

## **LECTURE 6**

### **THE RNAi MACHINERY AND REGULATION OF HETEROCHROMATIN**

# Small ncRNA and gene/chromatin regulation

*micro-RNAs = miRNAs*

*short interfering RNAs = siRNAs*

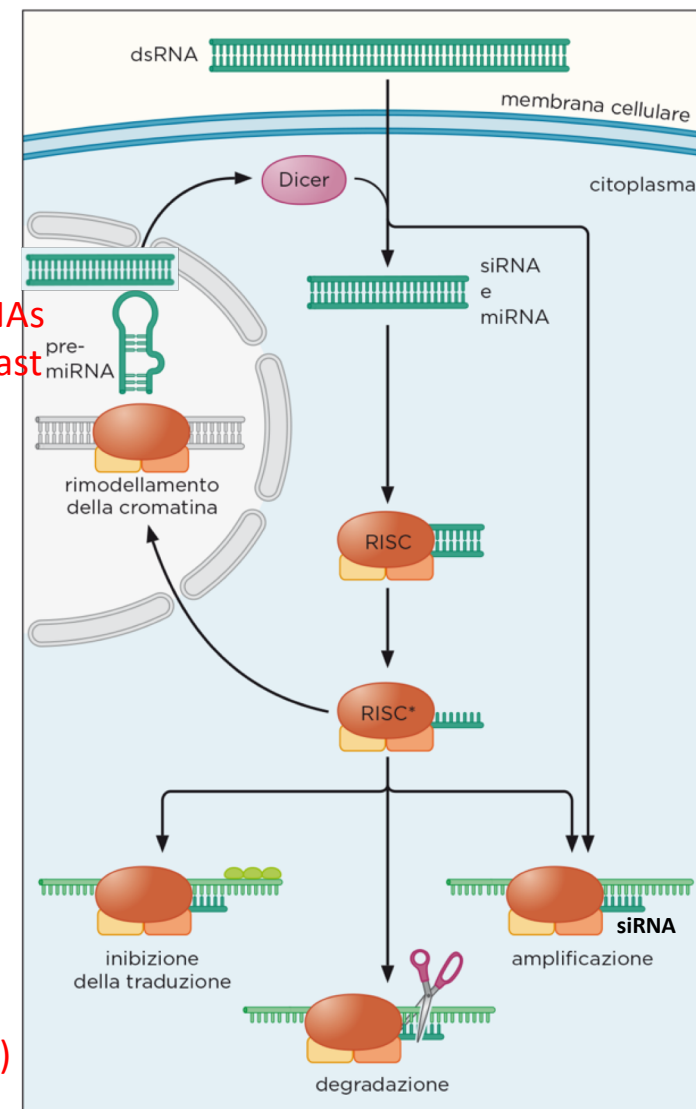
**miRNAs and siRNAs are generated by the same machinery**

1. Long precursor RNA
1. Processing into small RNAs by Type 3 RNase Dicer  
siRNAs and miRNAs (21-23 nt) still double-stranded
3. Processing by RISC complex  
(RNA induced silencing complex)
4. guide RNA → regualtory RNA  
passenger RNA → will be eliminated
5. RISC complex+guide RNA → regulatory function
  - A. **RNA degradation = siRNA effect (cutting = “slicing”)**
  - B. **inhibition of mRNA translation =mRNA effect**
  - C. **transfer to nucleus and chromatin regulation = siRNA mediated silencing**

miRNAs: always “trans”-acting on mRNAs

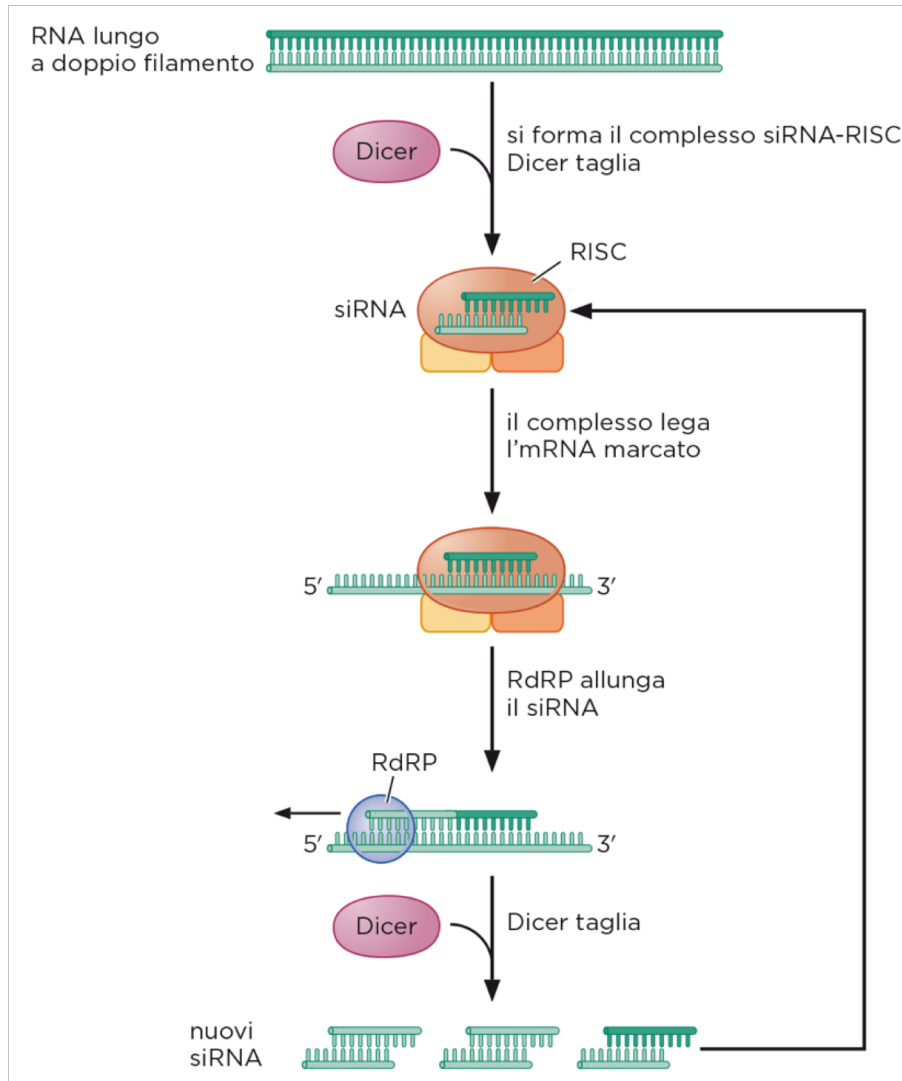
siRNAs: mostly “cis” acting on chromatin (S-pombe)

NO  
miRNAs  
in yeast





## RNA dependent RNA polymerase amplifies siRNAs

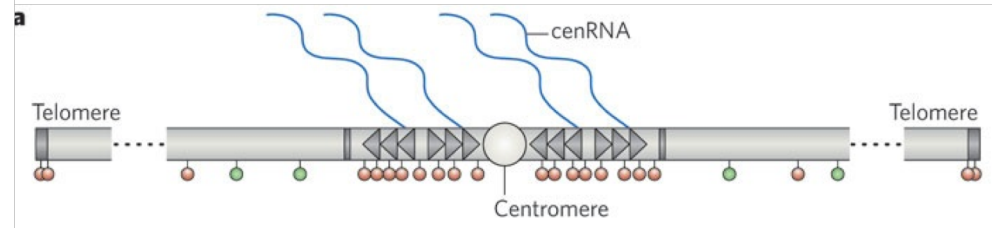
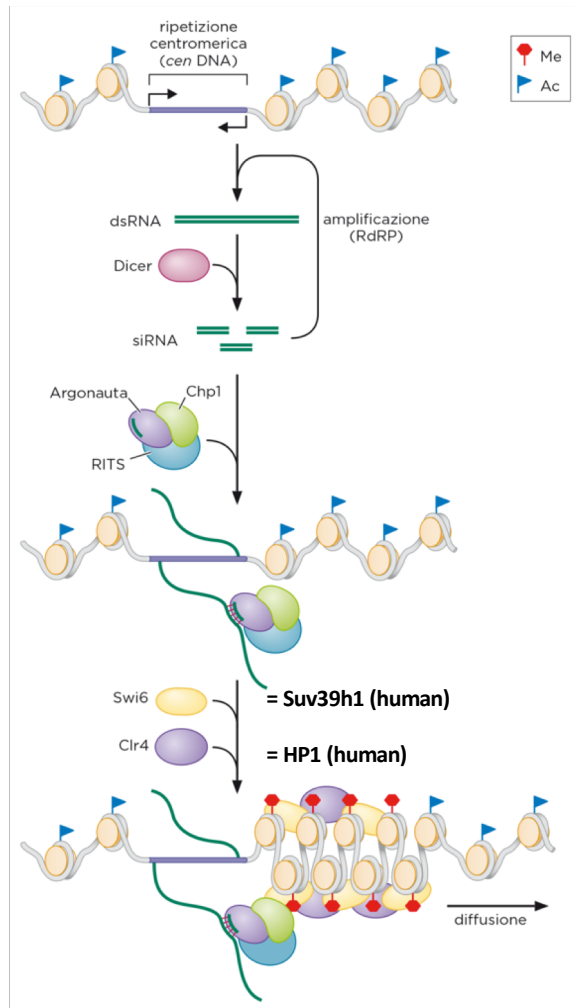


In plants, *S. pombe*, nematodes, human,  
NOT IN *S. CEREVISIAE*

NO RNA dependent RNA polymerase in Vertebrates

siRNAs as endogenous  
**epigenetic regulators**

# The first model for siRNAs and chromatin regulation (induction): *S. pombe*: siRNA mediated chromatin regulation (silencing)



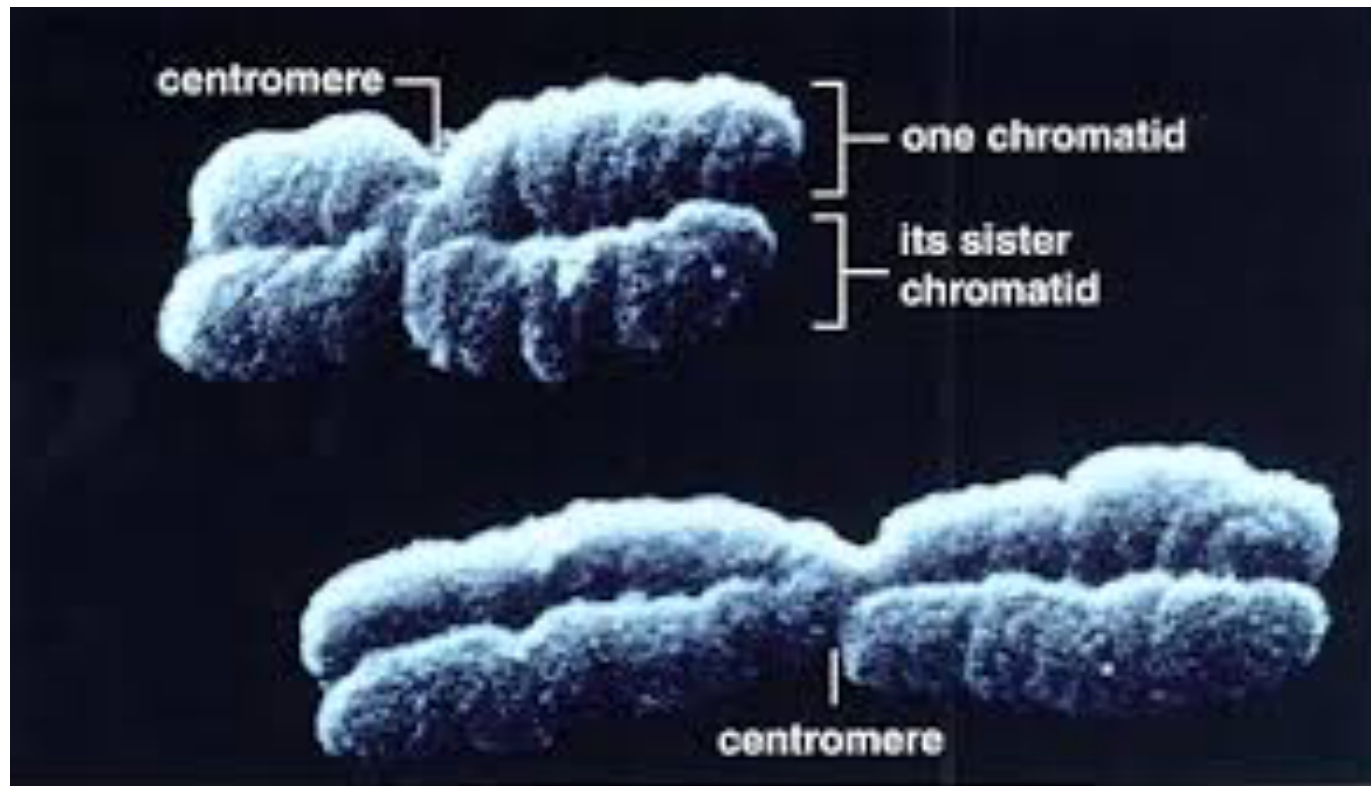
## Centromeres in *S. pombe*:

- Heterochromatin (H3K9me3, Clr4 (HP1))
- Reporter genes inserted: repression

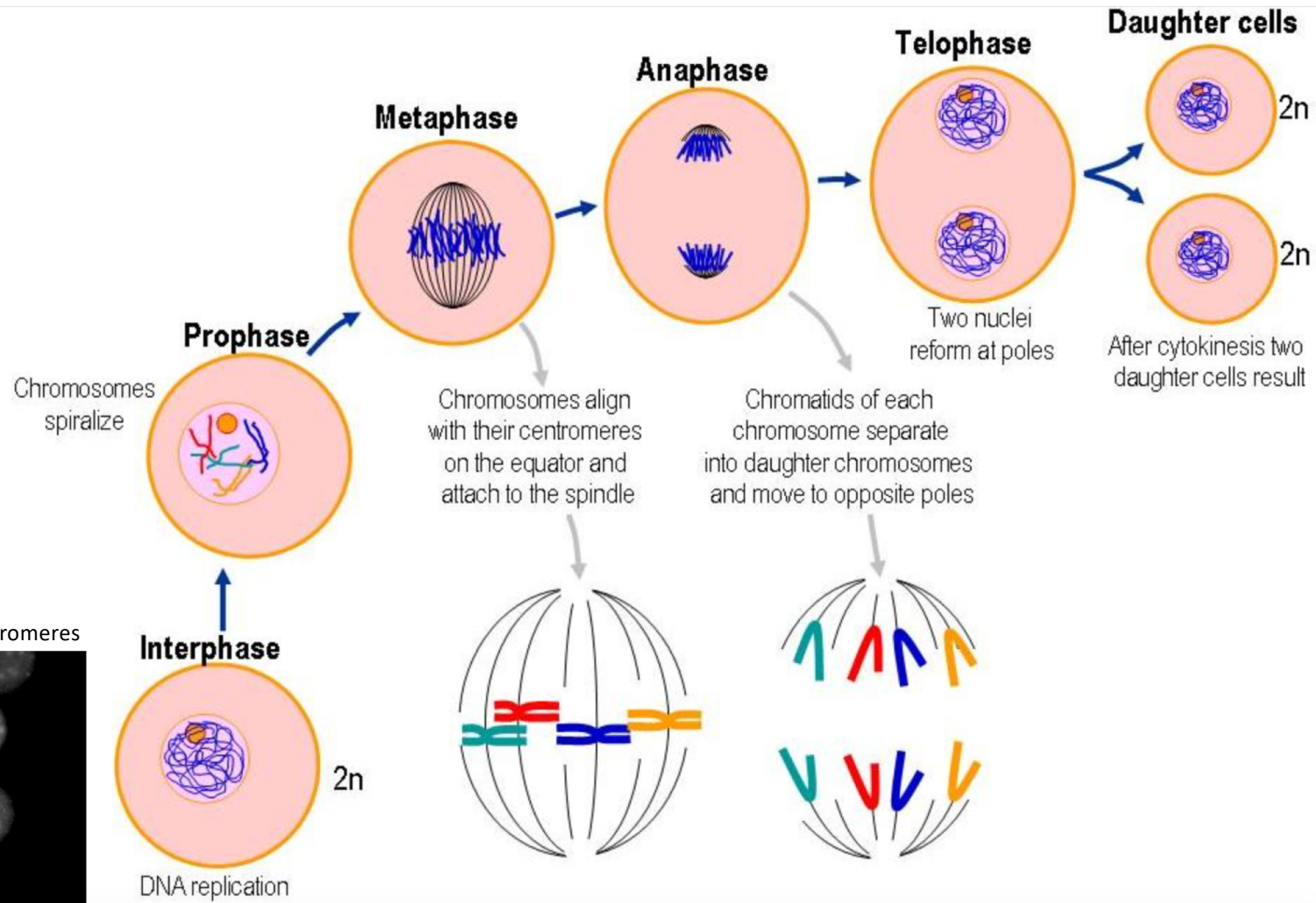
## -Discovery:

RNAi mutant result in loss of H3K9me3/Clr4 and reactivation of reporter gene that was inserted into centromeric region  
=RNAi mediated gene silencing

