RNAi-mediated epigenetic control of the genome

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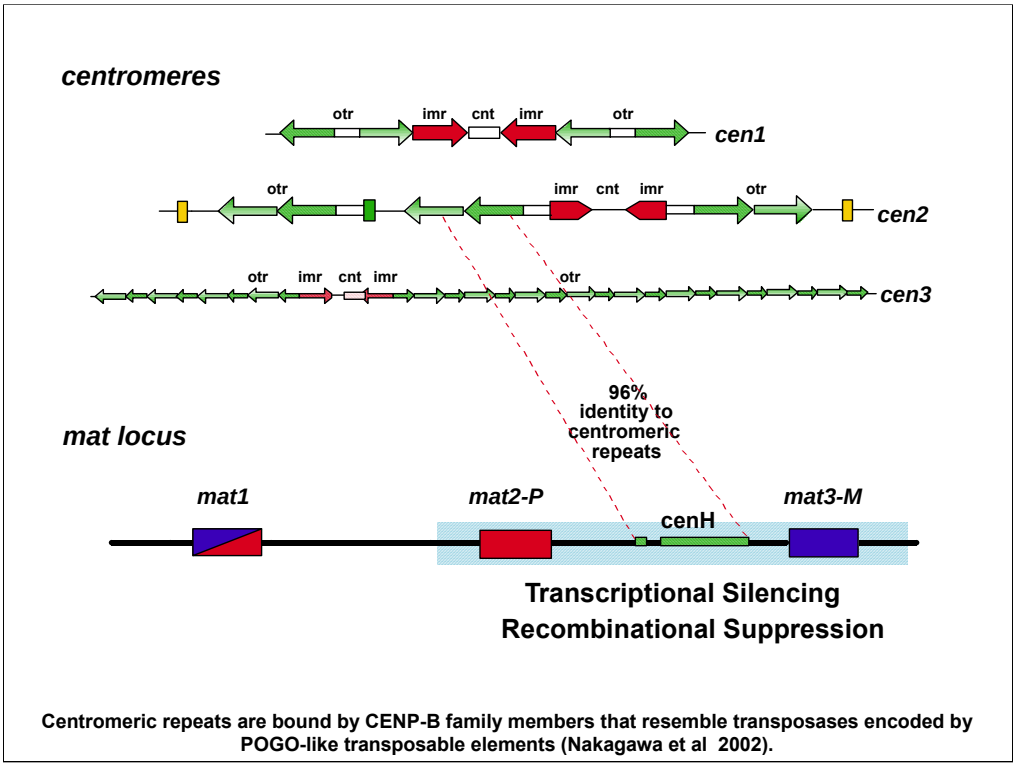
The organization of genomes into higher-order chromatin structures has number of biological implications

- Stable maintenance of gene expression patterns during development
- Maintenance of genomic integrity and prohibition of inter- or intrachromosomal recombination in repetitive DNA sequences
- Proper segregation of chromosomes
- Cancer and other human diseases (e.g. prostate cancer, leukemia, Wilm's tumor etc)

We are studying the epigenetic control of higher-order chromatin assembly that has number of biological implications. For example, these higher-order structures are essential for stable maintenance of gene expression patterns during development. Secondly, a large portion of our genome is made of repetitive DNA elements. These repeat sequences are major source of genomic instability. It is believed that higher-order structures help maintain genomic integrity by prohibiting inter- and intra chromosomal recombination in repeat sequences. These structures play an important role in segregation of chromosomes and defects in higher-order chromatin assembly can lead to cancer and other diseases.



Centromeric repeats are bound by CENP-B family members that resemble transposases encoded by POGO-like transposable elements (Nakagawa et al 2002).

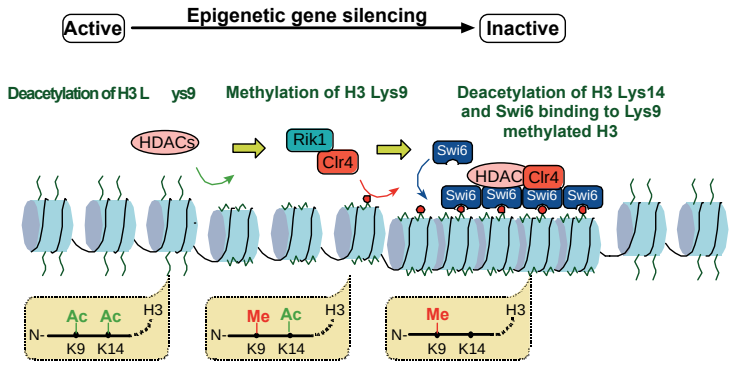
Fission yeast centromeres are large complex structures. Each centromere contains several of these repeat structures that are maintained in a transcriptionally repressed state. Mating-type region contains three genes. mat1 is active but this 20-kb domain containing mat2 and 3 loci is maintained in a repressed state. Importantly, a part of this interval between mat2 and 3 shares strong homology to centromeric repeats, which are believed to be remnants of transposable elements.

