNA allow the formation of centromeric heterochromatin over these repeats. This silent chromatin is required to mediate tight physical cohesion between sister-centromeres by maintaing a high density of cohesin on these centromere repeats. RNAi and silent chromatin components are therefore for normal chromosome segregation during mitosis and their disruption results defective centromere function.*

*The centromere specific histone H3 like protein CENP-A is a fundamental component in specifying the site of centromere assembly in most eukaryotes. CENP-A replaces normal histone H3 specifically at active centromeres and its incorporation and propagation at a particular site is subject to epigenetic regulation. How CENP-A is deposited and maintained only at active is not known but understanding this process is fundamental to dissecting the process of kinetochore assembly.*

## Fission yeast centromeres

Fission yeast centromeres span 35-110 kb and are composed of a central core region of non-repetitive DNA (*cnt*), flanked by inverted repeat regions – the innermost repeats (*imr*) and the outer repeats (*otr*). The DNA is encompassed in two distinct chromatin domains: the central core region, consisting of the *cnt* and *imr* sequences, upon which the kinetochore is

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assembled; and the heterochromatic outer repeat domains (silent chromatin). Thus the fission yeast centromeres resemble those of vertebrates, with the kinetochore embedded in a sea of heterochromatin. Marker genes placed within the fission yeast centromere are transcriptionally silenced. The degree and properties of the silencing vary with position in the centromere; strong silencing is observed at the outer repeats, whilst that in the central core is less robust.

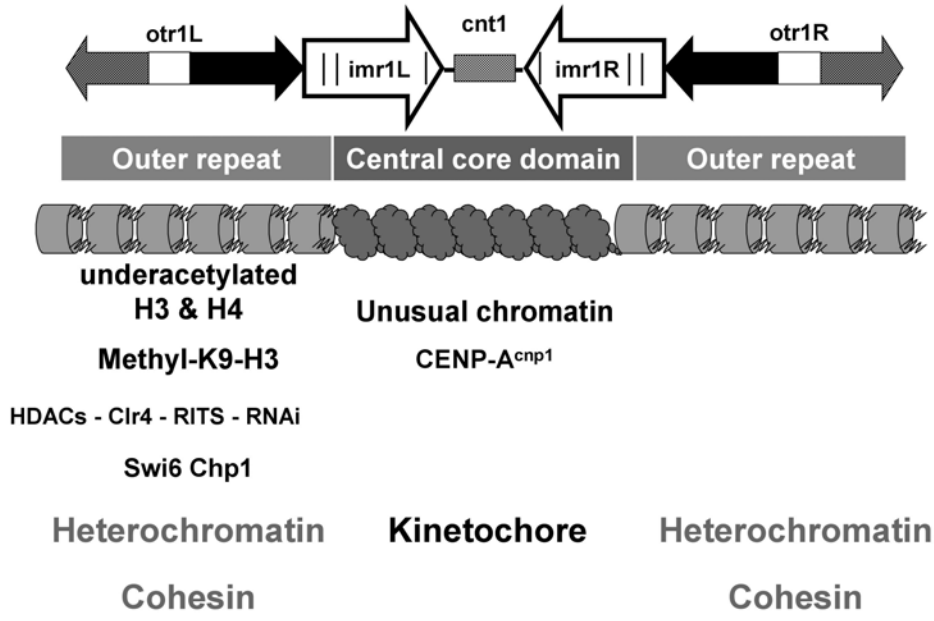


***Figure 1. Schematic representation of fission yeast centromere 1.***
*Centromere 1 is ~35 kb and consists of a central core (cnt) of non-repetitive sequence flanked by innermost repeats (imr) and outer repeats (otr) which together form an almost perfect inverted repeat around the central core. Insertion of marker genes anywhere in the centromere results in their transcriptional silencing. The centromere is divided into two domains: the central core domain (cnt and imr) and the outer repeat domain. Different classes of mutants affect silencing in each domain, and each is associated with a distinct set of proteins. Short vertical lines represent tRNA genes. The central core region has a unique chromatin structure. CENP-A$^{cnp1}$ replaces histone H3 in the central region, and upon this chromatin platform, the kinetochore is assembled. The outer repeats are packaged in nucleosomes which are underacetylated on the N-terminal tails, due to the action of the histone deacetylases. This allows di-methylation of lysine 9 of histone H3 by the histone methyltransferase Clr4 (Suv39), providing a binding site for the chromodomain proteins Swi6 (HP1) and Chp1. This Swi6-containing heterochromatin is responsible for the recruitment of a high density of cohesin to the outer repeat region which is important for proper biorientation of centromeres at mitosis. The assembly of heterochromatin is dependent on the RNAi machinery and siRNAs derived from centromeric transcripts. This involves the RNAse III-like endonuclease Dicer, the RITS complex (Chp1, Tas3, Ago1 and siRNAs), the RNA dependent-RNA polymerase Rdp1.*