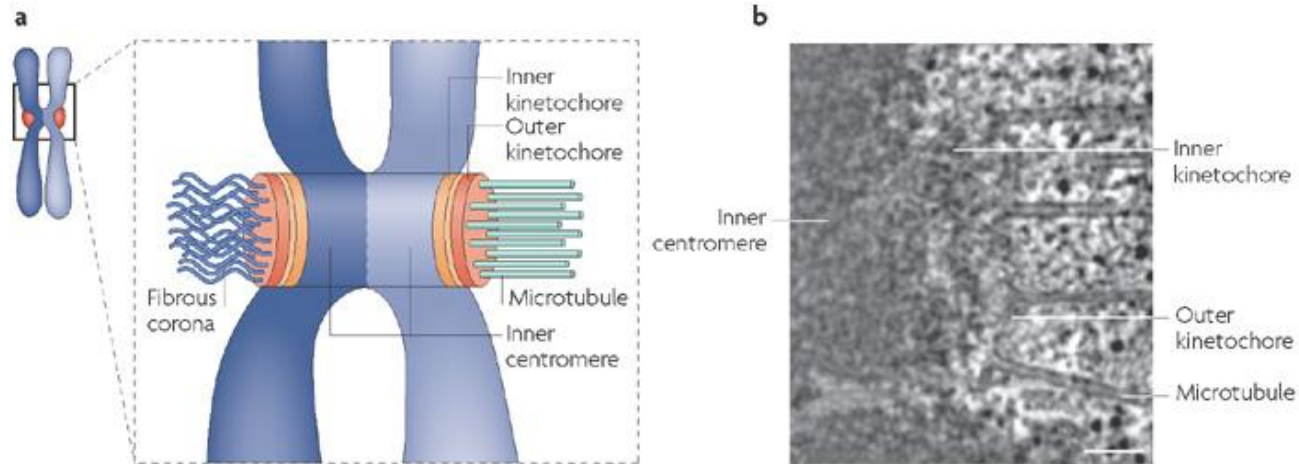
## Eukaryotic centromeres



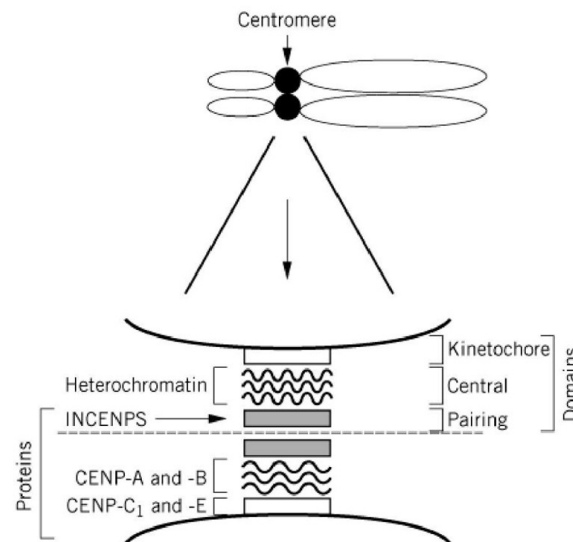
## Eukaryotic centromeres



## Functional regions at centromeres

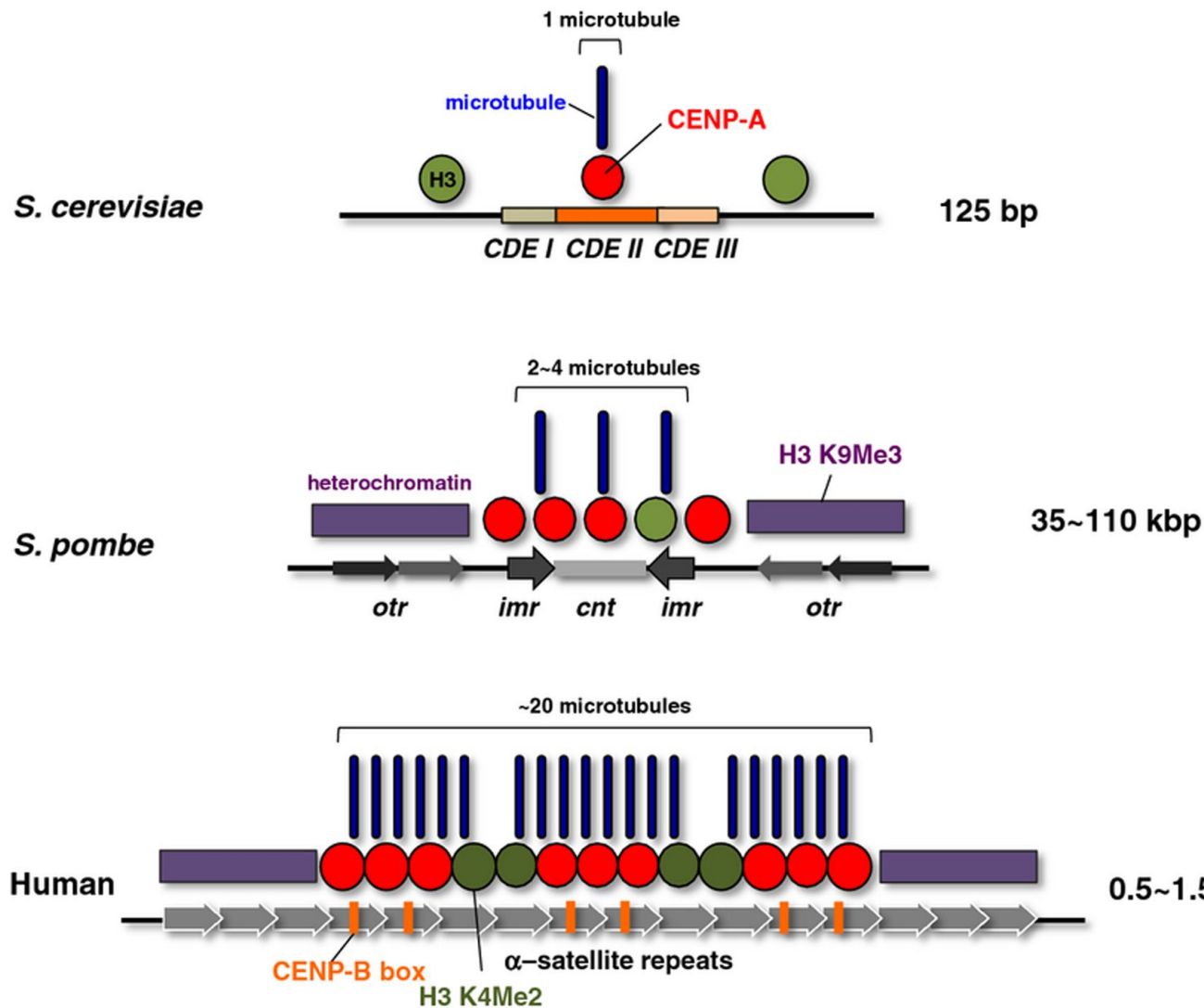


Nature Reviews | Molecular Cell Biology



**Centromeric DNA consists of constitutive heterochromatin**

# Centromere structure in diverse organisms



The CENPA gene encodes a centromere protein which contains a histone H3 related histone fold domain that is required for targeting to the centromere. CENPA is proposed to be a component of a modified nucleosome or nucleosome-like structure in which it replaces 1 or both copies of conventional histone H3 in the (H3-H4)<sub>2</sub> tetrameric core of the nucleosome particle.

Diversity of centromeric structure and CENP-A nucleosome distribution among eukaryotes.

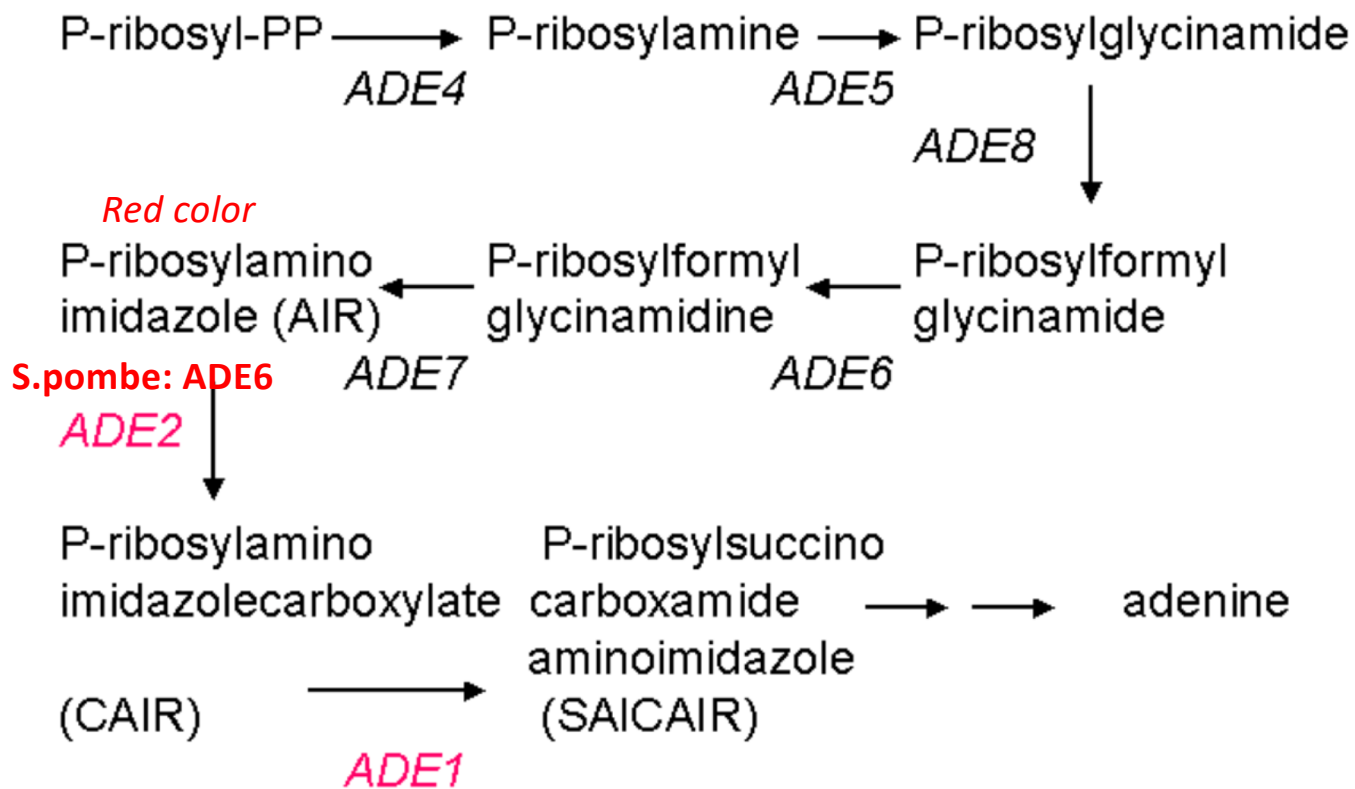
Schematic representation of centromeres in budding yeast (*Saccharomyces cerevisiae*), fission yeast (*Schizosaccharomyces pombe*) and human.

1. Budding yeast has a point centromere, which is defined by a 125-bp sequence and is occupied by a single CENP-A containing nucleosome. There are three conserved elements in budding yeast CEN DNA: CDE (centromere DNA element) I, CDEII and CDEIII
2. *Schizosaccharomyces pombe* and human have regional centromeres that are flanked by pericentromeric heterochromatin. In *Schizosaccharomyces pombe*, a multiple number of CENP-A (Cnp1)-containing nucleosomes are assembled onto unique sequences (cnt – centromeric sequence and imr – innermost repeats). Pericentric, heterochromatic repeats are called “outer repeats” (otr)
3. Human centromeres consist of  $\alpha$ -satellite DNA arranged in tandem into higher order repeats (each arrow), and some  $\alpha$ -satellite DNA contains CENP-B binding sites (CENP-B box). CENP-A localizes to a portion of these arrays. The number of microtubule attachment sites also varies among organisms. CENP-B is a highly conserved centromere protein in mammals and binds to a 17-bp motif in a CENP-B box. It has been shown that  $\alpha$ -satellite DNA with a CENP-B box is responsible for de novo centromere assembly in human somatic cells.

Satellite DNA consists of very large arrays of tandemly repeating, non-coding DNA. Satellite DNA is the main component of functional centromeres, and form the main structural constituent of heterochromatin.

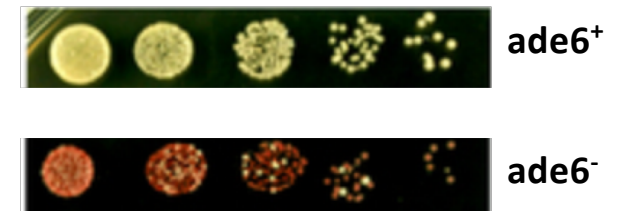


Ade6 mutants in *S.pombe* (ADE2 in *S. cerevisiae*) generate red-colored intermediates of the adenine biosynthesis pathway



The adenine biosynthesis pathway in *S.cerevisiae*

*S. pombe*



### Strategy:

Generate *S.pombe* strain with Ade6 mutation.

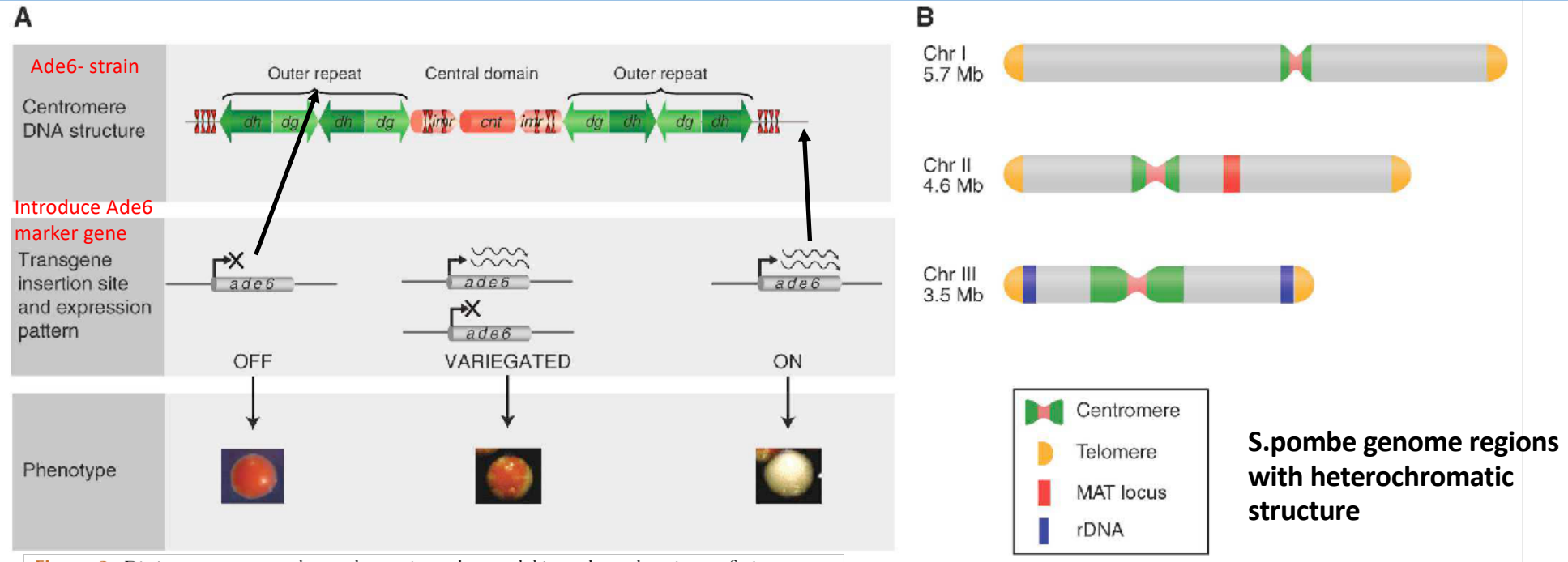
Re-introduce Ade6 maker into the genome.

If located in euchromatin: White colony

If located in pericentric heterochromatin: Red colony



# Centromere structure in *S.pombe*



**Figure 2.** Distinct outer repeat heterochromatin and central kinetochore domains at fission yeast centromeres. (A, top) Representation of a fission yeast centromere. The central domain (pink, kinetochore) is composed of *imr* and *cnt* elements, the outer repeats contain transcribed *dg* and *dh* repeats (green, heterochromatin). All three centromeres have a similar overall arrangement; however, the number of outer repeats differs: *cen1* (40 kb) has two, *cen2* (65 kb) has three, and *cen3* (110 kb) has approximately 13. Clusters of transfer RNA (tRNA) genes (double arrowheads) occur in the *imr* region and at the extremities of all three centromeres. (Middle) Schematically shows transcription patterns of marker genes placed within the outer repeats, central domain, or beyond the centromere. (Bottom) Images showing the phenotype of *S. pombe* colonies of *ade6*<sup>+</sup> transgenics inserted at various sites within the centromere. Cells expressing *ade6*<sup>+</sup> from a transgene inserted in sequences outside the centromere form white colonies. When *ade6*<sup>+</sup> is inserted at sites within the outer repeats, expression is silenced and red colonies are formed. Expression of *ade6*<sup>+</sup> from the central domain is typically variegated, resulting in red, white, and sectorial colonies. (B) A schematic representation of *S. pombe* chromosomes. The three chromosomes are depicted showing the four main regions of heterochromatin: centromere, telomere, *mat2/3*, and rDNA regions.

## Ade6 reporter gene system to measure chromatin structure:

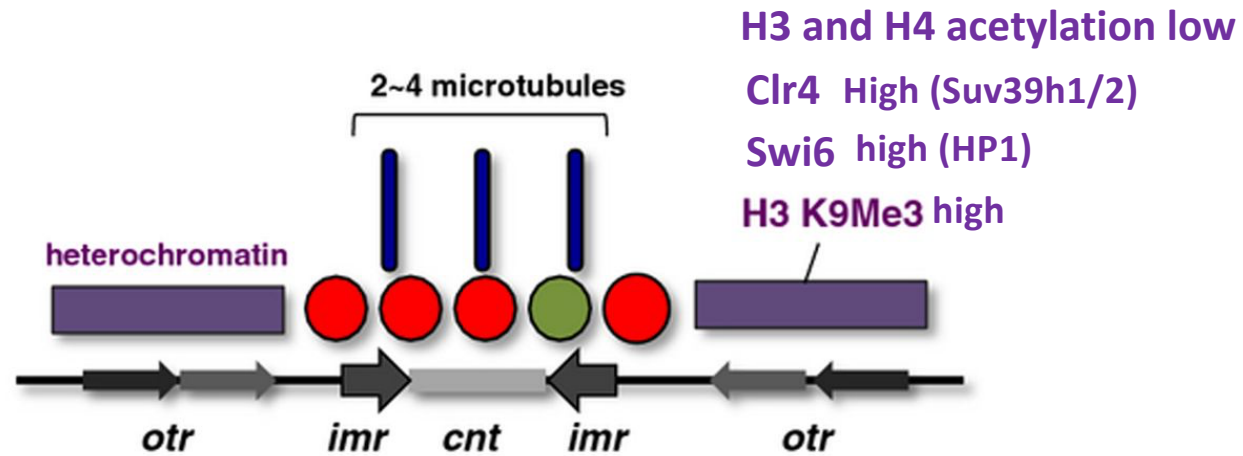
When expressed - euchromatin: white colony

When silenced - heterochromatin: red colony

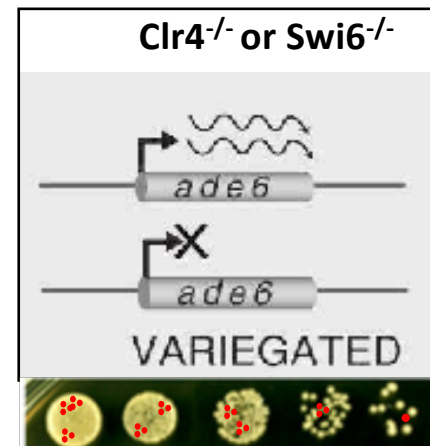
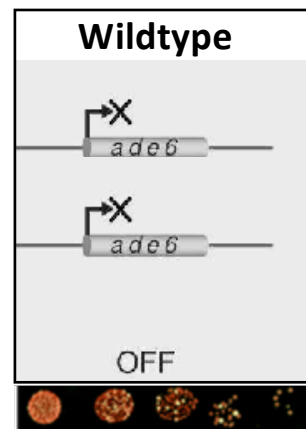
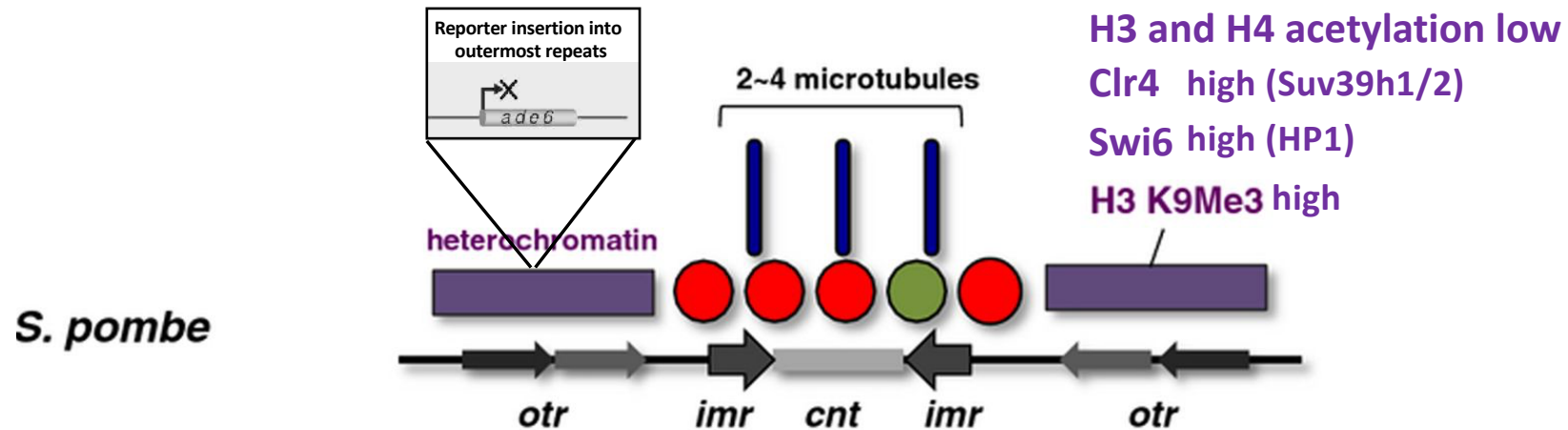
Ura4 reporter system: Ura4 converts agent to toxin → cell dies when Ura3 is expressed

## Centromere structure in *S. pombe*

*S. pombe*



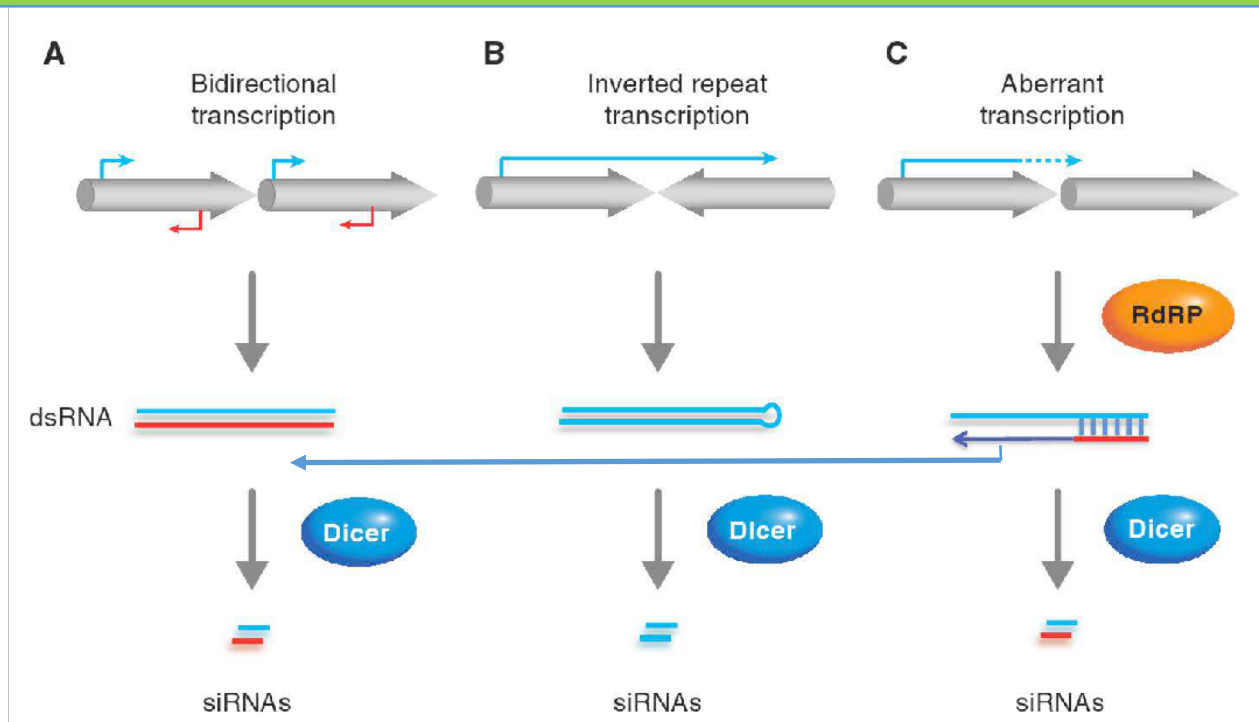
## Centromere structure in *S. pombe*



How is heterochromatin recruited  
To centromeres??