Factors involved in the mating-type region and centromeric silencing

Locus	Function	Motif/Similarity
Clr3	Histone deacetylase	Human HDAC4 and HDAC5, S. cerevisiae Hda1
Clr4	Histone H3 Lys9-specific methyltransferase	SET and chromo domains/ Human SUV39H1, Drosophila Suvar3-9
Clr6	Histone deacetylase	Human HDAC1 and HDAC2, S. cerevisiae Rpd3
Sir2	Histone deacetylase	Human and S. cerevisiae Sir2
Swi6	Chromatin modifier	Chromo and shadow domains/ Drosophila HP-1 and Polycomb
Chp1	Chromatin modifier	Chromo domain
Chp2	Chromatin modifier	Chromo and shadow domains
Rik1	Putative RNA binding protein	WD40 repeats

Several trans-acting factors that affect silencing at the mating-type region and centromeres have been identified. The identities of all these silencing factors are now known. A common theme that has emerged is that these factors are either involved in modification of histone tails or they are chromatin proteins. For example, Clr3 and Clr6 are histone deacetylases and Clr4 is a histone H3 Lys9-specific methyltransferase that belongs to Suv39 family of methyltransferases. Swi6 shares structural and functional similarities to HP1 proteins from other systems; and there are two other Swi6 like chromatin proteins called Chp1 and 2 that are also important for silencing.

Heterochromatin assembly is conserved from fission yeast to humans



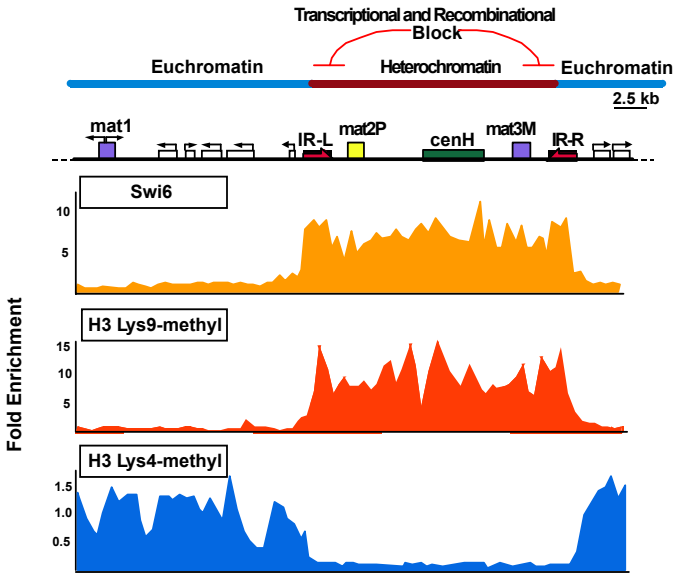
Recent work from our lab as well as others has defined temporal sequence of events, with regard to histone modificatios, that are essential for epigenetic gene silencing. We showed that HDACs and HMTases cooperate with each other to establish a histone code that is recognized by Swi6. In fact, we know that once Swi6 is localized, it presumably interacts with histone modifying proteins which then modify adjacent nucleosomes, creating another Swi6 binding site. This process is repeated many times leading to spreading of heterochromatin.

question

What prevents the spreading of repressive heterochromatin into neighboring euchromatic areas in a normal chromosomal context?

Knowing that heterochromatin can spread, an important question is how do cells prevent spreading of heterochromatin into nearby euchromatic regions containing important genes?

H3 Lys9 methylation and Swi6 are enriched throughout silent mating-type region surrounded by boundary elements

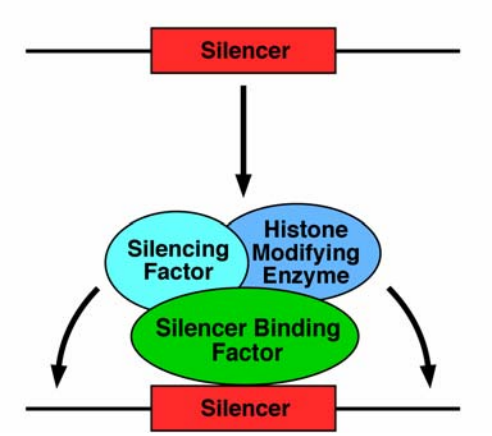


To address this important question, we prepared a high resolution map of heterochromatin complexes throughout 50-kb of mat locus. We found that Swi6 and H3 Lys9 methylation are preferentially enriched throughout the silent mating-type region while H3 methylated at Lys4, only few amino acids away, is specifically enriched at surrounding euchromatic regions. We also discovered that these distinct patterns of histone modifications are kept separate by these inverted repeats or what we call boundary elements surrounding the heterochromatin domain. Deletions of boundary elements cause spreading of Lys9 methylation and repressive chromatin to neighboring sequences, leading to epigenetic repression of these genes.