The central core region is packaged in a unique chromatin structure. Whilst bulk chromatin exhibits a canonical ladder pattern upon limited digestion with micrococcal nuclease, hybridisation with a central core probe gives a smear pattern. This unique pattern forms only when the central core is in a functional context: only on centromere plasmids with segregation function is the unique chromatin seen over the central core, and it is lost in mutants which affect central core silencing and function. The other major difference between central core chromatin and the rest of the genome is the presence of the histone H3 variant CENP-A$^{cnp1}$ which replaces (totally or partially) histone H3. Presumably this CENP-A$^{cnp1}$ is assembled into specialised

nucleosomes, and their structure or organisation cause the atypical nuclease sensitivity. Upon this specialised central core chromatin the kinetochore is assembled. Transcriptional silencing at the central core reflects the assembly of a fully functional kinetochore complex rather than canonical heterochromatin.

## Centrometric heterochromatin

The outer repeats are assembled in repressive heterochromatin. Histones H3 and H4 in the nucleosomes of outer repeat chromatin are underacetylated on lysines in their N-terminal tails, compared with euchromatin. This correlates with transcriptional silencing: a *ura4*$^{+}$ gene placed within the outer repeats is strongly silenced with very few colonies forming on media lacking uracil and many forming on media containing the counter-selective drug FOA. Hypoacetylation is important for centromere function as brief treatment with the histone deacetylase inhibitor TSA causes a heritable increase in acetylation of centromeric chromatin, expression of marker genes and a concomitant defect in chromosome segregation. The hypoacetylated state of the outer repeat chromatin is due to the activities of three histone deacetylases:

n addition to their hypoacetylation, outer repeat nucleosomes differ from their euchromatic counterparts in their methylation status. Lysine 9 of histone H3 is methylated in heterochromatic regions – the centromeric outer repeats and the silent mating type locus. The histone methyltransferase Clr4 (equivalent to metazoan Suv39) is responsible for methylation of histone H3 K9. This modification creates a binding site for the chromodomain protein Swi6 (equivalent to metazoan HP1). The absence of either Swi6 or Clr4 causes alleviation of outer repeat (and mating type) silencing, and Swi6 is specifically located at these regions. Another chromodomain protein, Chp1, is required for methylation of histone H3 lysine 9 and binds dimethylated H3 peptide in vitro. It is found in the RITS complex along with Tas3, Ago1 and siRNAs.

## Role of RNAi in formation of heterochromatin

The observations that marker genes inserted into centromeric heterochromatin in fission yeast were transcriptionally silenced and no steady state transcripts were detected coming from the outer repeat regions, suggested that the centromeres were transcriptionally inert. Ironically, it transpires that the formation of silent heterochromatin is actually dependant on transcripts from the outer repeat regions and the RNA interference machinery. Mutants in components of the RNAi machinery, Argonaute (Ago1), Dicer (Dcr1) and RNA-dependent RNA polymerase (Rdp1), alleviate silencing of marker genes in the centromeric outer repeats and display increased rates of chromaosome loss and lagging chromosomes. In these mutants, but not in wild-type, transcripts derived from the top and the bottom strands are detectable. The transcripts are partially complementary and could potentially form dsRNA which would be a substrate for the RNase III-related endonuclease Dicer.

Observations in several organisms suggest that the RNAi machinery is likely to have (up to at least) three silencing effects in the cell: the degradation of messenger RNA homologous to siRNAs (post-transcriptional gene silencing – PTGS – involving the RISC complex that includes Argonaute); chromatin-based silencing (either DNA methylation in plants, methylation of histone H3 lysine 9 and recruitment of chromodomain proteins such as Swi6 in fission yeast, or, both in human cells; and translational inhibition. Some proteins appear to have roles in only one of these processes, whilst others have roles in both mRNA degradation and the chromatin-dependent silencing pathway. The chromodomain protein Swi6 operates only in the chromatin-based pathway, RNAi components such as Ago1, Dcr1 and Rdp1 affect both pathways (e.g. Ago1 is thought to be a component of both RISC and RITS).

How is the transition made from acetylated euchromatin to the heterochromatic state? Two models can be envisaged for how siRNAs elicit the formation of heterochromatin. In one, homologous DNA is the target for siRNAs. The evidence for this DNA-RNA view comes from experiments in plants where dsRNAs homologous to a promoter region lead to chromatin-based silencing. More recently it has been shown that transfection of human cells with several siRNAs results in chromatin based silencing of a target gene. Since promoters themselves are not

expected to be transcribed, it is suggested that the siRNAs may be targeting DNA. The second model invokes RNA-RNA pairing between the siRNAs and homologous nascent transcripts.

In this second scenario, nascent transcripts might attract homologous siRNAs assembled in the RITS complex (Chp1, Ago1, Tas3), along with histone modifying enzymes – the histone deacetylase activities and the histone methyltransferase Clr4. This would be analogous to the situation with other chromatin modifying activities which are known to track along with elongating RNA polymerase II, for example, the SET1 and SET2 histone H3 lysine 4 and lysine 36 methyltransferases.