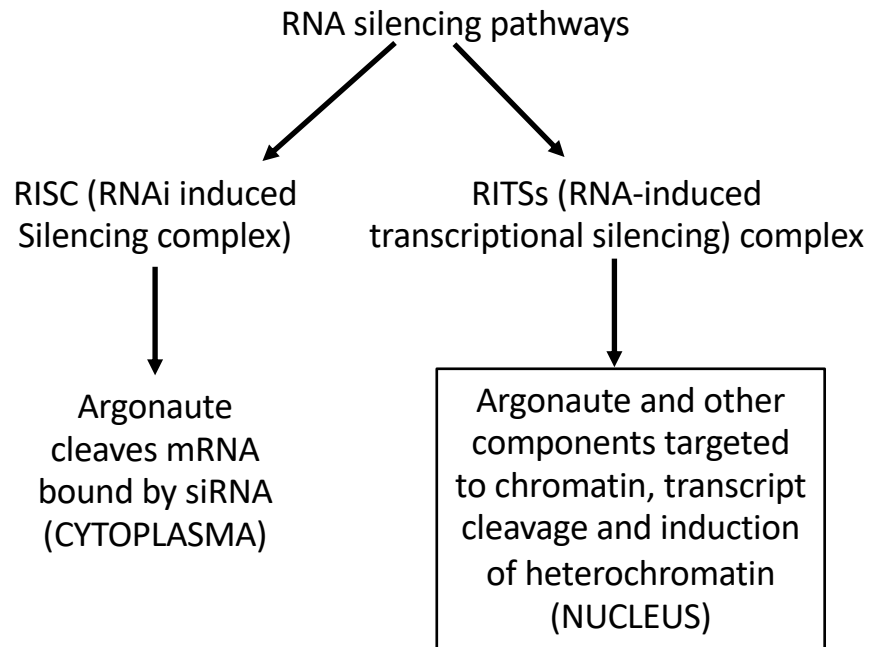
BUT ALSO IN RNAi MUTANTS (for example Dicer<sup>-/-</sup>)

## Sources of RNAs for siRNA generation



**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.

# RNAi silencing pathways



**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 <sup>a</sup>	CMT3	–	–	–
Tas3 <sup>b</sup>	–	AIN-1	GW182	TNRC6
Rdp1	RDR1 to 6	Ego-1, Rrf-1 to -3	–	–
Hrr1	SGS2/SDE3 <sup>c</sup>	ZK1067.2	GH20028p	KIAA1404
Cid12	–	Rde-3, Trf-4 <sup>c</sup>	CG11265 <sup>c</sup>	POLSC <sup>c</sup>
Swi6	LHP1 (TFL2)	Hpl-1, F32E10.6 <sup>d</sup>	Hpl-2, HP1a, b	HP1α, β, γ
Clr4	SUVH2 to 6	–	Su(var)3-9	Suv39h1 and 2
Rik1 <sup>e</sup>	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

<sup>a</sup>An 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.

<sup>b</sup>Tas3 is a GW motif protein. Members of this conserved family are found associated with Argonaute family members.

<sup>c</sup>Cid12 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.

<sup>d</sup>*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.

<sup>e</sup>*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.

# The RNAi machinery degrades RNAs derived from centromeres

Volpe et al. 2002  
Science

## Step1: *ura4<sup>-</sup>* strain

*ura4<sup>-</sup>* renders cells resistant to 5-fluoroorotic acid (5-FOA)

## Step2: insert a URA4 mini gene into chromosomal arm

(produces a short, non-functional URA4 RNA)

## Step3: insert a URA4 full length gene into centromeric

Positions (*cnt*, *imr*, *otr*)

(produces a full length, functional URA4 mRNA (=marker) when expressed)

→ In centromeric positions URA4 marker gene is silenced

→ =5FOA resistant

## Step4: Elimination of RNAi components and HC

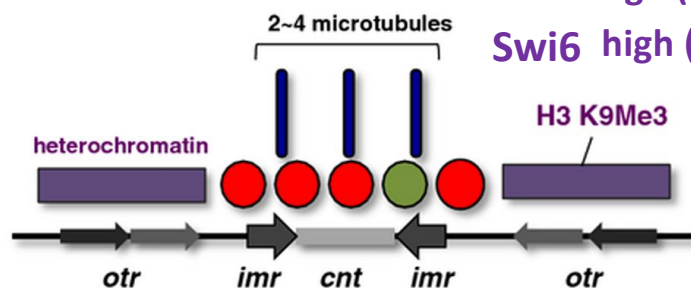
components

or: random mutagenesis screen

## Step5: Interesting yeast strains: 5FOA sensitive; Identify mutated genes

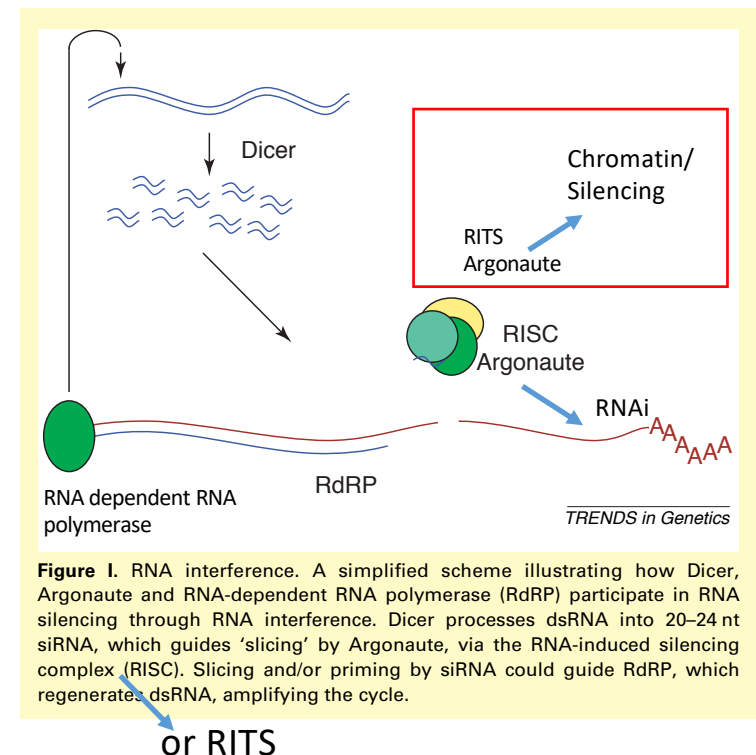
*Clr4* High (*Suv39h1/2*)  
*Swi6* high (HP1)

*S. pombe*



## Components of the RNAi machinery in *S. pombe*:

1. Dicer: cleavage of dsRNA into siRNAs
2. RISC/RITS with Argonaute (RNase domain): target RNA cleavage guided by siRNA
3. RNA dependent RNA polymerase: RdRP: reverse transcription of RNA, guided by siRNA → creates dsRNA → RNAi amplification



**Figure 1.** RNA interference. A simplified scheme illustrating how Dicer, Argonaute and RNA-dependent RNA polymerase (RdRP) participate in RNA silencing through RNA interference. Dicer processes dsRNA into 20–24 nt siRNA, which guides ‘slicing’ by Argonaute, via the RNA-induced silencing complex (RISC). Slicing and/or priming by siRNA could guide RdRP, which regenerates dsRNA, amplifying the cycle.

# The RNAi machinery degrades RNAs derived from centromeres

Volpe et al. 2002 Science

## Step1: *ura4<sup>-</sup>* strain

*ura4<sup>-</sup>* renders cells resistant to 5-fluoroorotic acid (5-FOA)

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## Step3: insert a URA4 full length gene into centromeric Positions (*cnt*, *imr*, *otr*) (produces a full length, functional URA4 mRNA (=marker) when expressed)

- In centromeric positions URA4 marker gene is silenced
- =5FOA resistant

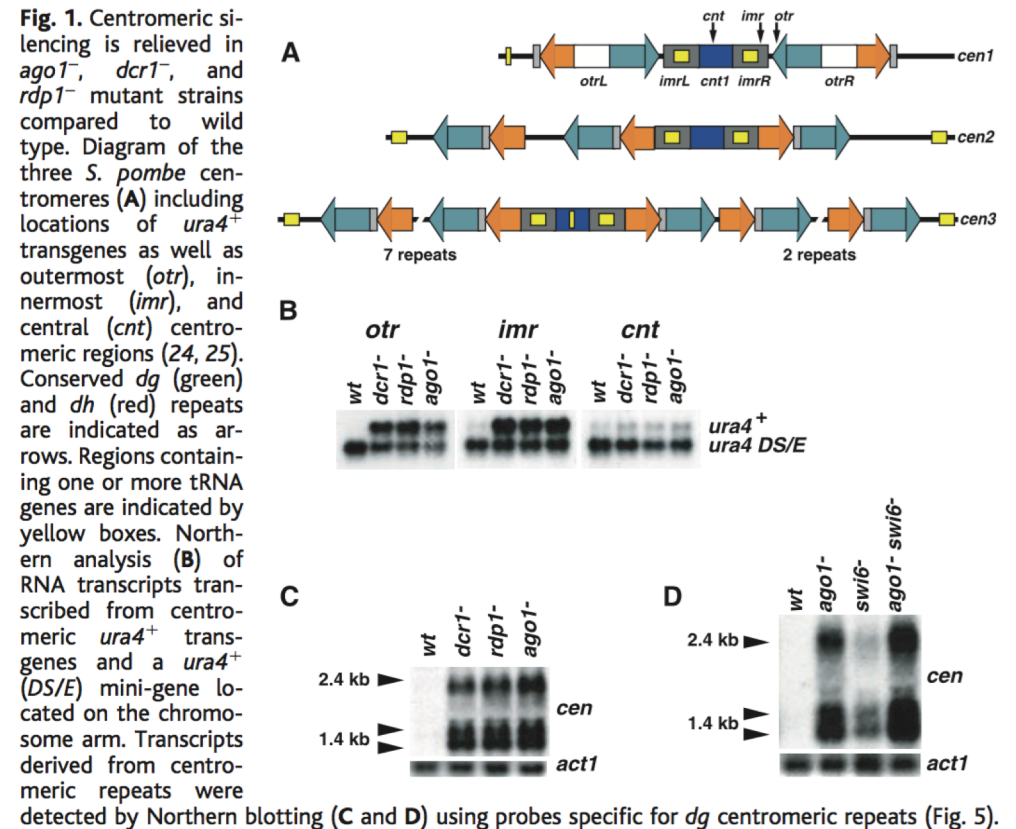
## Step4: Elimination of RNAi components and HC components

Or random mutagenesis screen

## Step5: Interesting yeast strains: 5FOA sensitive; identify mutated gene(s)

### Model system: 3 URA reporter genes:

1. URA4 reporter in centromere regions (*otr*, *imr*, *cnt*) → *ura4<sup>+</sup>*
2. URA4 minigene (shorter than centromeric URA4 located in a chromosomal arm (*ura4* DS/E)



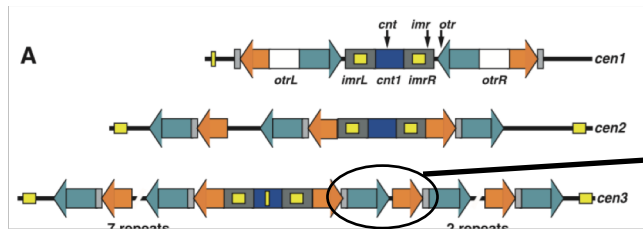
**B: loss of RNAi machinery results in increases URA4 reporter expression**

**C: loss of RNAi machinery results in increased centromeric transcript levels**

**D: loss of RNAi and loss of Swi6 results in increase centromeric transcript levels**

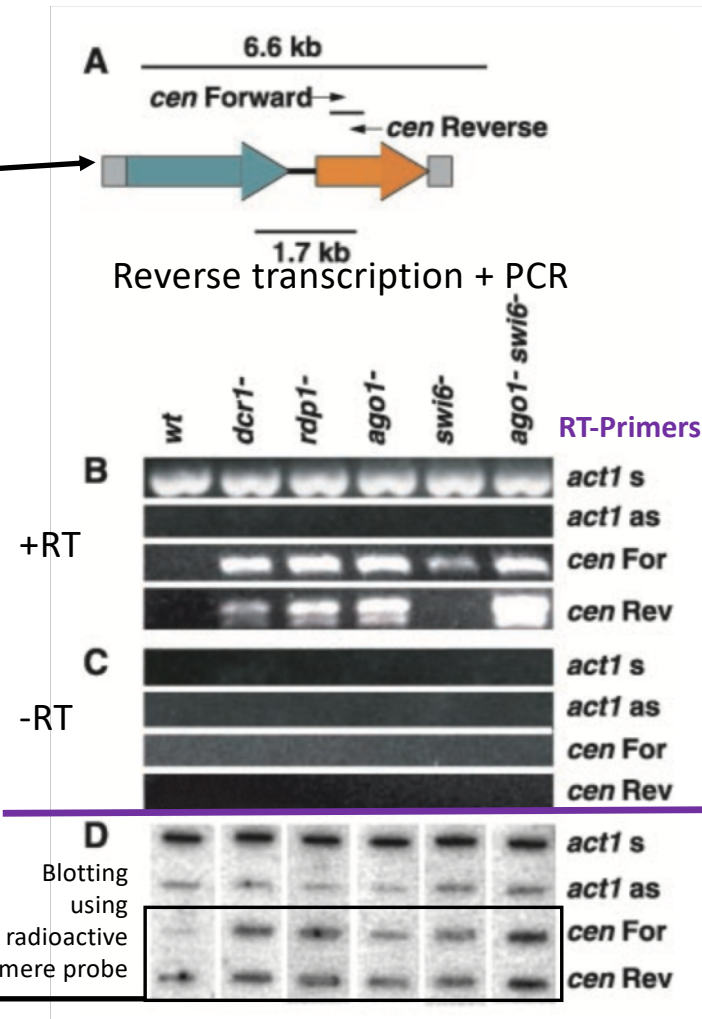


# The RNAi machinery degrades RNAs derived from centromeres



In wt cells production of centromeric reverse transcripts  
 Loss of RNAi machinery results in an increase of forward – reverse RNAs derived from pericentric repeats

Preparation of **nascent** RNA  
 RNA that is in progressing transcription. RNA still associated at chromatin  
 The centromeric forward strand is degraded in wt cells; but accumulates in RNAi<sup>-/-</sup> cells



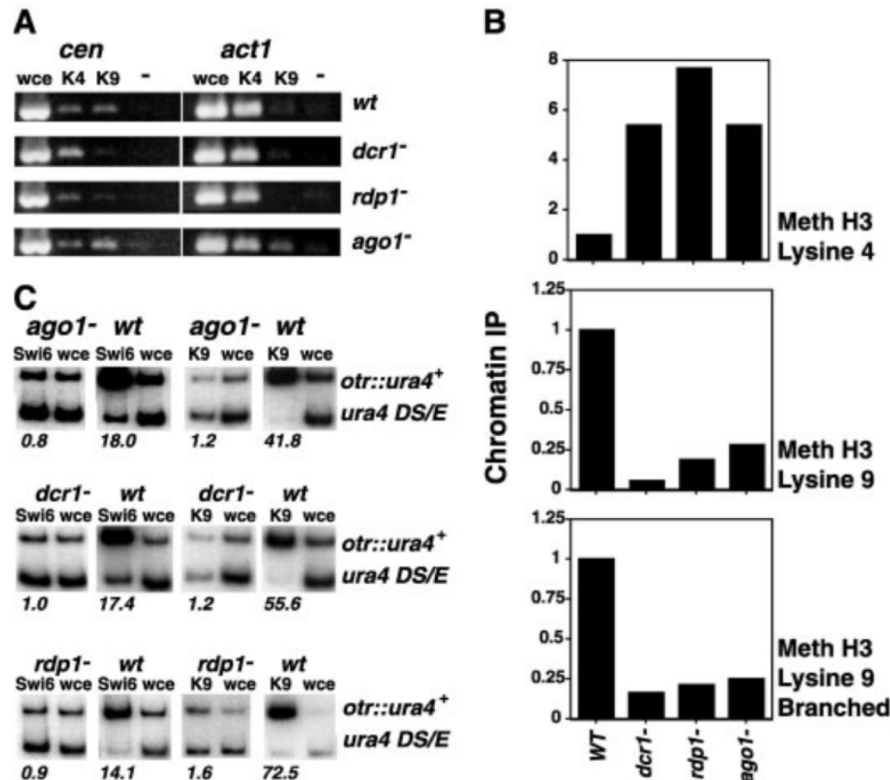
**Fig. 2.** Centromeric *otr* transcripts are both transcriptionally and posttranscriptionally regulated. **(A)** A schematic diagram showing the direction of transcription of forward and reverse transcripts corresponding to the *dh* repeat. Strand-specific RT-PCR analysis was performed in the presence **(B)** or absence **(C)** of reverse transcriptase. Samples were incubated with primers from the *dh* repeat complementary to either the forward (*cen* For) or reverse (*cen* Rev) centromeric transcripts in first strand cDNA synthesis reaction (primer locations are summarized in Fig. 5). Both primers were present in subsequent cycles of PCR amplification after heat inactivation of the reverse transcriptase. Treatment of control reactions lacking reverse transcriptase **(C)** was identical except that these samples were not subjected to first strand synthesis. Strand-specific control reactions were also conducted using primers specific for *act1* sense (*act1* s) or *act1* antisense (*act1* as) transcripts. Strand-specific analysis of nascent RNA transcripts was performed by nuclear run-on assay **(D)**. Radiolabeled nascent RNA purified from mutant and wild-type strains was hybridized to nylon membranes containing strand-specific probes made using the same primer pair as in **(A)**. These probes recognized either forward (*cen* For) or reverse (*cen* Rev) centromeric transcripts. Control probes recognized either sense (*act1* s) or antisense (*act1* as) actin transcripts.