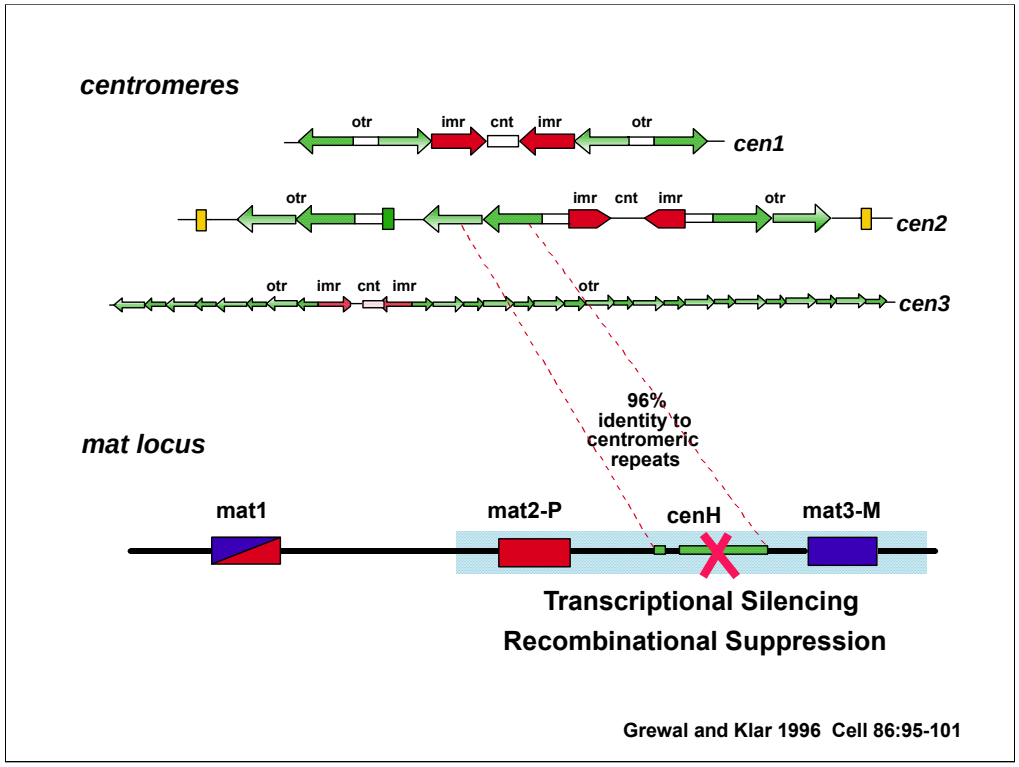
Question:

How are heterochromatin complexes targeted to specific locations in the genome??

Heterochromatin nucleation by DNA binding proteins

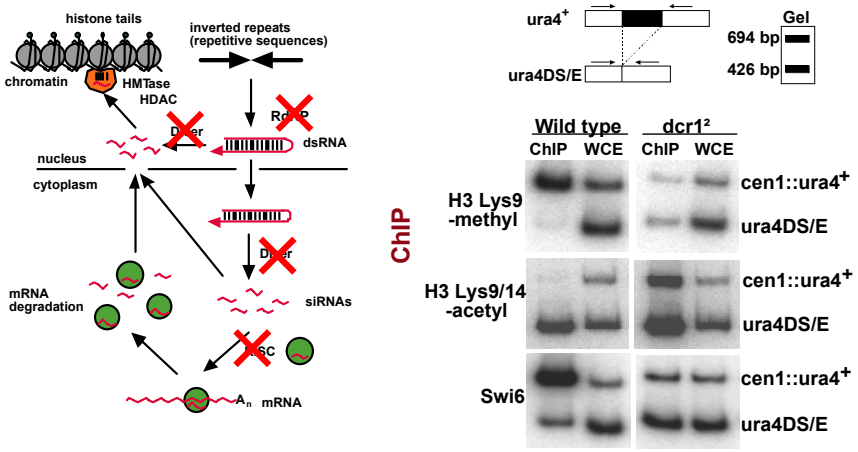


Heterochromatin complexes can be targeted by DNA binding proteins that recognize specific DNA sequences. These DNA binding protein often have modular structures. That is in addition to a DNA binding domain these proteins have effector domains which can recruit histone modifying activities and silencing proteins. However, it is known that repeat sequences of diverse sequence composition can initiate heterochromatin formation in higher eukaryotes.



We believe heterochromatization occurs in response to the presence of transposable elements that are dispersed throughout the genomes. In other words, the presence of these repeats at centromeres, one of which is also present at the mat locus, are key to the initiation of heterochromatin formation at these loci. The idea that these repeats are important for silencing is not new to us. In fact many years ago (in our papers Grewal and Klar 1996 and Grewal and Klar 1997), we showed that deletion of this repeat called cenH abolishes silencing throughout this entire domain.