## Synthetic Heterochromatin by expression of dsRNA

Does primary DNA sequence matter, or does any double stranded RNA have the potential to induce heterochromatin? Expression of a hairpin RNA homologous to a portion of the *ura4* gene induced silencing and heterochromatin formation on the *ura4*$^{+}$ gene in the genome but failed to produce these effects on a genomic copy of *ura4* that lacked this portion of the gene. This suggests that heterochromatin can potentially be formed on any piece of DNA simply by production of the appropriate dsRNA. The ability to produce 'synthetic' heterochromatin will be valuable in the dissection of heterochromatin assembly and centromere function.

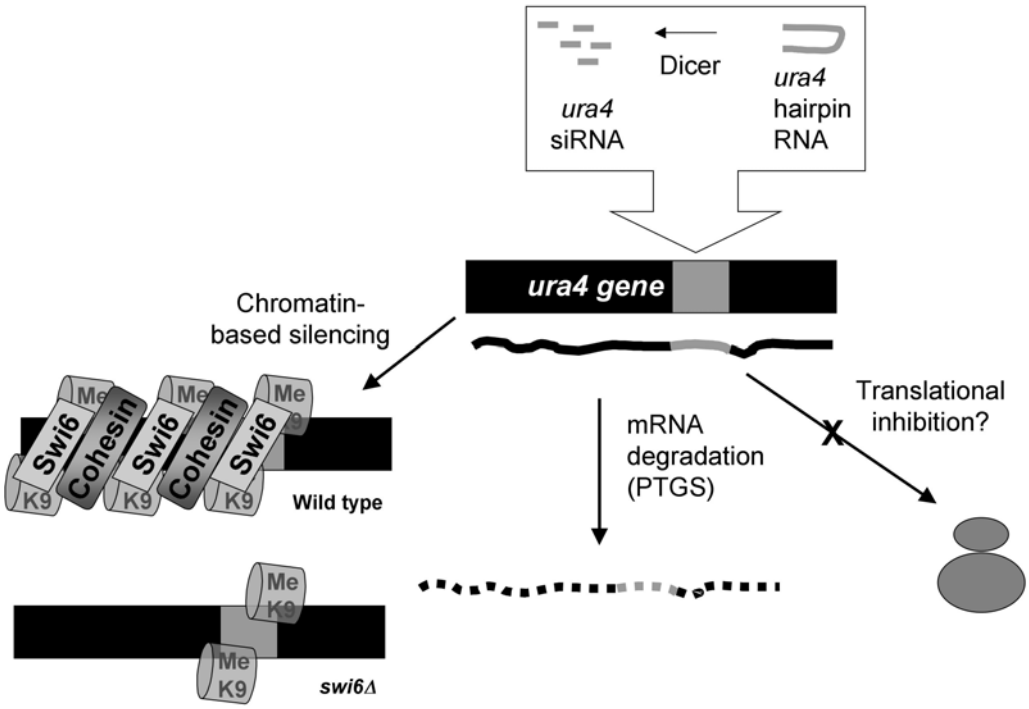


***Figure 2 Gene Silencing by RNA interference***
*Observations in several organisms suggest that the RNA interference machinery causes silencing by three different routes. This is illustrated for silencing by a plasmid-expressed hairpin RNA homologous to a 280 bp region of the ura4 gene in fission yeast. Box: dsRNA is cleaved by Dicer to produce siRNAs. Middle: Silencing by mRNA degradation or post-transcriptional gene silencing (PTGS). siRNAs are incorporated into the putative RNA-induced silencing complex (RISC, which includes Argonaute – Ago1 in fission yeast), which causes degradation of mRNA homologous to the siRNAs. Left: Chromatin-based silencing. In plants this involves DNA methylation to silence genes. In fission yeast, siRNAs incorporated into the RITS complex (Ago1, Chp1 and Tas3) lead to methylation of lysine 9 of histone H3, binding of Swi6 and recruitment of cohesin, at homologous regions. Assembly of heterochromatin requires the histone deacetylases, the histone methyltransferase Clr4, Rik1 and Rdp1. In wild-type cells, spreading of heterochromatin occurs beyond the region of homology. In swi6□ cells, lysine 9*

*methylation occurs only at the region of homology, indicating that Swi6 is required for spreading. Right: microRNA-dependent translational inhibition contributes to silencing in some organisms, but it is not known whether this occurs in fission yeast.*

In wild-type cells expressing the *ura4* hairpin, sequence upstream of the homologous region also become modified with lysine 9 methylation. This spreading phenomenon fails to occur in swi6□ cells suggesting that Swi6 is required for the propagation of silent chromatin marks.

## Overview

We are now utilising various tools and assays in fission yeast to elucidate how an siRNA signal is transmitted to bring about the assembly of silent chromatin on homologous chromatin. We are also investigating how this silent chromatin contributes to centromere integrity and thus the normal segregation of chromosomes during cell division.