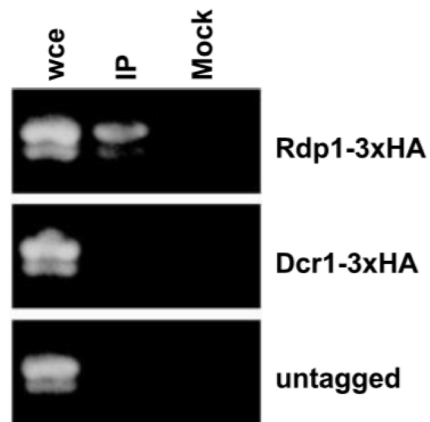
## Loss of the RNAi machinery results in the loss of H3K9me3 at outermost repeats

**Fig. 3.** Chromatin structure at centromeric repeats is altered in *ago1*<sup>-</sup>, *dcr1*<sup>-</sup>, and *rdp1*<sup>-</sup> mutants. Chromatin immunoprecipitation (ChIP) was performed on extracts from formaldehyde fixed mutant or wild-type cells. DNA fragments purified from whole-cell extracts (wce) or co-precipitated with antibodies to K4 or K9 histone H3 were amplified by PCR using primers specific for centromeric *dg* repeats (Fig. 5) or *act1* (A). Quantitation was performed using quantitative PCR (B) and normalized to actin (K4) and mating type region (K9) controls, which were amplified

from the same ChIPs in each genotype (21). DNA fragments from wce or co-precipitated with antibodies to Swi6 or K9 histone H3 were amplified by multiplex PCR using *ura4*-specific primers (C). These primers amplify both a *ura4*<sup>+</sup> transgene located in the *otr* of *cen1* (*otr::ura4*<sup>+</sup>) and the *ura4* DS/E minigene (*ura4* DS/E) located on the chromosome arm. Relative levels were estimated using a FUJI phosphoimager and are indicated below each lane.

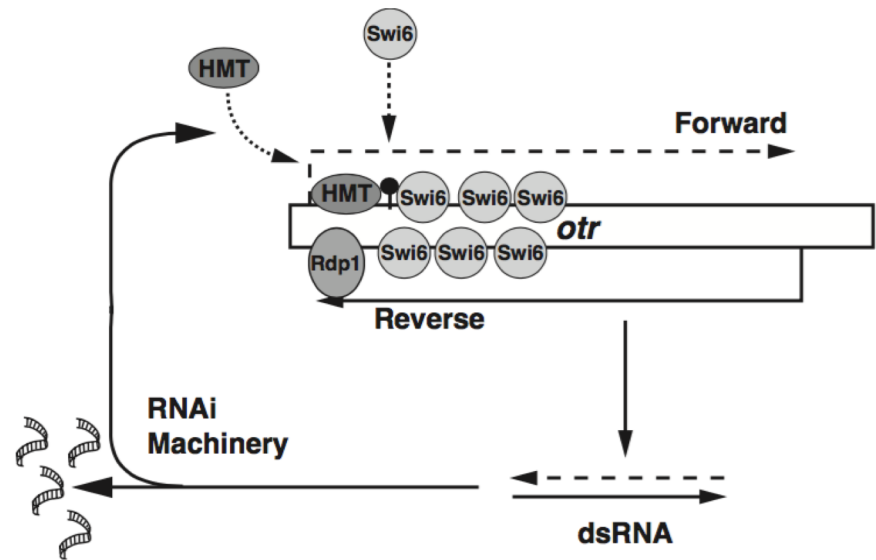


## Loss of the RNAi machinery results in the loss of H3K9me3 at outermost repeats



**Fig. 4.** Rdp1, but not Dcr1, is bound to centromeric chromatin. Chromatin immunoprecipitation (ChIP) was performed on extracts derived from triple-HA tagged Dcr1 or Rdp1 strains. DNA fragments from wce or co-precipitated with antibodies raised against the triple-HA tag were amplified by PCR using centromere-specific primers and compared to untagged strains. Mock reactions were identical except without the addition of primary antibody.

**Fig. 6.** The RNAi machinery is required for the initiation and maintenance of the heterochromatic state of centromeric repeats. Reverse strand centromeric transcription occurs in wild-type cells and is degraded posttranscriptionally by the RNAi machinery. Low-level transcription from the forward strand and/or amplification by Rdp1 results in generation of dsRNA, which is converted to siRNA by RNAi. Rdp1, bound to the chromatin, promotes targeting of histone modifications to specific sequences via siRNA, resulting in maintenance of the heterochromatic state (HMT, histone methyl transferase).



## 4 critical steps in heterochromatin formation at centromeres in *S. pombe*

1. Pericentric sense anti-sense transcription

2. RNAi mediated degradation of pericentromeric dsRNA

3. Amplification of siRNA loop by RNA dependent RNA polymerase

4. Heterochromatinization

**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>
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–	–	Prg-1 and 2, and 19 others	Aubergine/ Sting	Piwi to Piwi4
Chp1 <sup>a</sup>	CMT3	–	–	–
Tas3 <sup>b</sup>	–	AIN-1	GW182	TNRC6
Rdp1	RDR1 to 6	Ego-1, Rrf-1 to -3	–	–
Hrr1	SGS2/SDE3 <sup>c</sup>	ZK1067.2	GH20028p	KIAA1404
Cid12	–	Rde-3, Trf-4 <sup>c</sup>	CG11265 <sup>c</sup>	POLS <sup>c</sup>
Swi6	LHP1 (TFL2)	Hpl-1, F32E10.6 <sup>d</sup>	Hpl-2, HP1a, b	HP1α, β, γ
Clr4	SUVH2 to 6	–	Su(var)3-9	Suv39h1 and 2
Rik1 <sup>e</sup>	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

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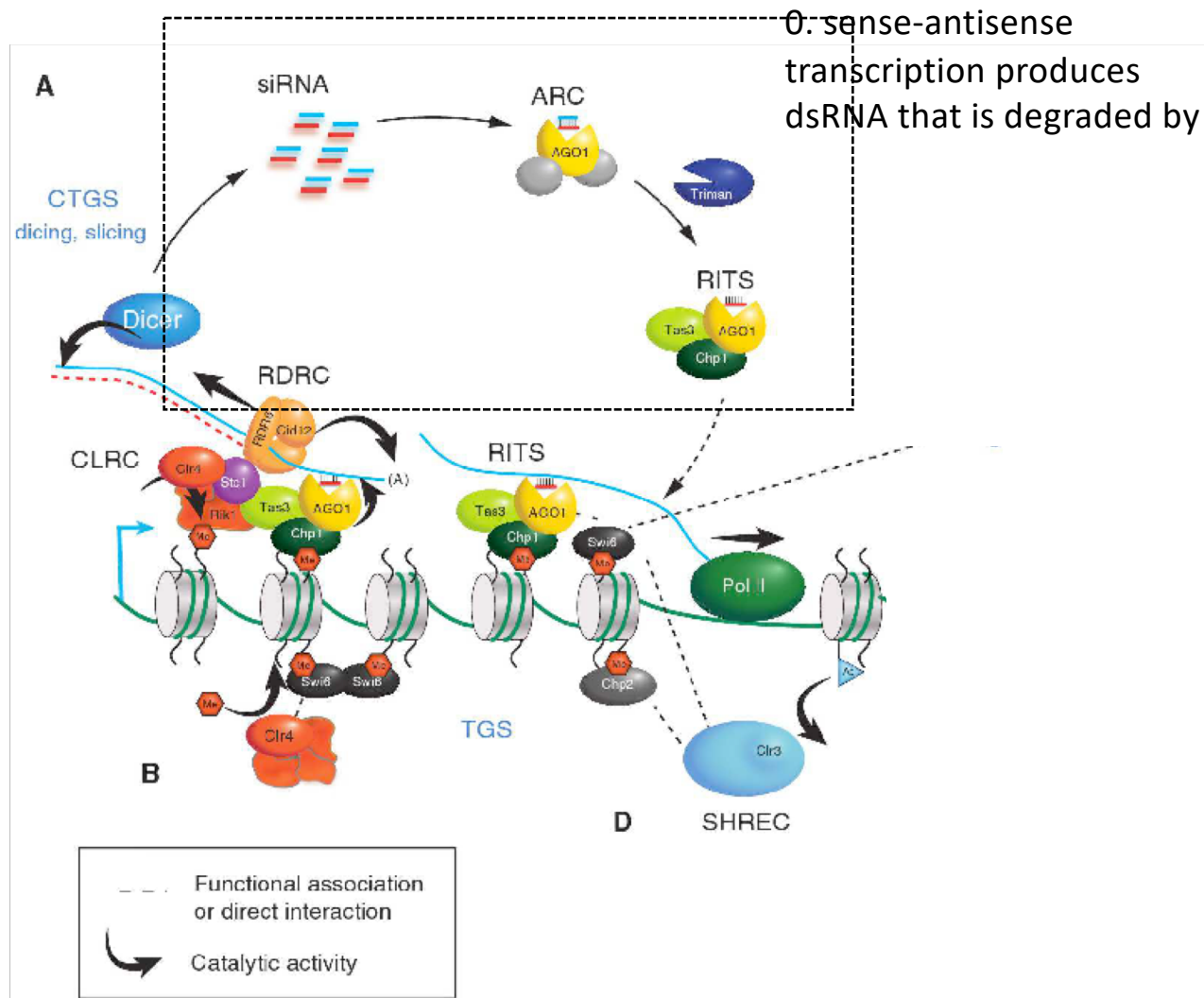
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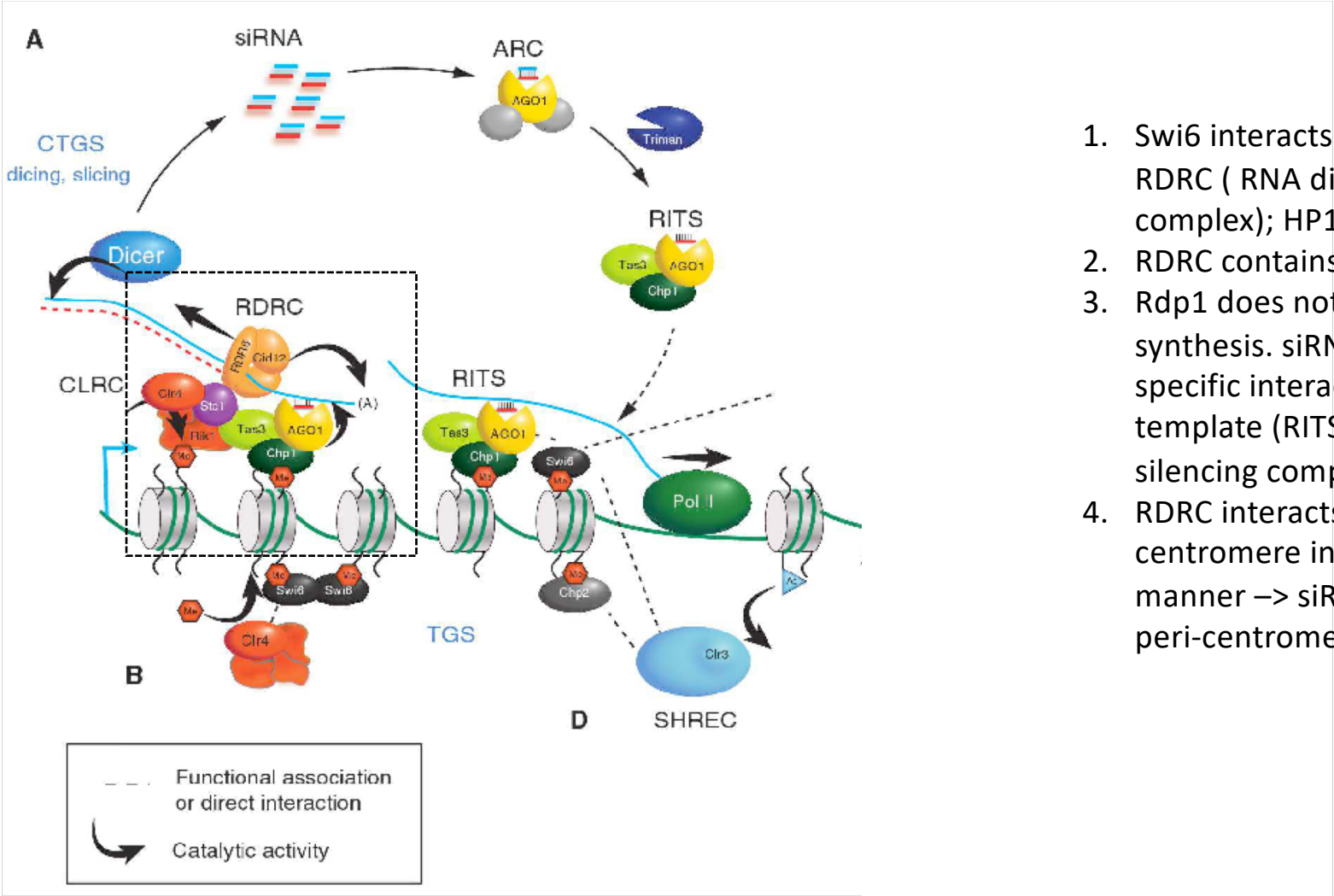
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## Step 2: RNAi – dsRNA formation – RITS assembly



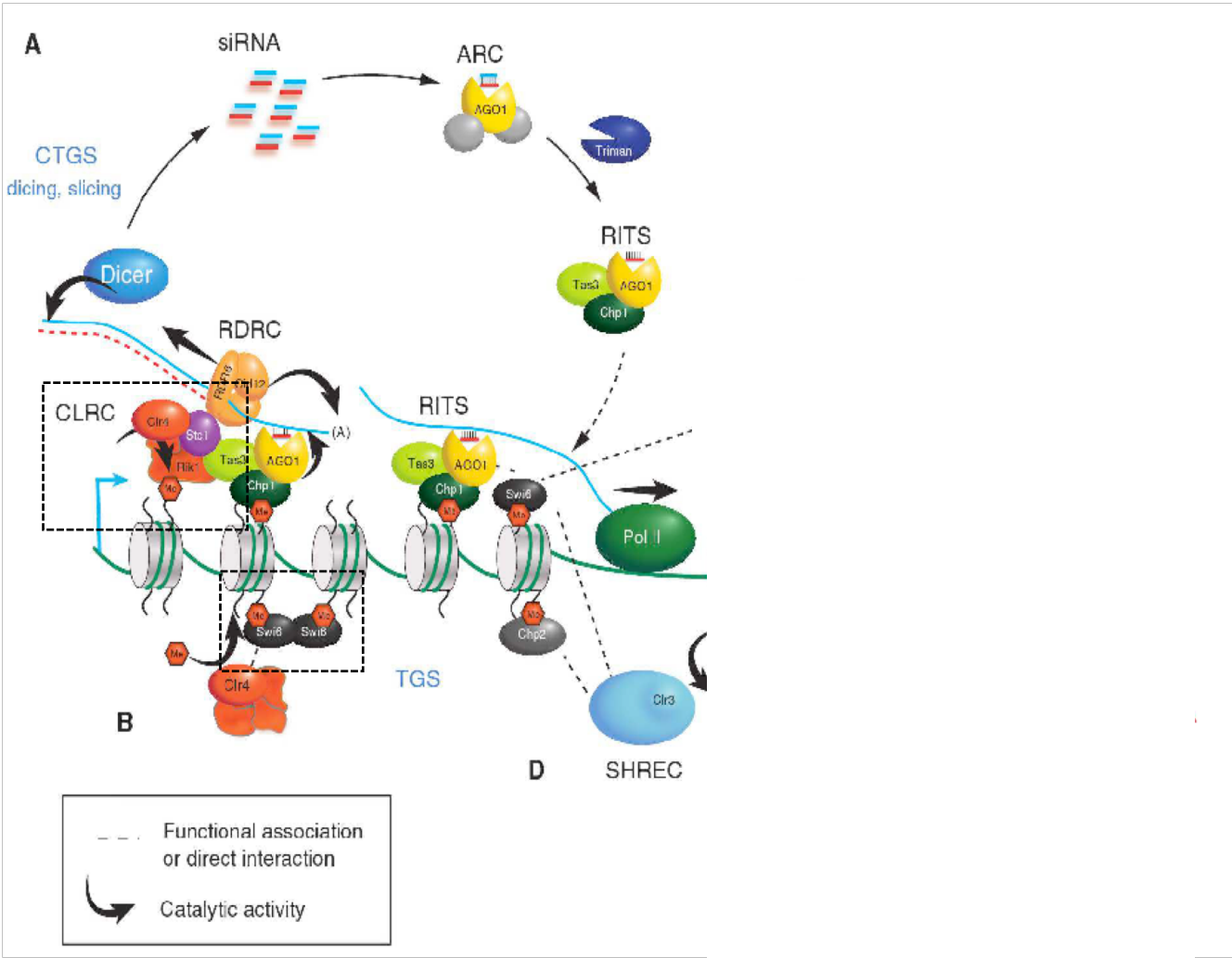
1. Dicer processed pericentric dsRNAs generating siRNAs (nucleus)
2. siRNA incorporated into the RITS complex consisting of **Ago1**, Chp1 and Tas3
3. RITS used siRNA to target nascent transcripts from centromeres (base pairing) and induces heterochromatin formation
4. Chp1 contains a chromodomain that binds to H3K9me3 that was previously imposed by **Clr4**
5. Clr4 has a chromodomain that binds H3K9me3 and Clr4 interacts with RITS
6. H3K9me3 and recruitment of **Swi6** that stabilizes the RITS complex
7. Chp2 chromodomain protein binds H3K9me3 and recruits the SHREC HDAC complex (**H4K14 specific Clr3 HDAC**) that shuts down transcription in S-Phase

**Step 3: RNA dependent RNA Pol activity**



1. Swi6 interacts with Ers1 that binds to the RDRC ( RNA directed RNA polymerase complex); HP1 has affinity for RNA
2. RDRC contains Rdp1 and other components.
3. Rdp1 does not require a primer for dsRNA synthesis. siRNA/RITS gives information for specific interaction of Rdp1 complex with template (RITS: RNA-induced transcriptional silencing complex).
4. RDRC interacts with chromatin at centromere in a siRNA and Clr4 dependent manner → siRNAs and Clr4 target RDRC to peri-centromeres

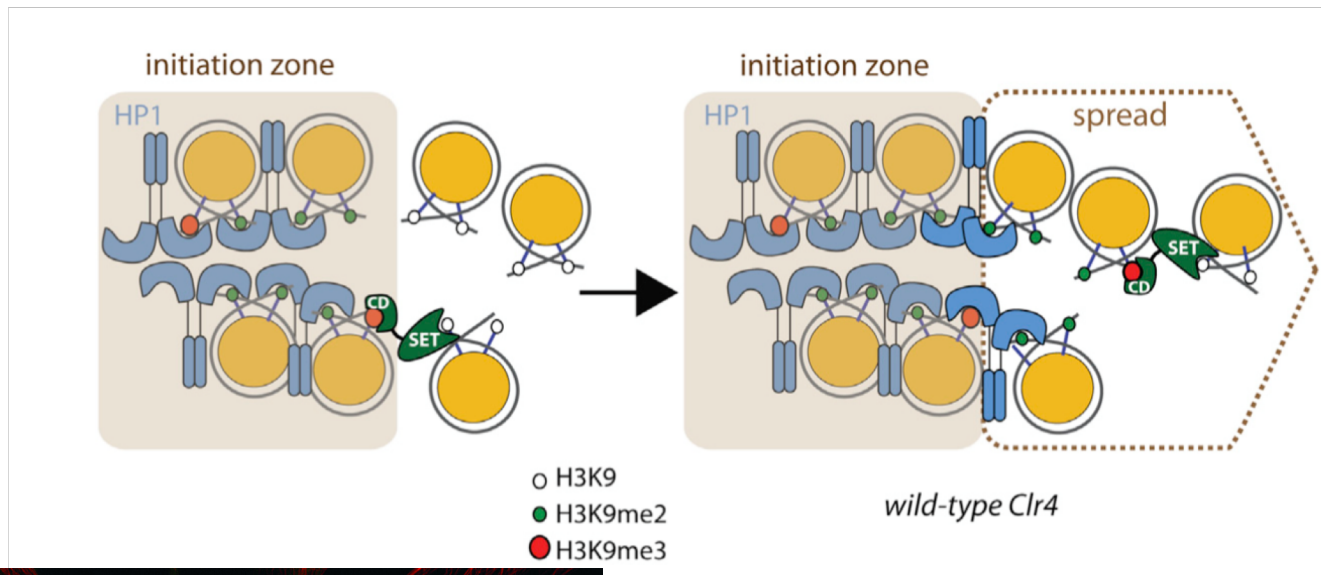
**Step 4: Heterochromatinization (Clr4, Swi6)**