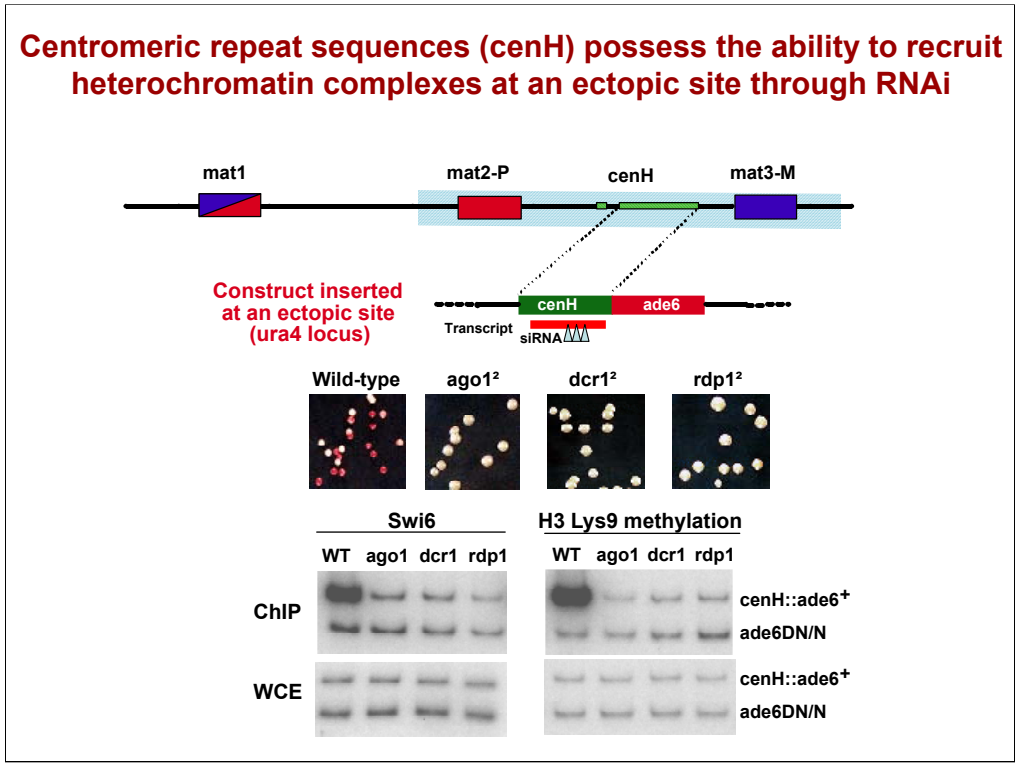
Components of RNAi machinery are required for targeting of histone modifying activities



Mutations in S. pombe homologs of Dicer, RDRP, and Argonaute affect H3 Lys9 methylation, H3 acetylation and Swi6 localization at centromres

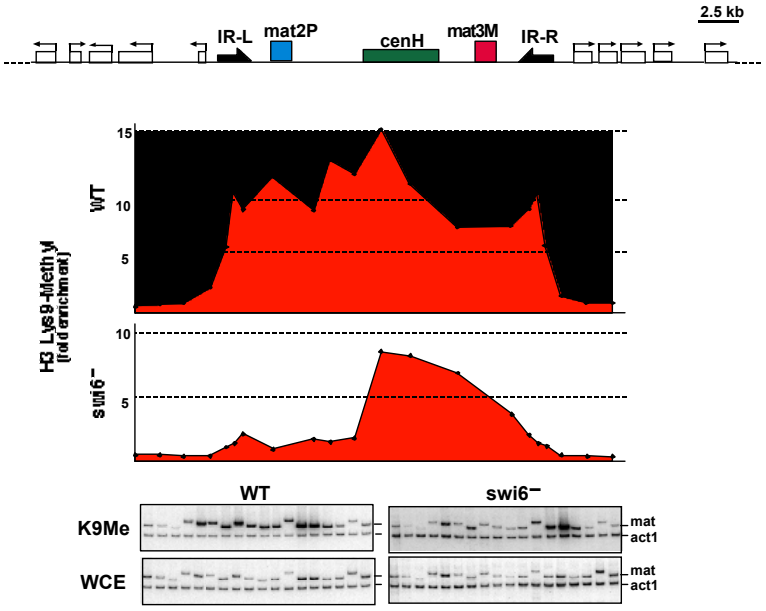
Even more importantly, we recently found that RNAi machinery essential for protecting genome from invasion by viral or transposable elements is required for chromosomal targeting of histone modifying activities. This slide shows classic RNAi pathway proposed by Greg Hannon at CSHL and others. Double stranded RNA produced directly or by RDRP is chopped into small pieces by Dicer. Small RNAs become part of RISC complex which degrades corresponding transcripts. We believe these small RNAs provide specificity for targeting of heterochromatin complexes to where this repeat is present in the genome. As shown in the right side panel, chromatin immunoprecipitation (ChIP) analysis showed that deletions of dicer, RdRP and Argonaute abolish histone H3 lysine methylation and Swi6 protein at centromeres, which are hall marks of heterochromatin. Moreover, these mutations cause increase in acetylation of histones that correlates with loss of heterochromatin at centromeres.

The realization that RNAi is important for heterochromatin formation was not as straightforward. Before Argonaute proteins were shown to be required for RNAi, these proteins had been shown to be important for the maintenance of stem cells. We had knocked out ago1 to study its affect on classic stem cell lineage pattern of mating-type switching. While performing genetics crosses, I however noticed that mutant cells show high incidence of chromosome segregation defects. Considering that chromosome segregation defects are hall mark of silencing defective mutants, we decided to analyze the role of RNAi machinery in centromeric silencing.