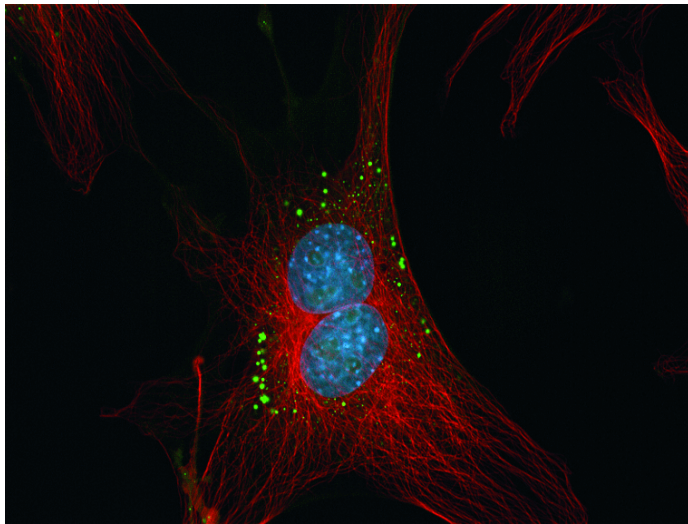
1. Clr4 is required for stabilizing RITS at centromere. → some Clr4-H3K9me3 is required to start heterochromatin formation. Note: loss of RNAi leads to loss of centromeric silencing → RNAi is required to boost H3K9me3/Swi6
2. Clr4 is a component of the CLRC complex
3. CLRC and RITS interact with each other via Stc1. Note: in RNAi-/- cells Stc1 levels are reduced → siRNAs contribute to complex structure that favors the action of Stc1 in ensuring the interaction between RITS and CLRC (ev. via other factors)
4. Efficient H3K9me3 by Clr4; Swi6 is recruited



## Step 4: Heterochromatinization (Clr4, Swi6)



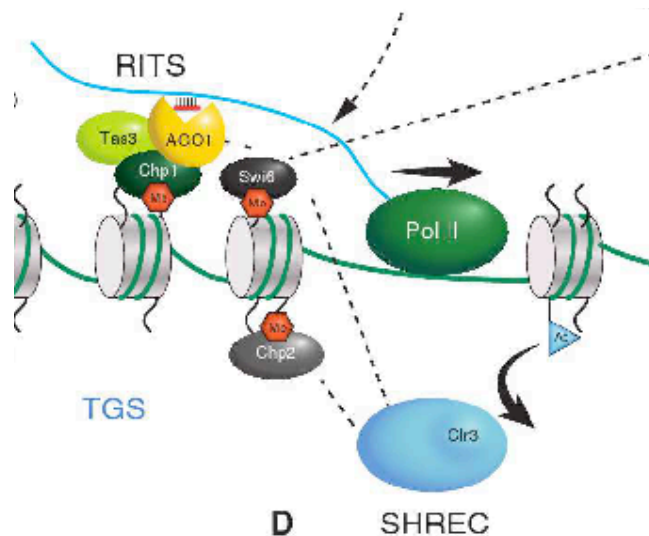
Clr4 mediates H3K9me3; Clr4 has a chromo-domain that interacts with H3K9me2/3  
Swi6 interacts with H3K9me2/3 and forms homodimer with another Swi6; Clr4 bind H3K9me2 + interacts with Swi6.  
Spreading of Clr4/Swi6 along chromatin  
Swi6 mediates nucleosome bridging and shortening of nucleosome to nucleosome distance → efficient chromatin compaction



MECHANISM CONSERVED FROM *S.pombe* → humans

Clr4	Suv39h1/h2
Swi6	HP1
H3K9me3	H3K9me3

## The role of RNA polymerase in centromeric silencing



Transcription by RNA-polymerase is essential for heterochromatin formation

Evidence: Mutations in RNA-Pol in *S.pombe*:

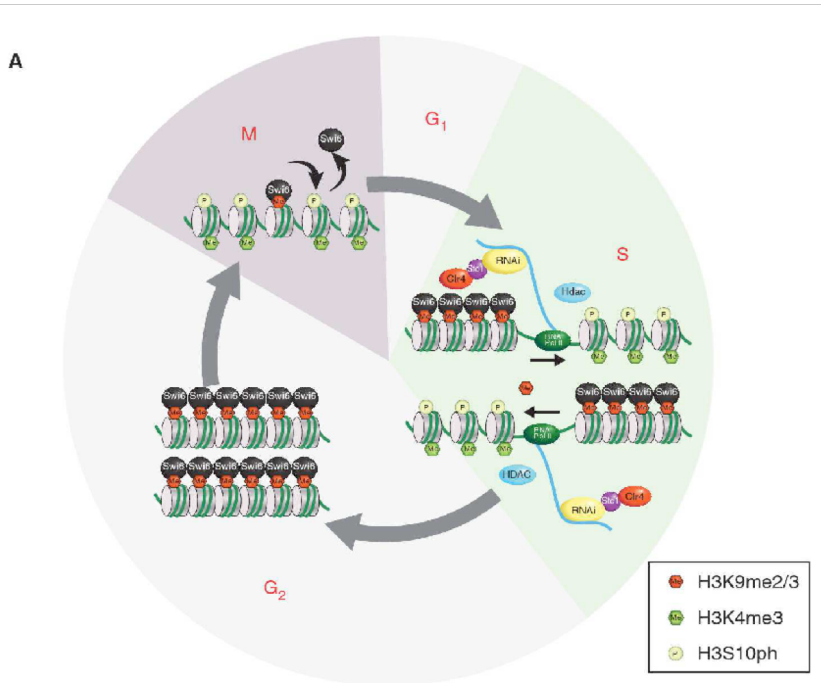
Rpb7-1: Reduced levels of transcription at pericentric repeats → less siRNAs → loss of silent chromatin

Rpb2-m203: RNA is produced, but not processed into siRNAs → reduced heterochromatin

- RNA polymerase is not exclusively responsible for transcription, but also has a role in integrating transcription with the downstream processing of the produced RNA
- Remember the RNA-Pol holoenzyme is a huge complex that integrates many proteins: i.e. FACT: chromatin modelling factor that colocalizes to centromeres i.e. Spt6 (histone chaperon): RNA pol interactor that is required to establish H3K9me3
- precise mechanisms: unknown – the complexity of protein interaction with the RNA-Pol holoenzyme is extremely high



# The maintenance of heterochromatin through numerous cell cycles



**Figure 4.** Cell-cycle regulation of centromere heterochromatin assembly. (A) Heterochromatin located at chromosomal centromeres becomes differentially methylated and phosphorylated on histones throughout the cell cycle as indicated. These modifications control the binding of the heterochromatin protein Swi6. During mitosis Swi6 is displaced by H3S10 phosphorylation. Swi6 binding is reestablished during subsequent DNA replication (S phase) when a more accessible chromatin structure permits RNA Pol II to transcribe centromeric DNA. This, in turn, recruits the RNAi machinery to direct H3K9me methylation. (B) Replication-coupled RNAi model (Li et al. 2011). This figure



Swi6 (HP1) binding to chromosomes is regulated during the cell cycle

At the enter of M-Phase H3K10 is phosphorylated at the entire chromosomes  
Resulting in the disattachment of Swi6 (HP1) from H3K9me3

Swi6 re-enters at high efficiency in S-Phase

Importantly: RNA Pol is associated with repeat sequences mainly in S-Phase (not at genes)  
→ **burst of pericentric transcription**

→ Rapid recruitment of the RNAi-machinery by nascent transcripts

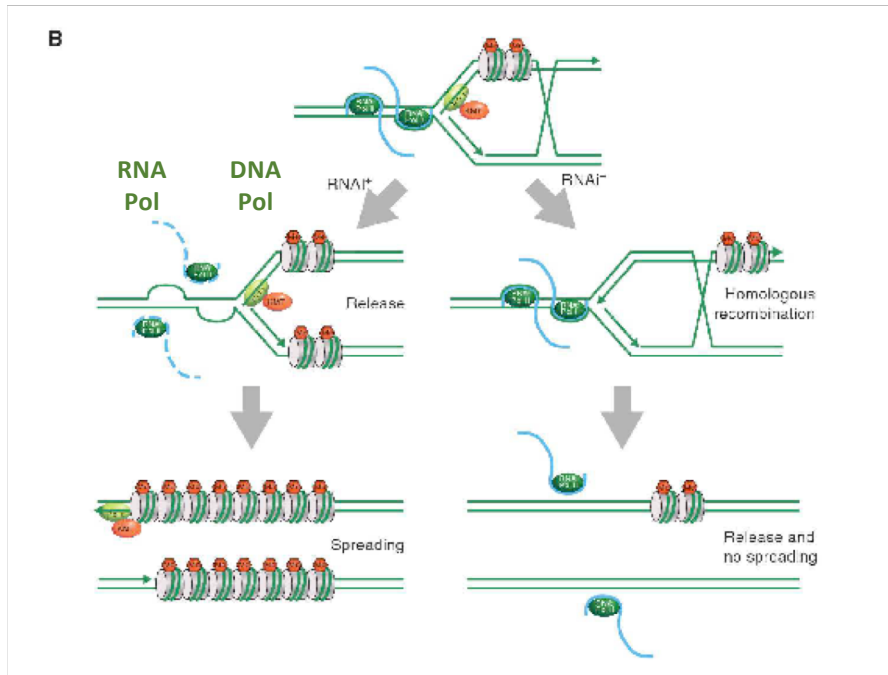
→ Burst in heterochromatin formation!!!

→ G2: reduced transcription → heterochromatin already formed

Note: Stc1 interacts with RITS (Ago1) and the CLRC complex (Clr4)

Note: SHREC complex contains the HDAC Clr3 that is important to subsequently shut down transcription

# The maintenance of heterochromatin



H3K9me methylation. (B) Replication-coupled RNAi model (Li et al. 2011). This figure illustrates an alternative model for how RNAi works at centromeres. Here, RNAi serves to release RNA Pol II from chromatin to avoid collision with DNA replication machinery during S phase. See text for further details. (A, Adapted from Djupedal and Ekwall 2008.)

**RNAi serves to release RNA polymerase from chromatin**



Swi6 (HP1) binding to chromosomes is regulated during the cell cycle

NOTE: RNA Polymerase can encounter DNA Polymerase → collision of major protein complexes that are linked to open DNA double strands (fragile DNA) → sterical stress on DNA → risk of breakage → genomic instability

**RNAi mutants result in stalling DNA replication forks and activate recombination**

Pol epsilon has a role in recruiting the CLRC complex (Clr4) –

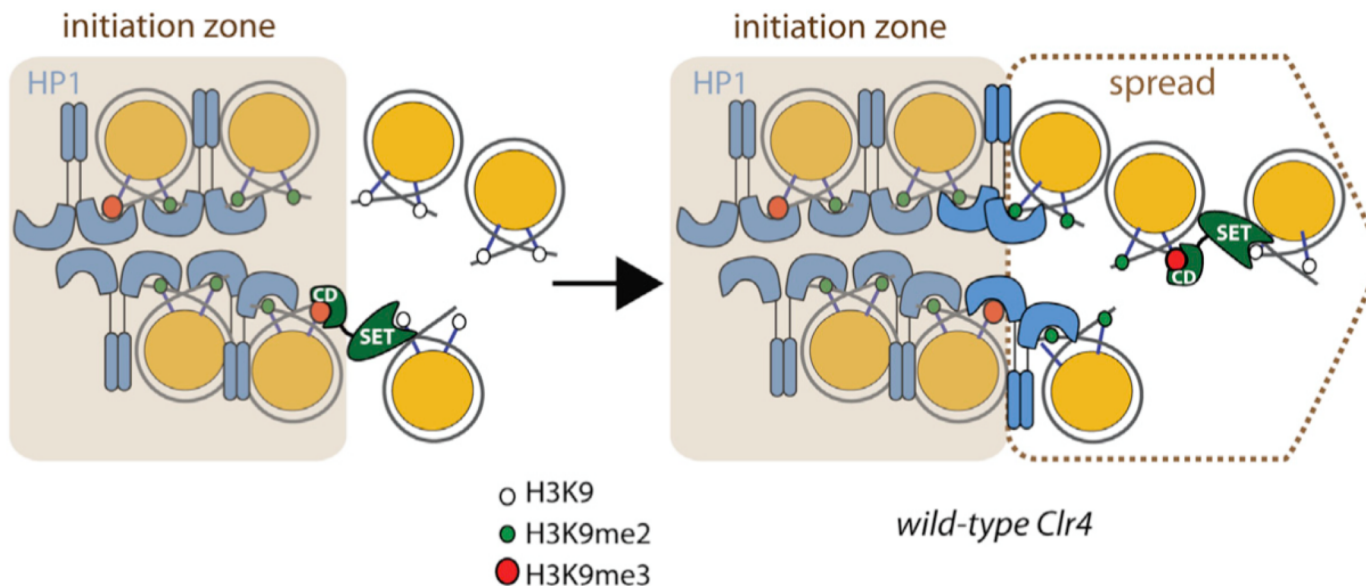
H3K9me3 → recruitment of RNAi machinery

RNA dependent RNA polymerase → amplification of RNAi pathway

→ Heterochromatin formation (SHREC and CLRC complex)

→ Silencing of transcription

## HOW IS CHROMATIN SPREADING LIMITED



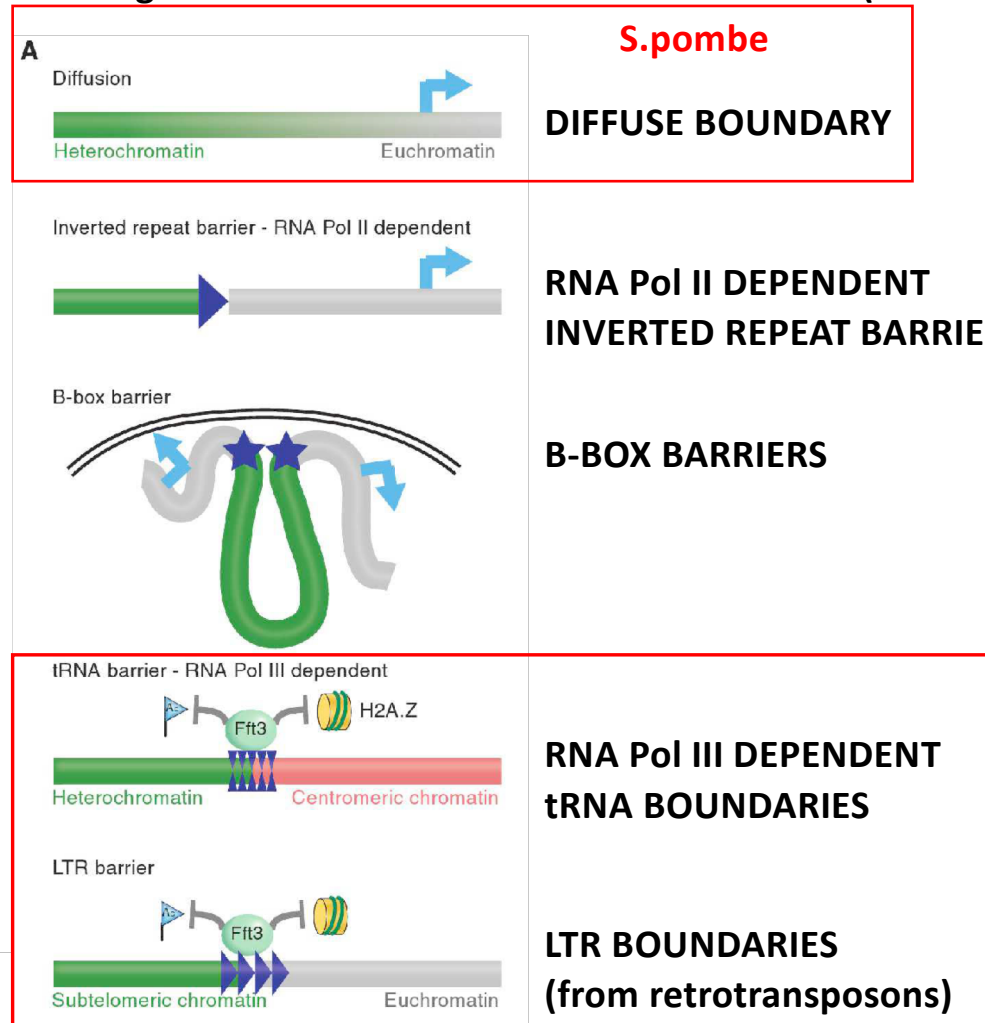
1. Clr4 mediates H3K9me3; Clr4 has a chromodomain that interacts with H3K9me3
2. Swi6 interacts with H3K9me3 and forms homodimer with another Swi6
3. Swi6 directly interacts with Clr4
4. These interactions mediate the spreading of Clr4/Swi6/H3K9me3 along chromatin.

Swi6 mediates nucleosome bridging and shortening of nucleosome to nucleosome distance  
→ Chromatin compaction and SPREADING

HOW CAN THIS PROCESS BEING STOPPED????

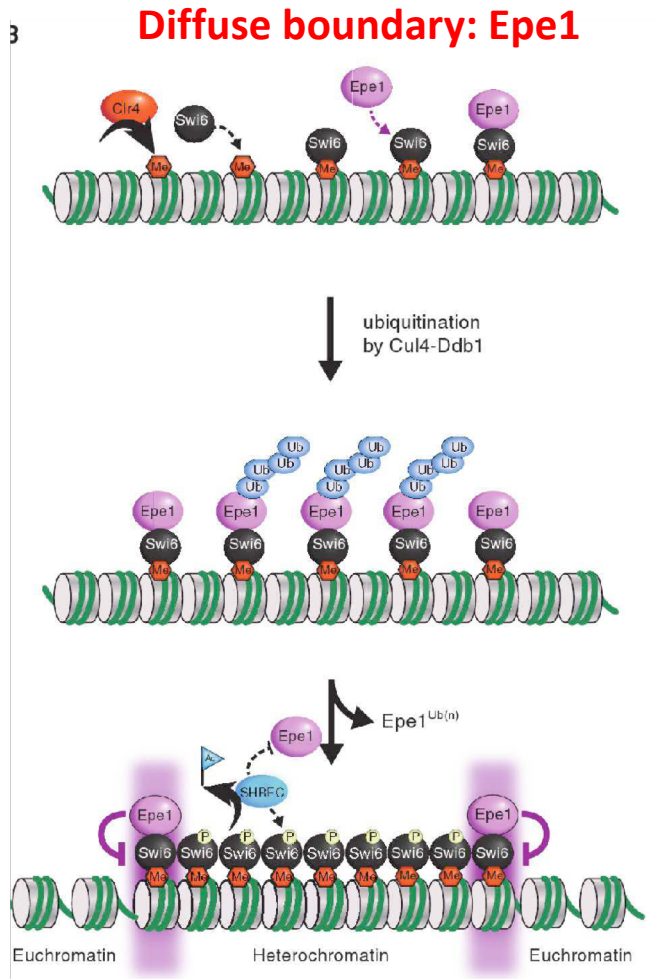
# HOW IS CHROMATIN SPREADING LIMITED

Boundary regions separate genome segments with different chromatin structure (euchromatin/heterochromatin)



**Figure 5.** Chromatin boundaries and the boundary mechanism involving Epe1 (for enhancement of position effect) in *S. pombe*. (A) A schematic representation of different types of boundary elements in *S. pombe*. (B) The mechanism of boundary function by

## HOW IS CHROMATIN SPREADING LIMITED: Epe1



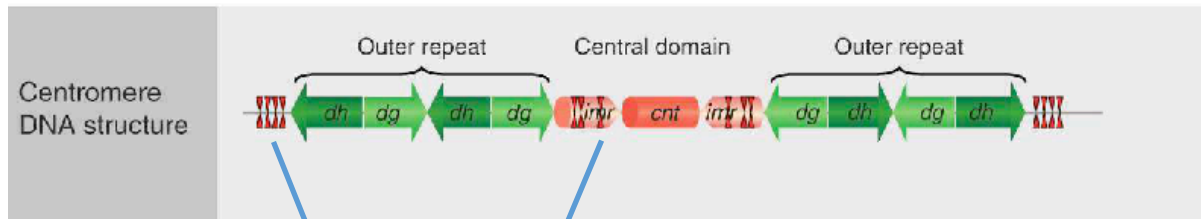
Epe 1 is an “anti-silencing” factor = counteracts heterochromatin building.

***S. pombe* Epe1-/- : more heterochromatin spreading**

1. Epe1 forms a complex with Swi6 in heterochromatic regions
2. However: **Epe1 is ubiquitinated and degraded by a cullin dependent ubiquitinase in regions with heterochromatin and remains only at boundaries between heterochromatin and euchromatin**  
(not known how ubiquitination is prevented at boundaries)
3. After removal of Epe1, Swi6 becomes phosphorylated (casein kinase 2)
4. Swi6 phosphorylation causes the recruitment of the SHREC complex → heterochromatin spreading and reduced Eps1 levels

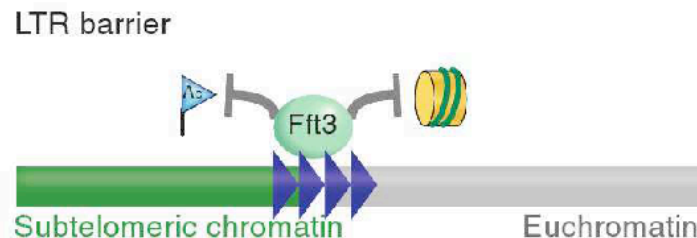
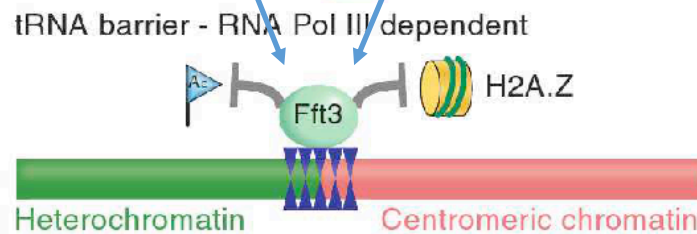
types of boundary elements in *S. pombe*. (B) The mechanism of boundary function by Epe1. Epe1 associates with Swi6, however, when the antisilencing factor is ubiquitinated by the Cul4-Ddb1 ligase and degraded in the heterochromatin region to allow for heterochromatin assembly. However, at boundaries, Epe1 is somehow protected from degradation, thus restricting the spreading of heterochromatin. Phosphorylation of Swi6 contributes to the dissociation of Epe1 at heterochromatin, while promoting the association with the HDAC complex SHREC in maintaining histone hypoacetylation. (A. Adapted from [Scott et al. 2007](#).)

# HOW IS CHROMATIN SPREADING LIMITED: tRNA clusters and Fft3



## tRNA boundaries

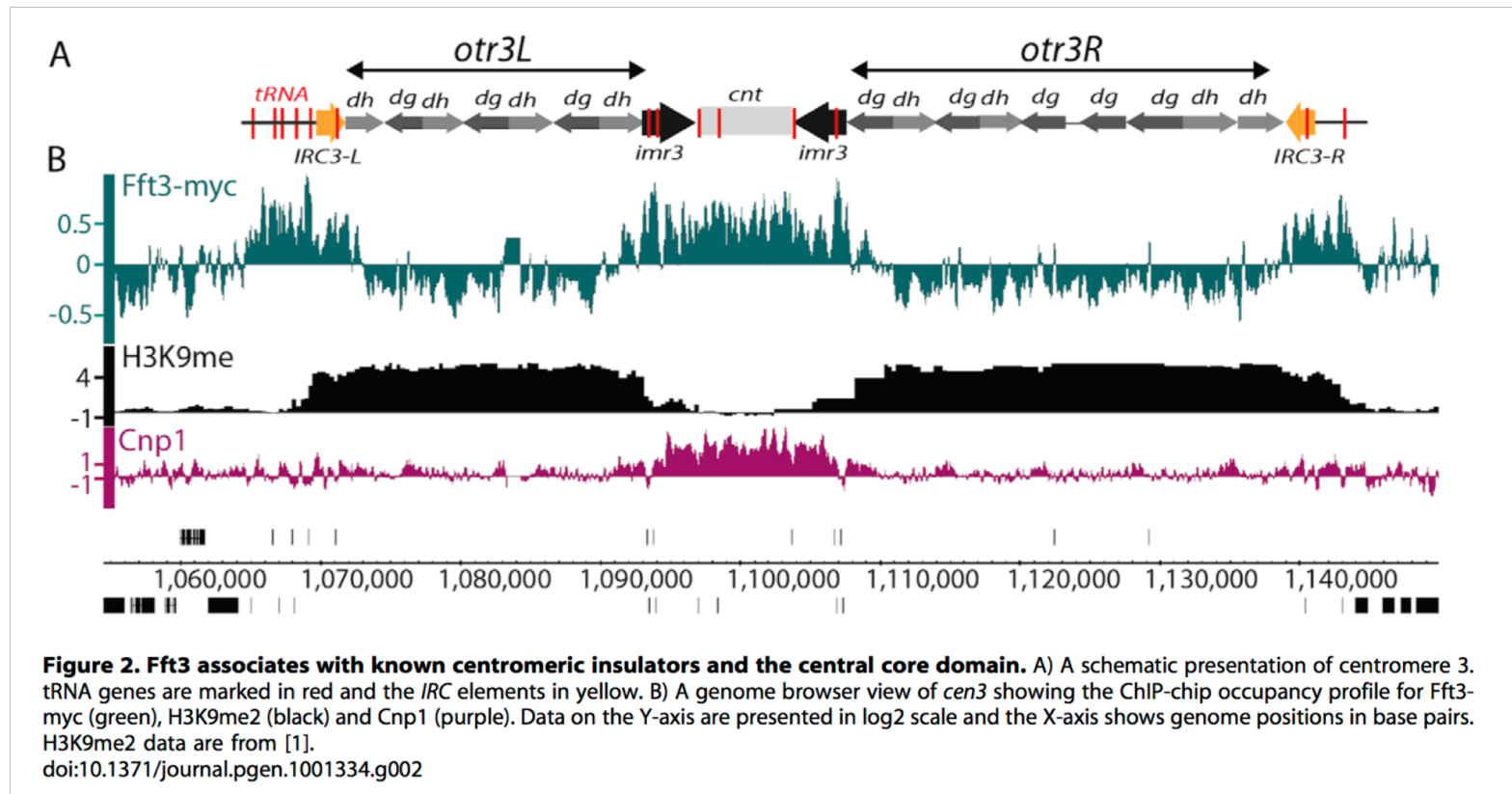
**A factor that prevents the spreading of euchromatin into heterochromatin**



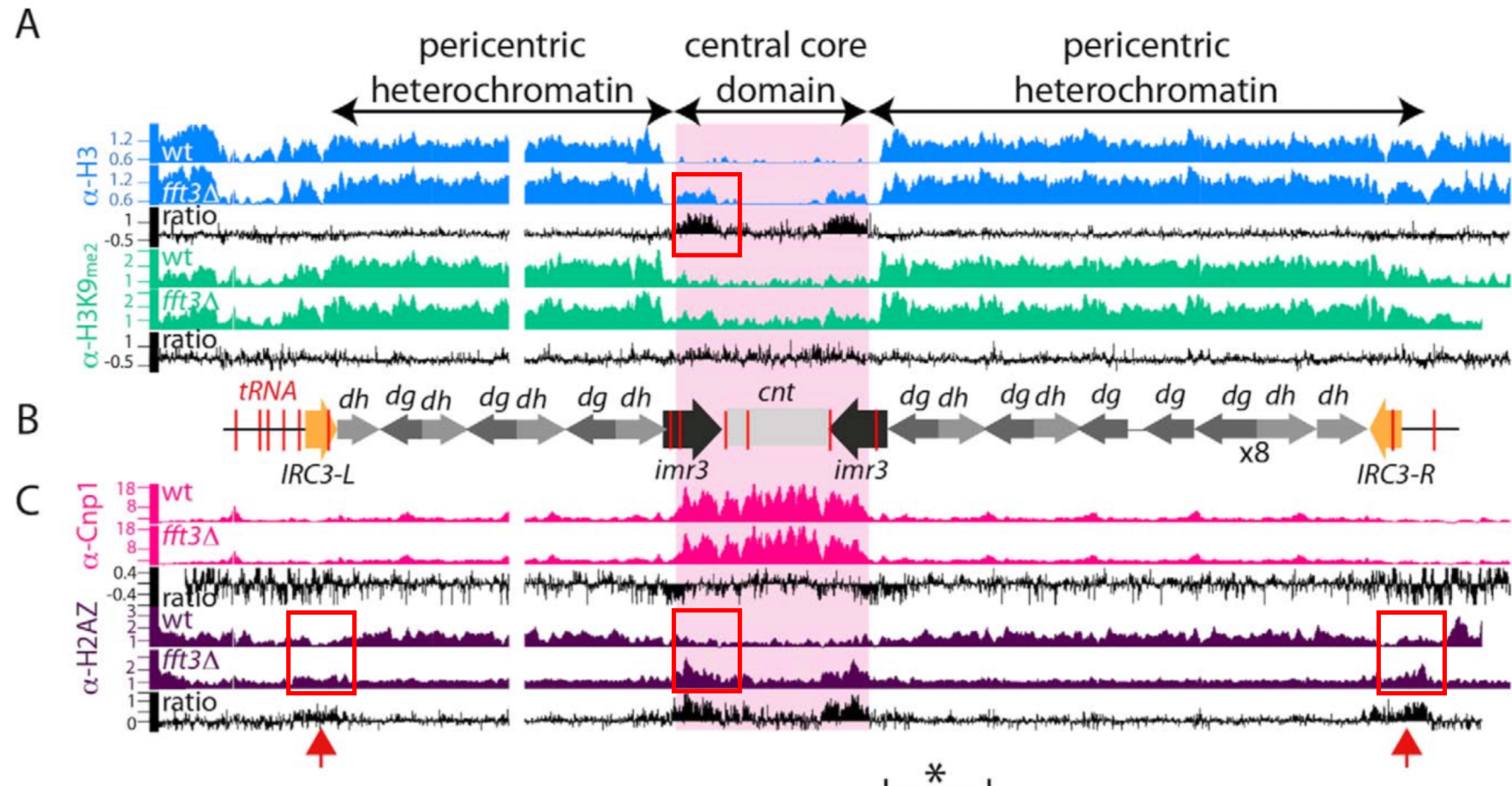
1. Despite their proximity to the repressive pericentric heterochromatin, the tRNA genes were actively transcribed by RNA Polymerase III (RNAPIII) = Euchromatin. Deletion of the tRNA clusters resulted in expansion of heterochromatin into the CENP-A chromatin—indicating that the tRNA genes functioned as chromatin barriers at the centromere.
2. Centromeric repeats represent HETEROCHROMATIN
3. Fft3 is restricted to tRNA genes (mechanism not known)
4. Fft3 is localized to the centromeric tRNA and subtelomeric LTR boundary regions, where it prevents euchromatin formation in the centromeric domain and subtelomeric regions (Fft3=nucleosome remodelling factor; ATPase domain)
5. Loss of Fft3 → extension of euchromatin into centromeric regions (H3/H4 acetylation and H2AZ = histone variant in euchromatin)
6. Mechanism ??? , also works at other sites → LTR barriers at subtelomeres



## HOW IS CHROMATIN SPREADING LIMITED: tRNA clusters and Fft3



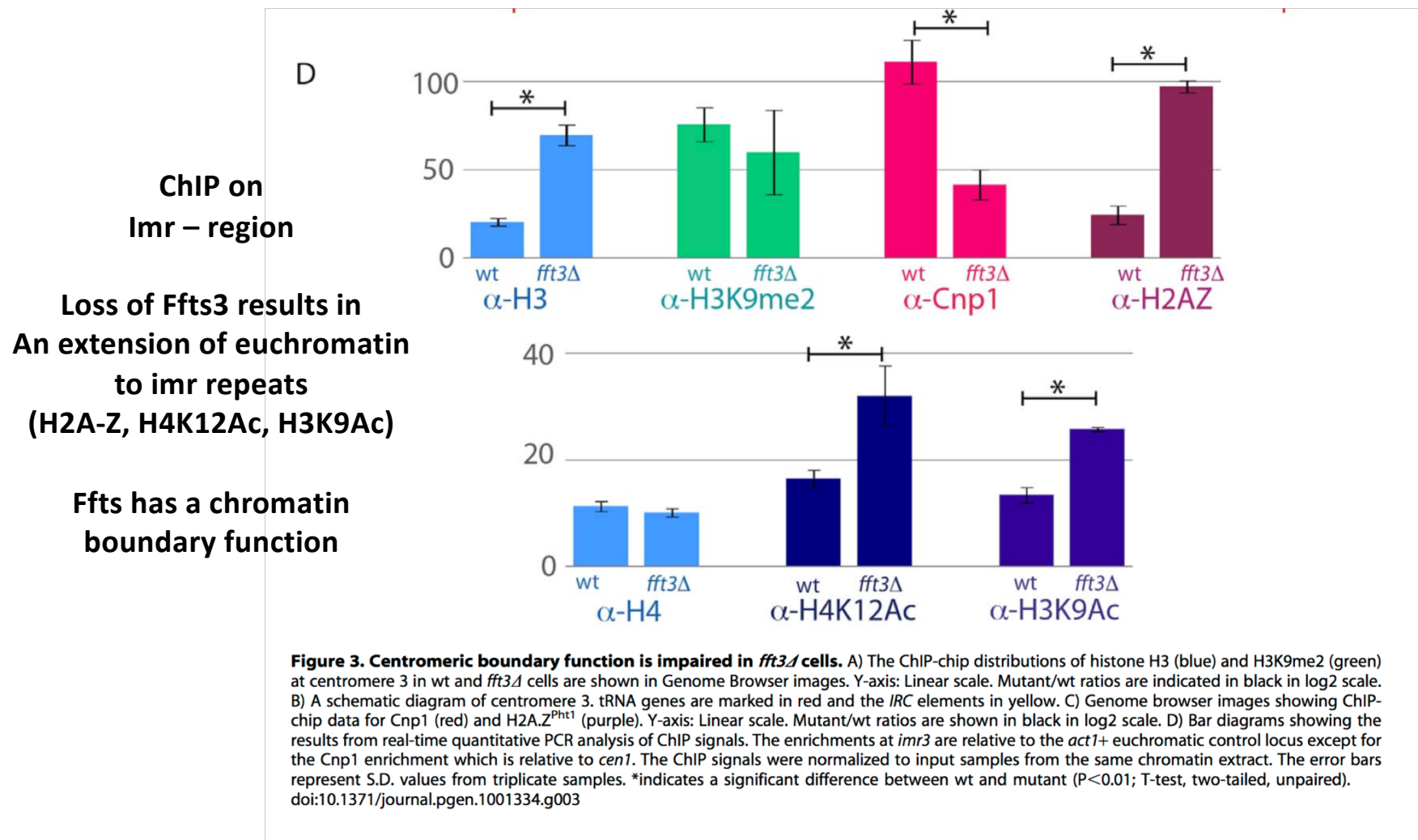
## HOW IS CHROMATIN SPREADING LIMITED: tRNA clusters and Fft3



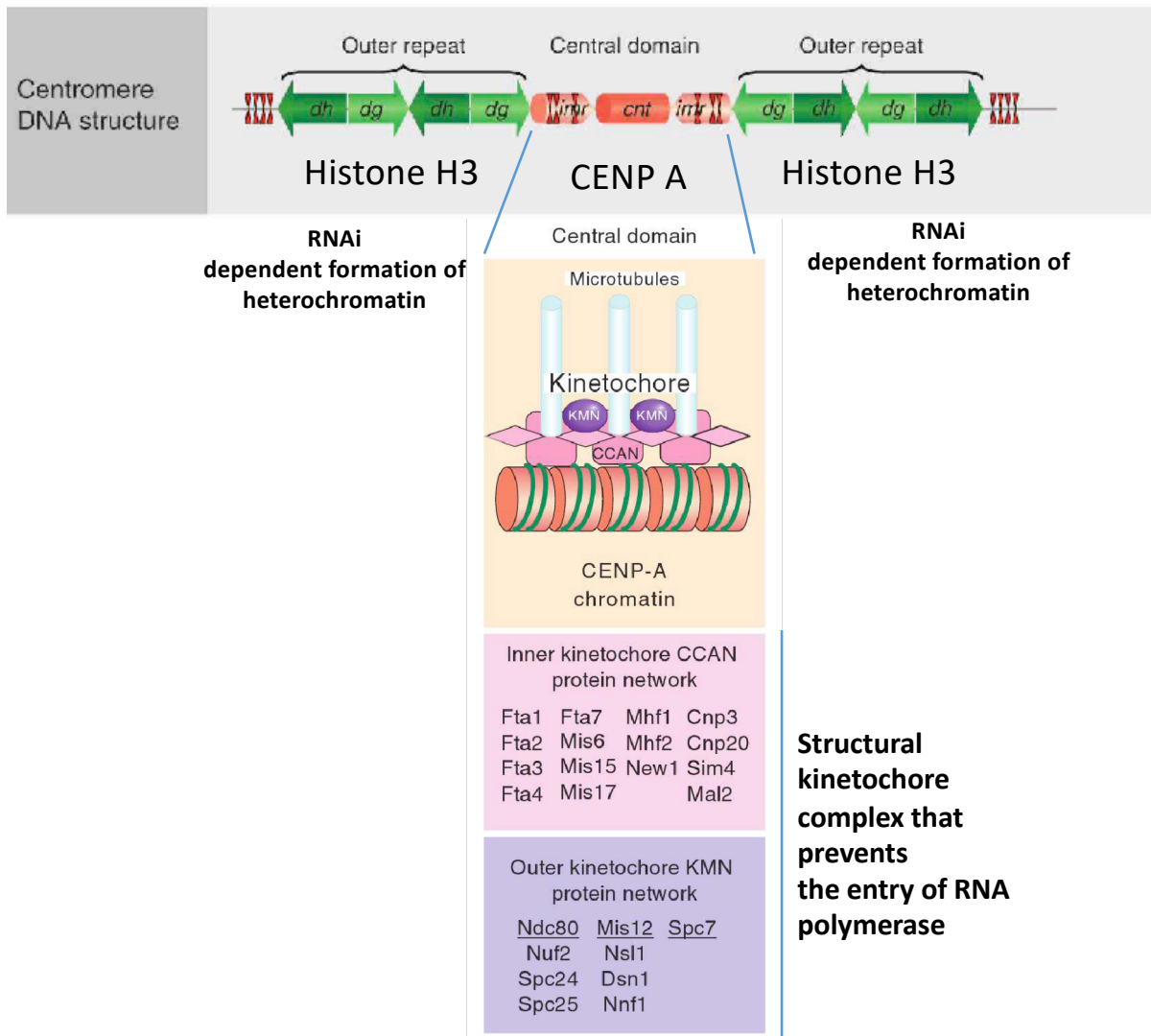
Normally H2A.Z is absent from all centromeric regions, including both the Cnp1 containing inner domain and the pericentric heterochromatin. H2A.Z marks active genes. Surprisingly, absence of Fft3, the H2A.Z levels increase by 4-fold at *imr3* and increase in IRC-L