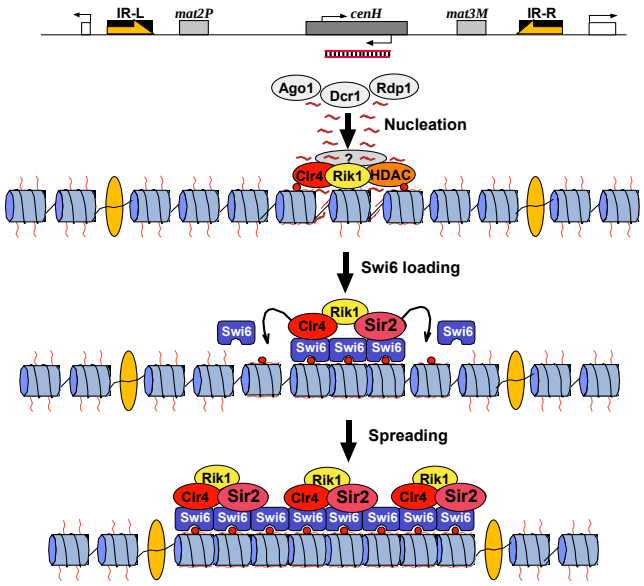
We reasoned that if repeats and RNAi machinery are important for heterochromatin formation, this centromere homology region should be able to induce silencing at an ectopic site, and silencing at ectopic site would depend upon RNAi machinery. To test this, cenH sequences fused to ade6 marker were inserted at ura4 site. We found that ade6 is repressed in wild-type cells as indicated by red color of colonies and this repression is abolished in RNAi mutants, as indicated by white color of colonies. Moreover, ChIP analysis showed that Swi6 and Lys9 methylation are enriched at ectpoic site in wild-type cells but not in RNAi mutants.

cenH is a heterochromatin nucleation center and spreading of H3 Lys9 methylation to surrounding sequences depends on Swi6



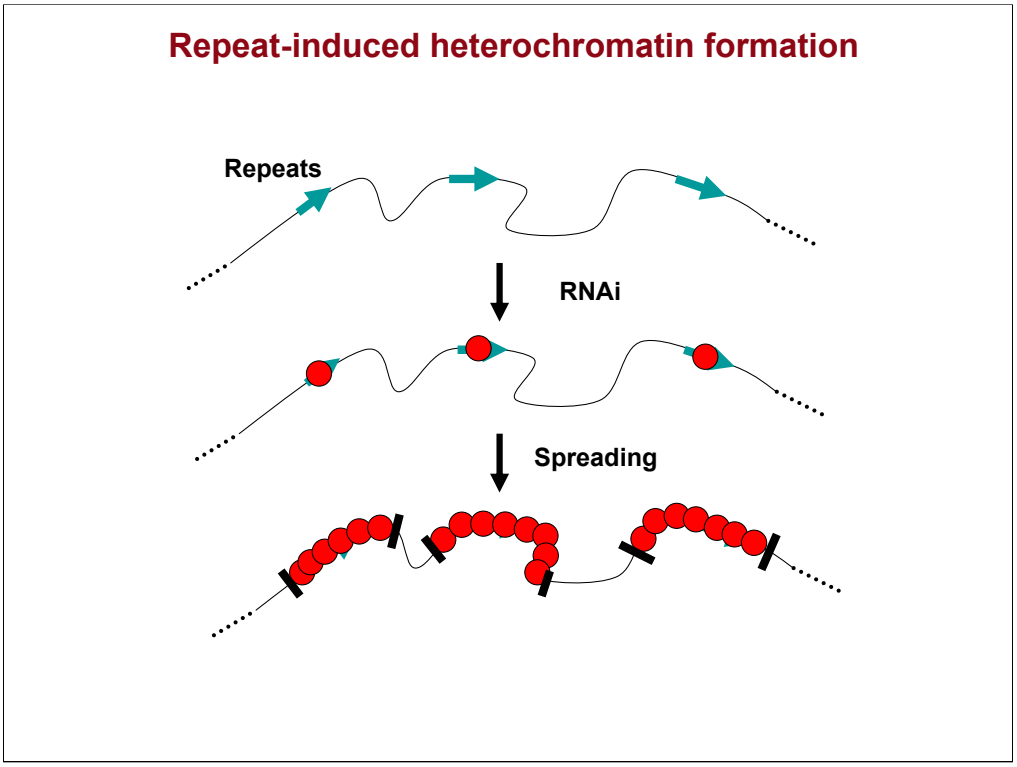
We next found that cenH repeat is indeed a nucleation center for heterochromatin formation. We found that although H3 Lys9 methylation is present throughout the silent mating-type interval in wild-type cells, it is strictly restricted to cenH region in swi6 mutant cells. In other words, H3 Lys9 methylation can be recruited to cenH region independent of Swi6 but its spreading and maintenance at neighboring regions requires Swi6 protein. Collectively, our results suggest that cenH is an RNAi-dependent heterochromatin nucleation center.