## HOW IS CHROMATIN SPREADING LIMITED: tRNA clusters and Fft3



# WHY IS CENTROMERIC HETEROCHROMATIN IMPORTANT?



**At the central domain the majority of histone H3 is exchanged for Cnp1 = CENP A**

→ **ca 19-24 CENP A nucleosomes per centromere**

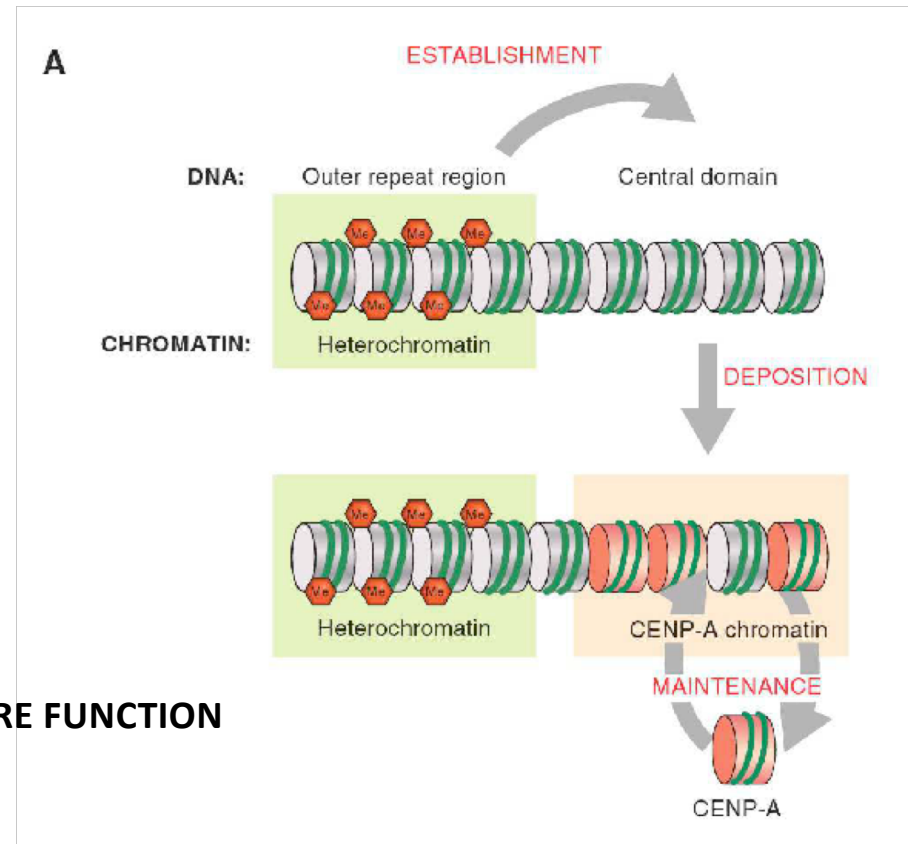
The CENPA gene encodes a centromere protein which contains a histone H3 related histone fold domain that is required for targeting to the centromere. CENPA is proposed to be a component of a modified nucleosome or nucleosome-like structure in which it replaces 1 or both copies of conventional histone H3 in the (H3-H4)<sub>2</sub> tetrameric core of the nucleosome particle. Silencing in central domain is thought to be mediated by the altered nucleosome composition and kinetochore construction (sterical hindrance)

Recruitment of CENP A requires **RNAi** and the presence of **heterochromatin**

## WHY IS CENTROMERIC HETEROCHROMATIN IMPORTANT?

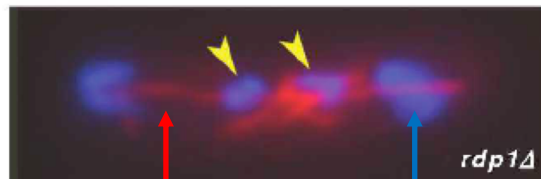
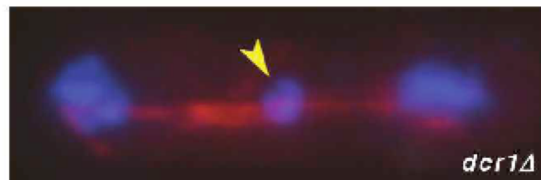
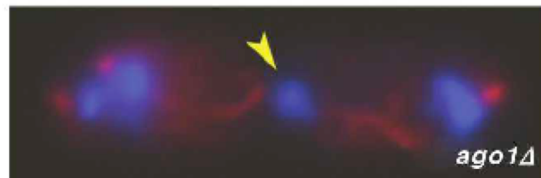
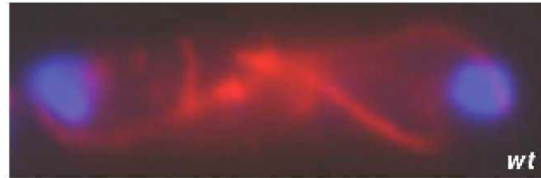
- Plasmid with only central repeats cannot be maintained in *S. pombe*
- No CENP-A incorporation
- Addition of outer repeat + central repeats: CENP A incorporation and plasmid maintenance
- Heterochromatin at outer repeats stabilize centromere function.

**HETEROCHROMATIN IS IMPORTANT TO ACTIVATE CENTROMERE FUNCTION**



## WHY IS CENTROMERIC HETEROCHROMATIN IMPORTANT?

### A Anaphase cells



Mitotic spindle

chromosomes

→ lagging chromosomes

Loss of\_  
RNAi  
machinery  
Ago1  
Dcr1  
Swi6  
Clr4

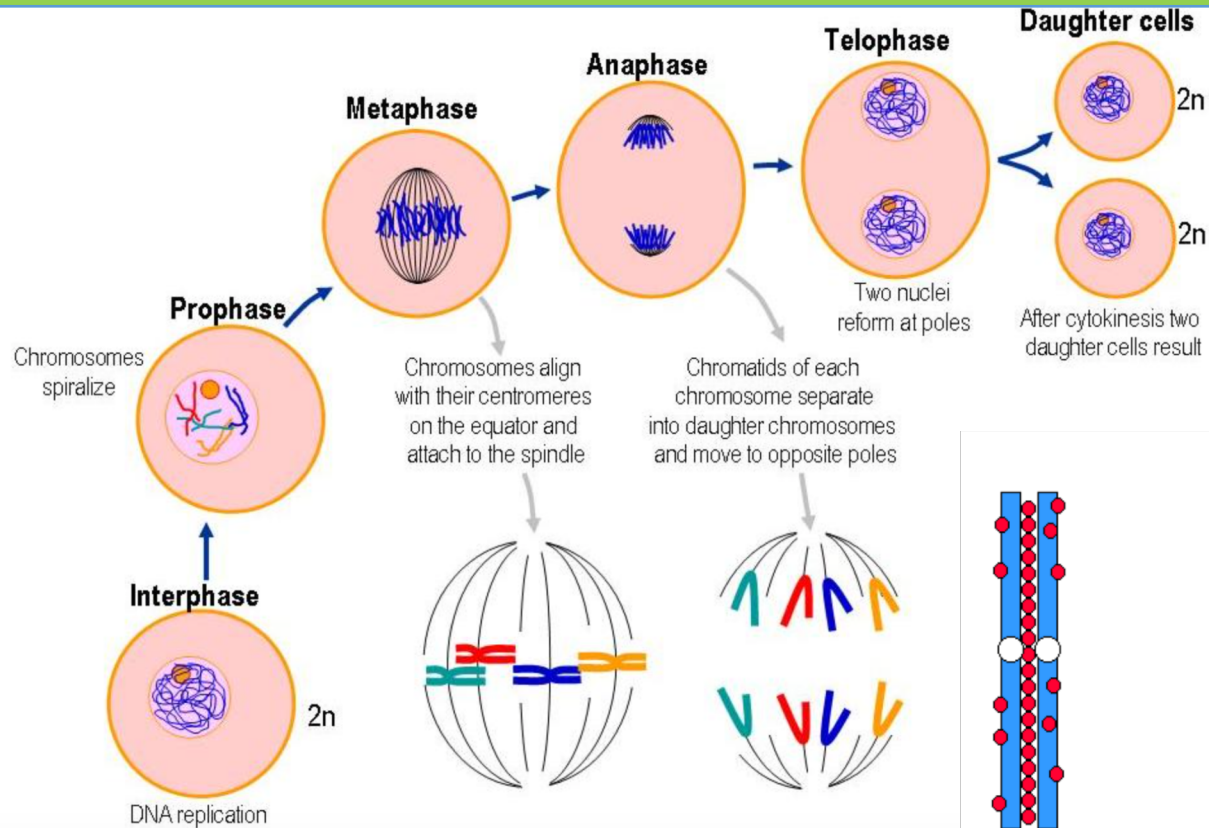


Chromosome segregation defects:

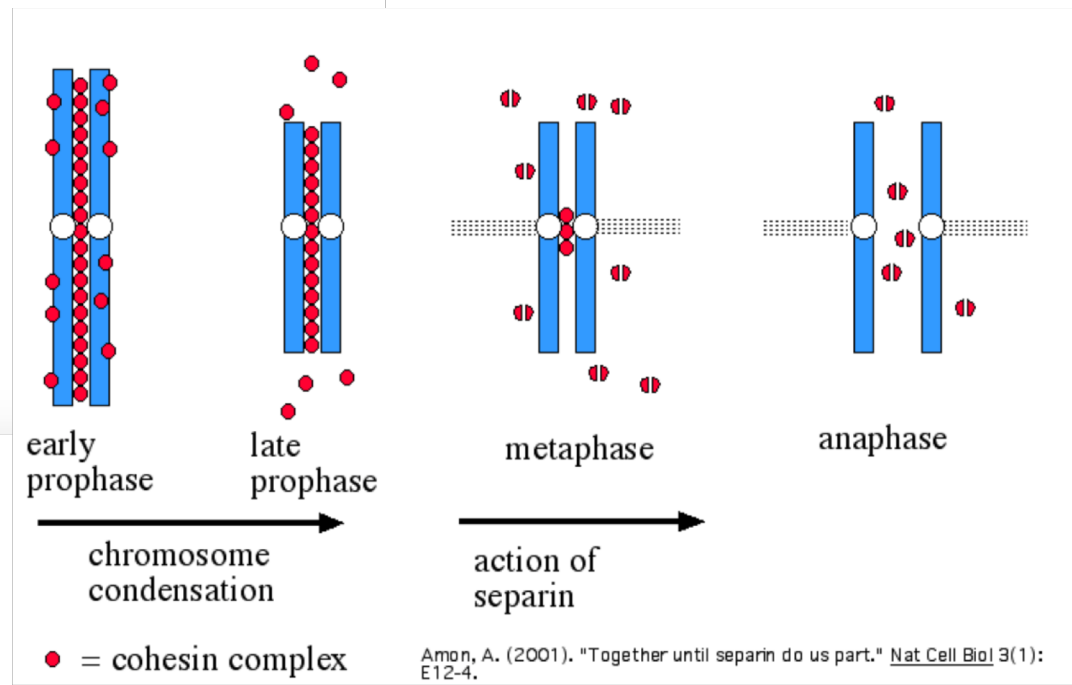
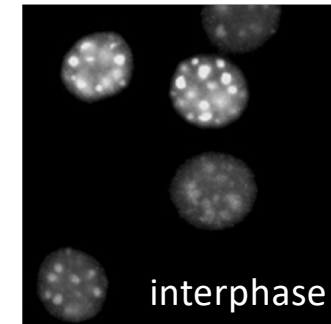
- Loss of chromosomes
- Lagging anaphase chromosomes
- Sister chromatid cohesion defects

**Heterochromatin is essential for controlled chromosome segregation**

# Eukaryotic centromeres hold together sister chromatids until chromosome segregation

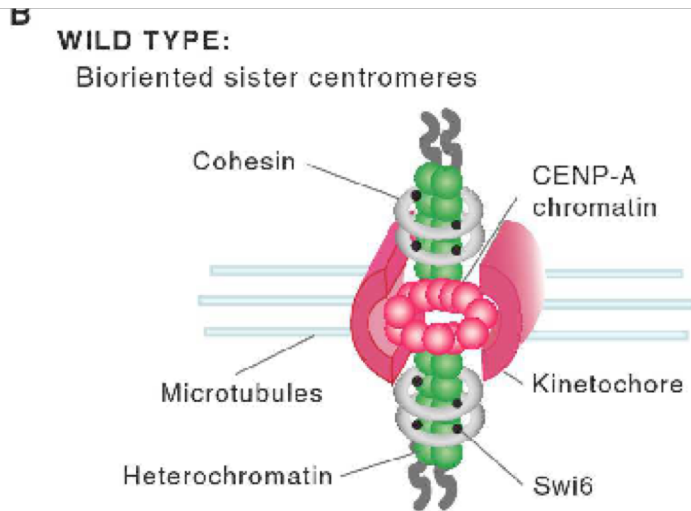


Chromocenters,  
aggregation of centromeres

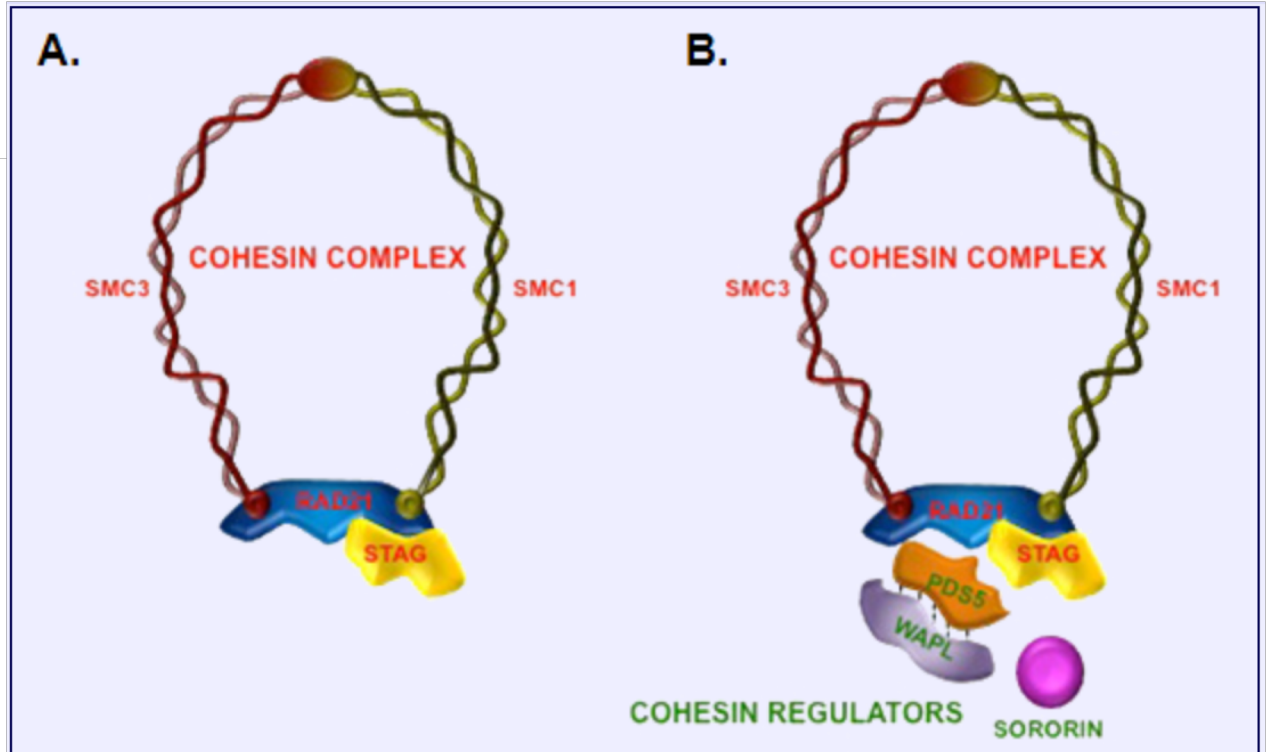
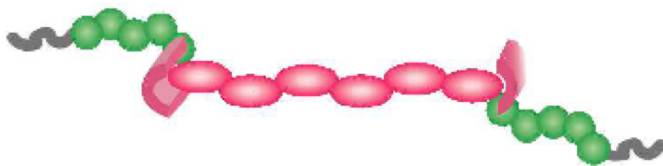


## WHY IS CENTROMERIC HETEROCHROMATIN IMPORTANT?

Cohesin forms a ring around sister-chromatids to organize chromosome segregation  
cohesin at centromere is very resistant and is thought to hold chromosomes well together until the spindle is perfectly attached to kinetochores



Defective heterochromatin:  
Merotellically oriented single centromere



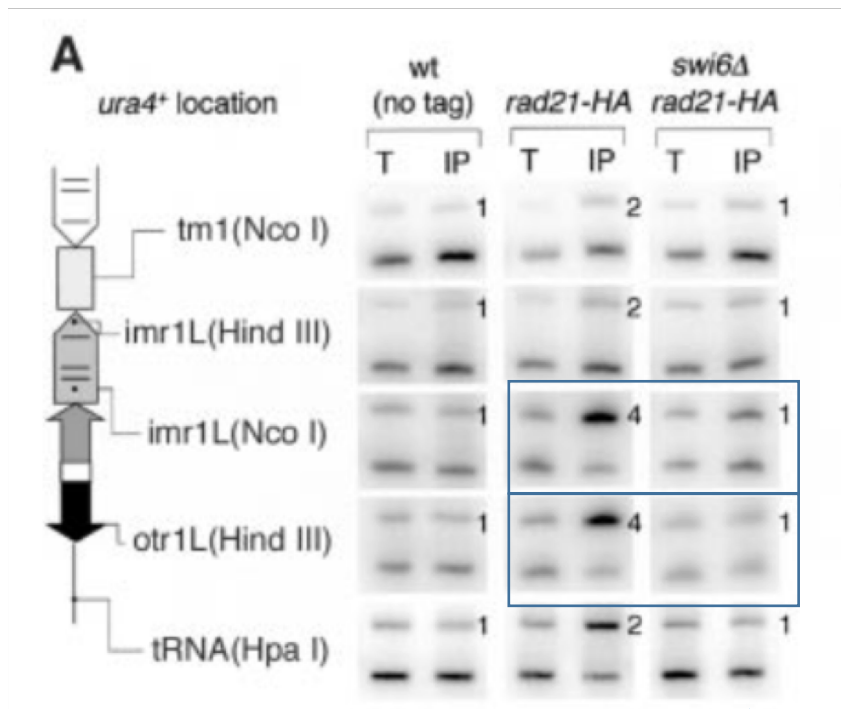
Centromeric heterochromatin is required to load cohesin to centromeres



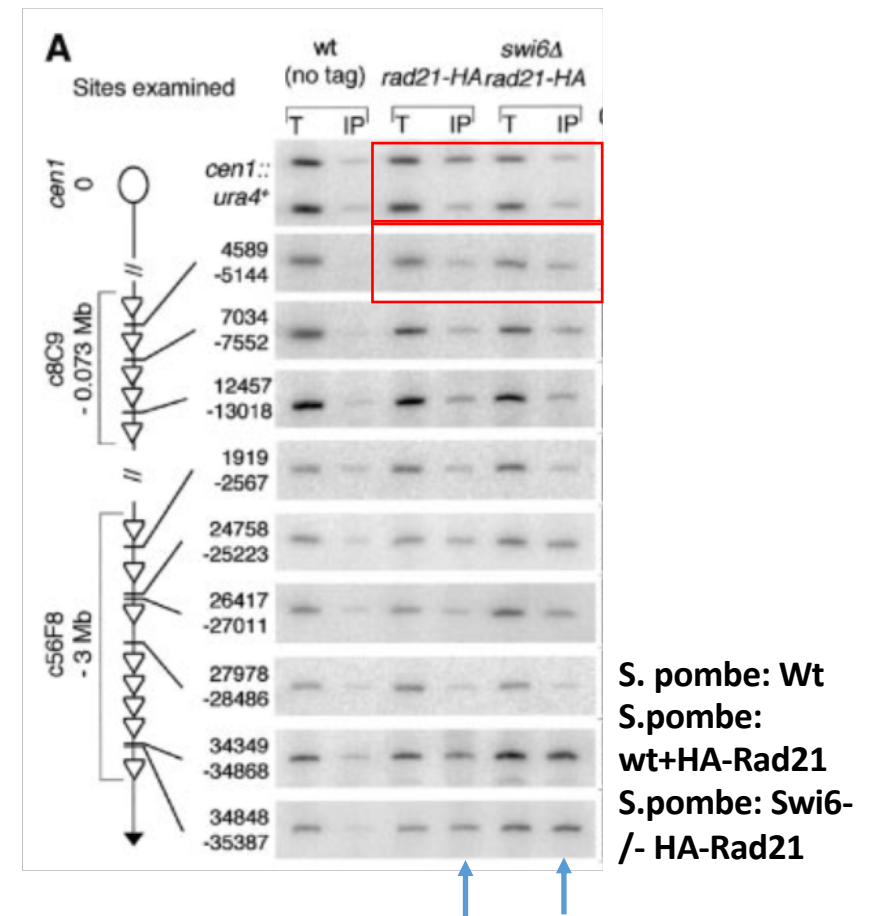
## WHY IS CENTROMERIC HETEROCHROMATIN IMPORTANT?

Swi6 is required to recruit cohesin subunit Rad21 to centromeres.....

→ Centromeric heterochromatin is required to recruit cohesin to centromeres

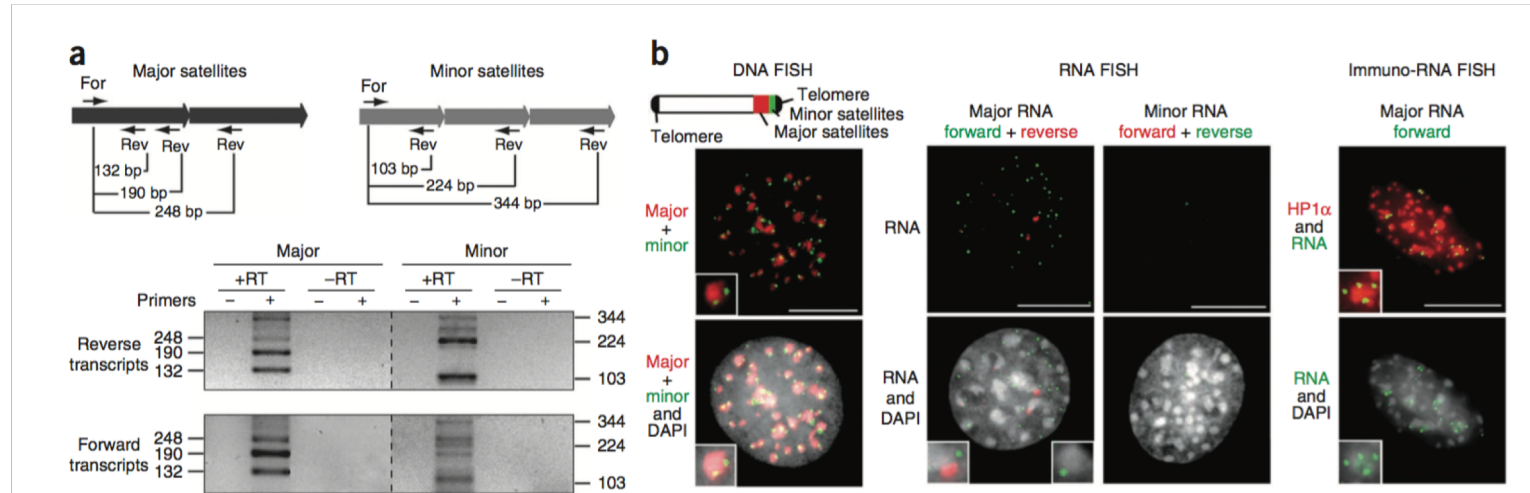
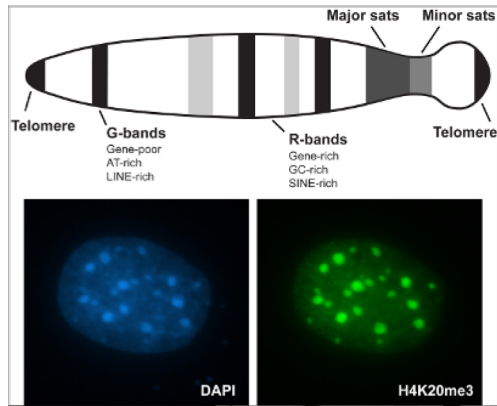


S. pombe: Wt  
S.pombe: wt+HA-rad21  
S.pombe: Swi6-/- HA-rad21

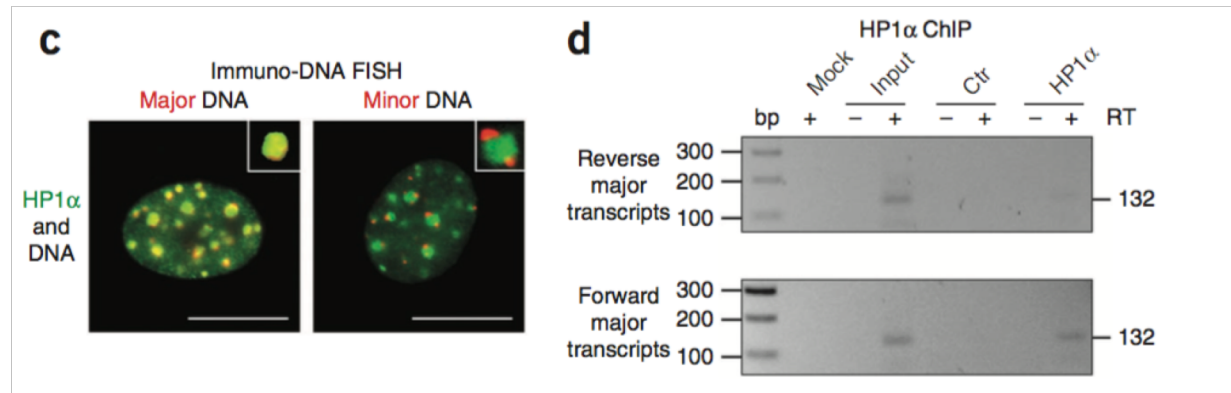


... but Swi6 is not important to recruit cohesin subunit Rad21 to other chromosomal positions

# Pericentric heterochromatin in vertebrates: the mouse as a model system



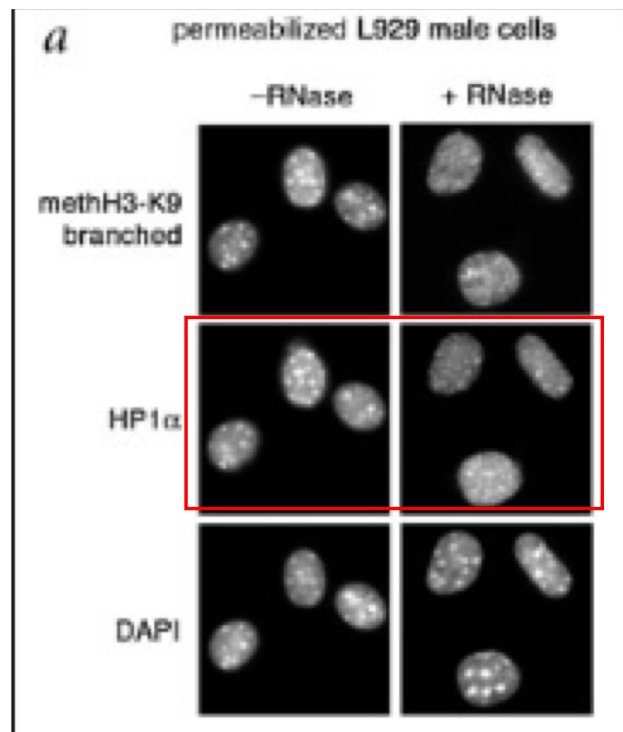
Suv39h1/h2 → H3K9me3 + HP1  
H3K9me3 calls in Suv420h  
Suv420h1/h2 → H4K20me3  
DNA methylation  
Bi-directional transcription of satellite repeats



Mouse pericentric repeats are transcribed in sense anti-sense orientation

## Pericentric heterochromatin in vertebrates: the mouse as a model system

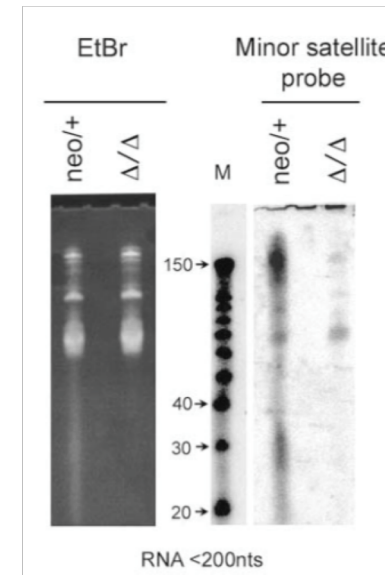
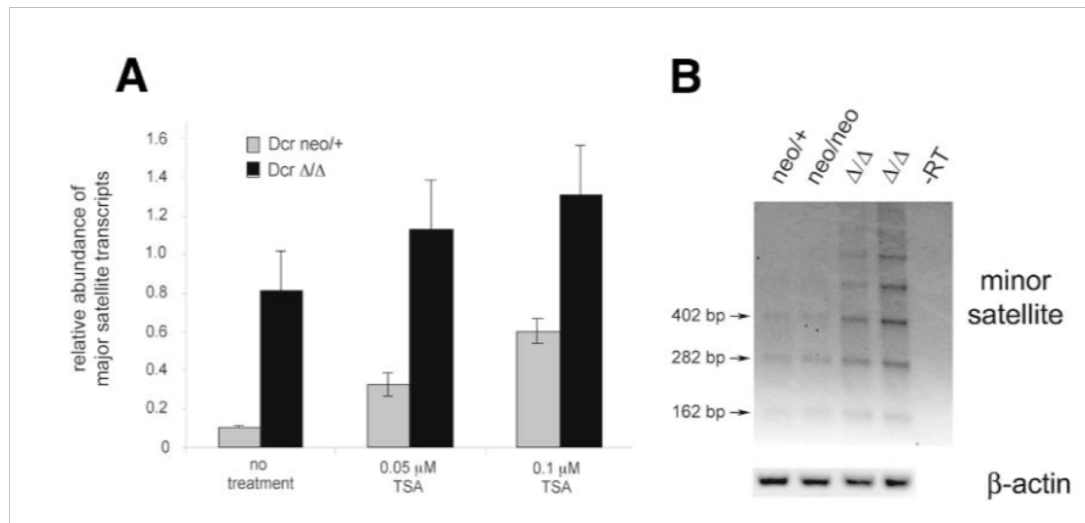
### A role of pericentric RNAs in heterochromatin formation



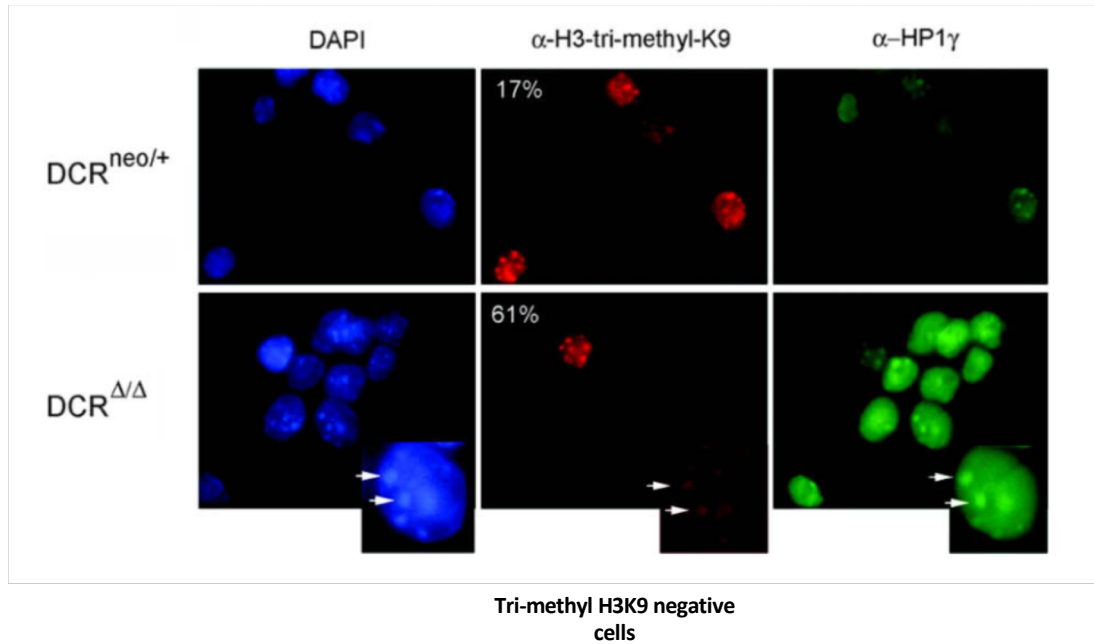
Digestion of RNAs from permeabilized cell results in a loss of HP1 from pericentric heterochromatin

## A role for Dicer at mouse centromeres??

Loss of dicer: more major and minor satellite transcripts (long)....and less small minor-satellite RNAs



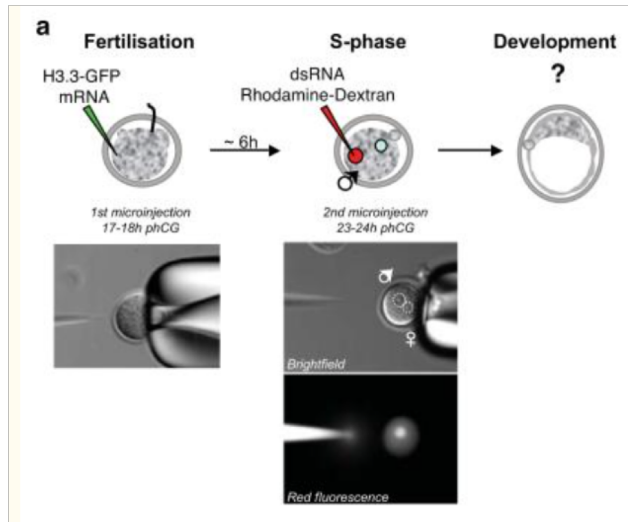
## A role for Dicer at mouse centromeres??



Loss of dicer reduces  
H3K9me3 and HP1 heterochromatin but  
**DOES NOT HAVE AN IMPACT ON  
CHROMOSOME SEGREGATION**

**DICER IS DISPENSABLE FOR  
HETEROCHROMATIN FORMATION**

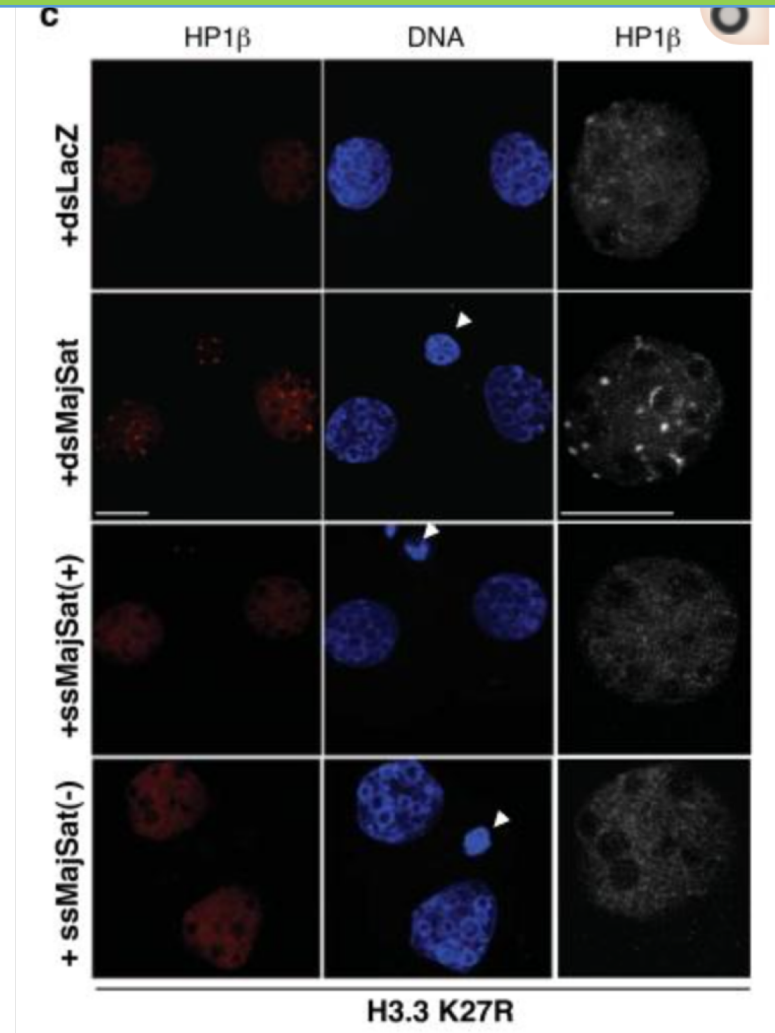
## A role for Dicer at mouse centromeres??



Injection of mutant H3.3 (histone variant) → loss of HP1 accumulation at pericentric heterochromatin

In injection of dsRNA from pericentric major satellite Repeats → rescue of HP1 at pericentric heterochromatin

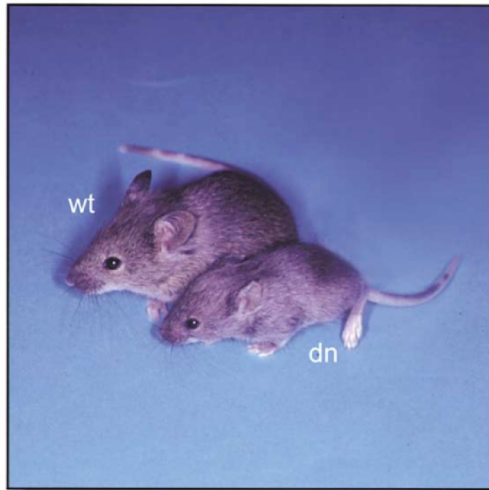
→ Evidence for RNAi in mammalian centromeric HC formation





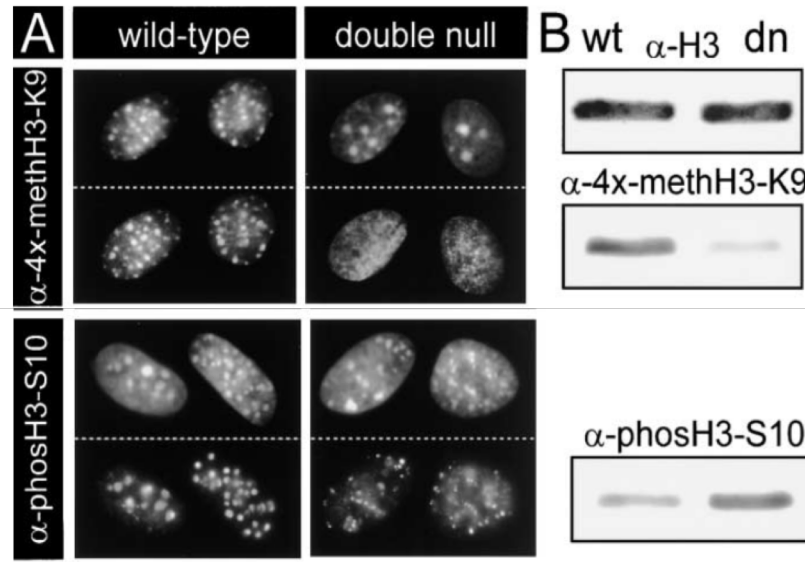
## A role for Dicer at mouse centromeres??

### Suv39h1/h2 KO



Suv39h1/h2 dn mice are smaller

Loss of pericentric H3K9 tri-methylation  
And abnormal H3S10 phosphorylation



FACS – scan: loss of Suv39h1/2 results  
in aneuploidy – increased chromosome  
numbers