Mechanism of heterochromatin assembly at the mat locus



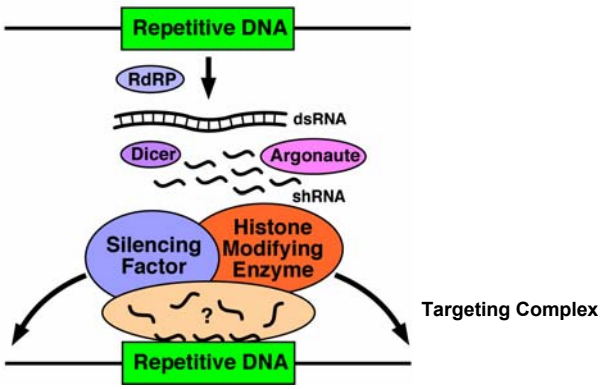
cenH repeat and RNAi cooperate to initiate heterochromatin formation that then spreads to a 20-kb domain, leading to epigenetic silencing of developmentally important mating-type loci

Collectively, our analyses have led to following model for heterochromatin assembly at mat locus. This panel on top shows silent mat region. We believe RNA transcripts produced from cenH are processed by RNAi machinery and help target methyltransferase and deacetylase activities. This initial recruitment is proposed to nucleate heterochromatin by establishing histone code for binding of Swi6. Once recruited Swi6 serves as a platform for recruitment of histone modifying activities that modify adjacent nucleosomes creating additional Swi6 binding sites. This process is repeated many times leading to spreading of heterochroman till they hit boundary elements. In other words, heterochromatin assembly occurs in two steps, first RNAi dependent initiation of heterochromatin and the second RNAi-independent spreading. The spreading of heterochromatin causes epigenetic silencing of mating-type genes. It is remarkable that heterochromatin structures nucleated at a repeat element controls expression of developmentally important genes.



One key advantage of RNAi mediated heterochromatin targeting is that any repeat capable of generating dsRNA could serve as a heterochromatin nucleation center. The spreading of heterochromatin from these repeats to surrounding sequences would have the potential to silence nearby genes, similar to cenH repeat mediated silencing of mating-type genes.

Model for RNAi-mediated targeting of heterochromatin

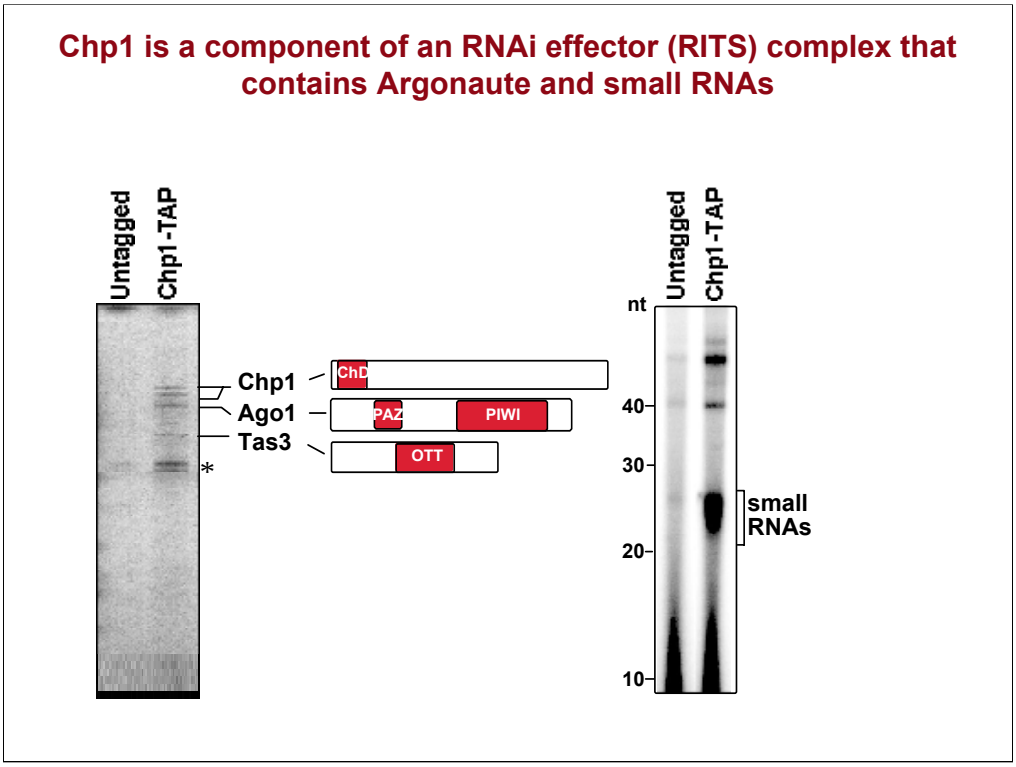


How do RNAi and small RNAs target heterochromatin? We previously hypothesized that a targeting complex, which uses small RNAs to provide specificity for its targeting to specific loci, would be play an important role in heterochromatin nucleation through recruitment of histone modifying activities and silencing proteins.

Chp1

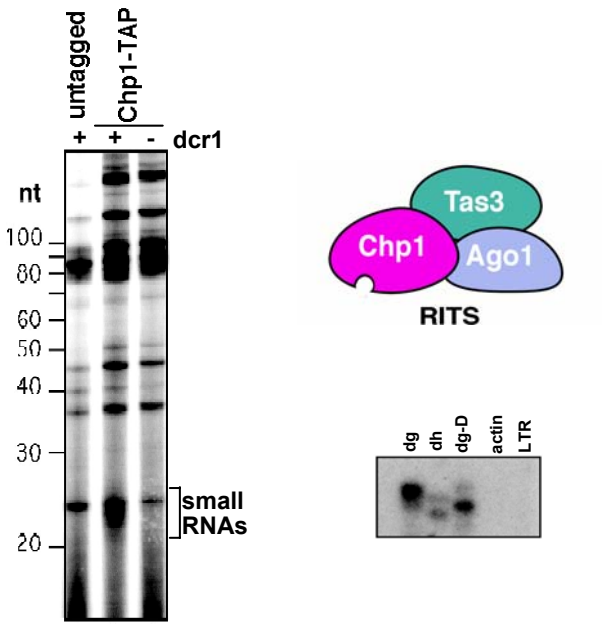
- Chp1 localizes to centromeric regions
- Chp1 is required for establishment of heterochromatin specific histone modification pattern
- chp1 mutant cells display phenotypes identical to cells carrying deletions of ago1, dcr1 or rdp1, factors involved in RNAi

To identify the components of this RNAi-mediated heterochromatin targeting complex we reasoned that such factors would act in early steps during heterochromatin assembly and would be required for the establishment of heterochromatin specific histone modification pattern. Moreover, such factors might bind to heterochromatic regions. In this regard, one of the proteins, Chp1, involved in heterochromatin formation is particularly interesting. It contains….(see slide)

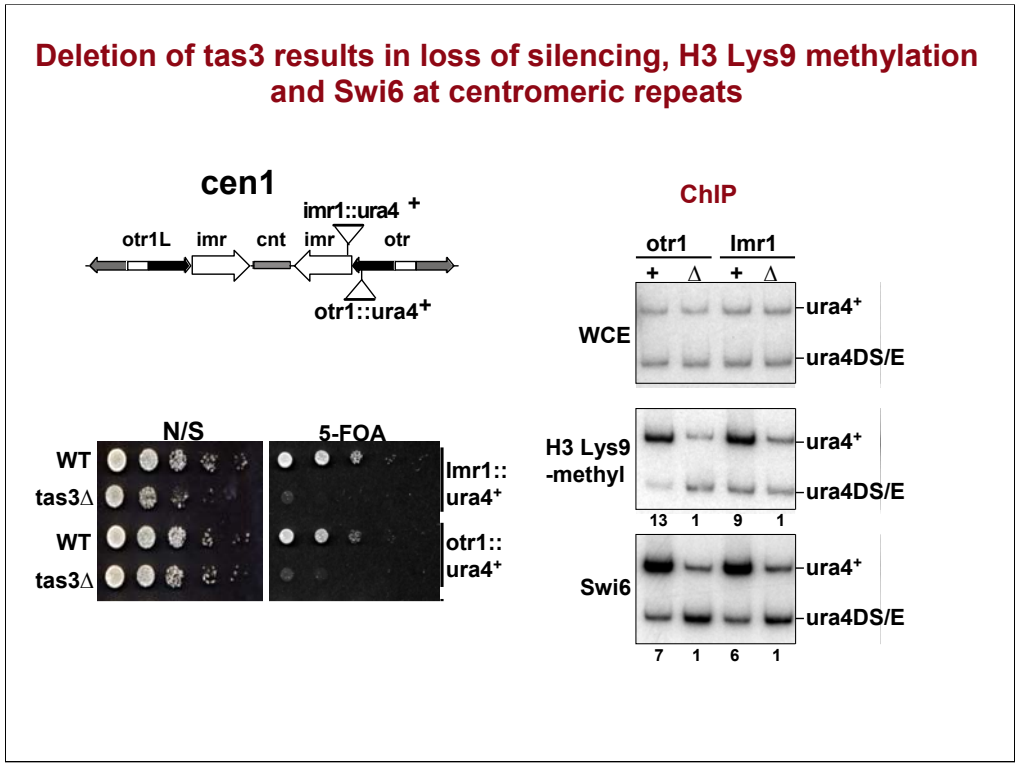


To characterize the possible involvement of Chp1 we, in collaboration with Dr Danesh Moazed's laboratory at Harvard Medical School purified this protein and identified its interacting factors. Purification of the Chp1 revealed that it associates with Argonaute involved in RNAi and a novel protein that we named Tas3. Moreover, purified Chp1 complex, which we have named RITS, contains small RNAs ranging in size from 22-25 nucleotides.

Small RNAs associated with RITS complex are Dicer products derived from centromeric repeat sequences

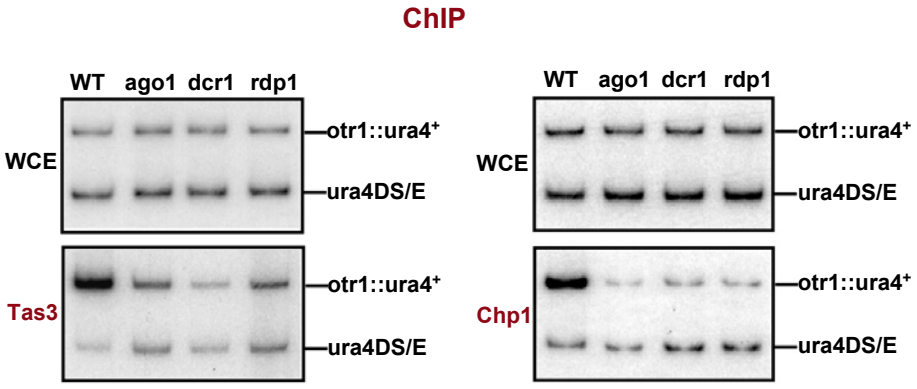


These small RNAs are not detected when complex is purified from dicer deletion strains, and these RNAs hybridize to PCR fragments derived from dg and dh centromeric repeats first identified by Yanagida lab. Interestingly, these small RNAs did not hybridize to LTR that were recently suggested to recruit heterochromatin in an RNAi-dependent manner. Interestingly, the proteins components of RITS are assembled into complex I dicer mutant cells but complex fails to localize to centromeres, as shown in next slide.



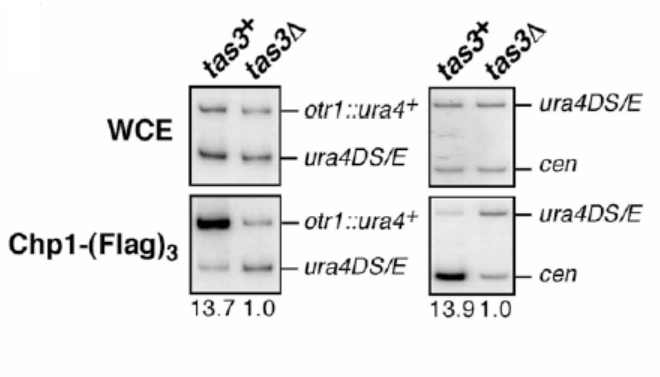
Chp1 and Ago1 are required for heterochromatin formation at centromeres. We investigated whether Tas3, a novel protein associated with RITS, also affects heterochromatin assembly. We found that deletion of Tas3 abolished silencing of markers inserted at the inner and outer centromeric repeats. Moreover, as we reported previously in the case of RNAi mutants, deletion of tas3 did not affect silencing at the mating-type region. ChIP analyses showed that deletion of tas3 abolishes histone H3 Lys9 methylation and Swi6 localization at ura4 marker inserted at imr and otr centromeric repeats. So like Chp1 and RNAi machinery Tas3 is required for heterochromatin assembly at centromeres.

Localization of RITS at centromeres requires RNAi



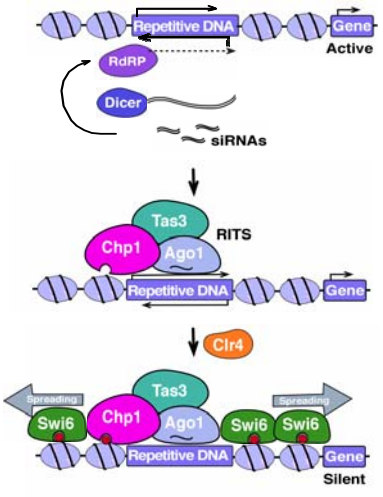
We next investigated if Tas3 binds to centromeric repeats and whether its localization is dependant upon RNAi pathway. Interestingly, like Chp1, Tas3 is localized to centromeres. More importantly, the localization of both Tas3 and Chp1 to centromeric repeats require RNAi machinery.

Tas3 is required for localization of Chp1 at centromeric repeats



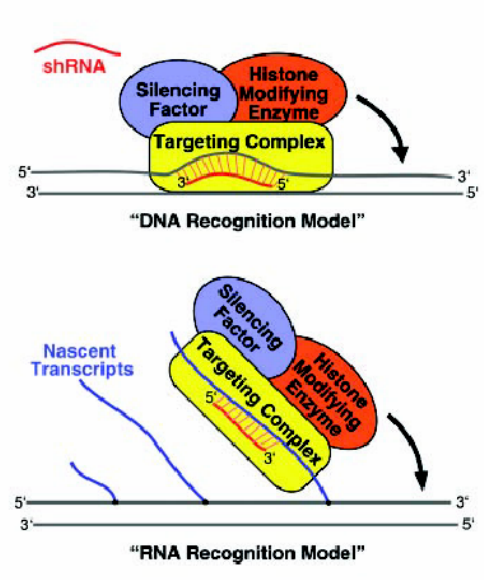
We also investigated whether Tas3 protein is required for Chp1 localization. Indeed, deletion of tas3 resulted in loss of Chp1 at centromeres as shown by ChIP analysis.

RITS-mediated targeting of heterochromatin



This slide shows model for RNAi-mediated heterochromatin formation.

Models for RNAi-mediated sequence specific initiation of heterochromatin assembly



Grewal and Moazed Science 2003

A key question is how do small RNAs provide specificity for targeting of heterochromatin complexes to specific sequences. By analogy to targeting of mRNAs by the RISC complex we suggest that RNAi-mediated recruitment of heterochromatin targeting complex involve pairing between siRNAs with either DNA or nascent RNA transcripts at the target locus.

Acknowledgments

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