Characterisation of factors affecting RNAi-directed heterochromatin assembly in fission yeast

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ABSTRACT

Centromeres are the regions on each chromosome responsible for the faithful transmission of genetic material during mitosis and meiosis. The failure of correct chromosome segregation can drive anueploidy and may contribute to tumour formation.

The fission yeast, *Schizosaccharomyces pombe,* provides an excellent model for the dissection of centromere structure and function due to its genetic tractability and comparatively small genome size. The three centromeres of *S. pombe* are similar to those found in more complex eukaryotes; they are complex repetitive structures which bind multiple microtubules at mitosis. In particular, eukaryotic centromeres contain large blocks of heterochromatin. The assembly of heterochromatin requires an orchestrated array of chromatin changes including histone deacetylation, methylation and the recruitment of proteins that bind these modifications. In fission yeast, heterochromatin assembly requires an intact RNA interference (RNAi) pathway. Non-coding RNA transcripts originating from centromeric repeats are found to be associated with protein complexes that mediate RNAi. These transcripts are processed and the resulting siRNAs direct heterochromatin assembly over the homologous repeats and induce transcriptional silencing.

The *centromere:suppressor of position effect* (*csp*) mutants were previously isolated as specifically alleviating silencing within the centromeric outer repeats. The initial aim was to identify and characterise additional factors by analysing several mutations in unknown genes and then investigate their role in centromeric heterochromatin formation and integrity. Complementation with genomic libraries followed by sequencing allowed the identification of *csp7, csp9, csp10* and *csp12* as alleles of *rdp1*$^+$, *ago1*$^+$, *cid12*$^+$ and *arb1*$^+$ respectively.

Cid12, which encodes a putative poly(A) polymerase and associates in a complex with Rdp1, was chosen for more detailed analyses. Mutations in the predicted catalytic domain of Cid12 which would be expected to

abrogate its function were generated (*Cid12*^{dada}). Recombinant wild type and mutant protein were produced and employed in *in vitro* assays designed to test the biochemical function of Cid12. To date no convincing poly(A) polymerase activity has been detected however, assays indicate that Cid12 may possess nuclease activity. Affinity selection indicates that Cid12 associates with many proteins. Such interactions may be constitutive or transient. It is possible that Cid12 is only active in the context of these proteins and/or that it has unusual, unknown requirements with respect to substrate specificity. These analyses are described in detail and demonstrate that Cid12 plays a central role in heterochromatin assembly at centromeres.

ABBREVIATIONS

6AU	6-azauracil
ARC	Argonaute siRNA chaperone complex
ATP	adenosine triphosphate
ars	autonomous replication sequence
bp	base pairs
BSA	Bovine Serum Albumin
cDNA	complementary deoxyribonucleic acid
ChIP	chromatin immunoprecipitation
CENP	centromere protein
cloNAT	nourseothricin
CLRC	Clr4-Rik1-Cul4 complex
DAPI	4,6-diamino-2-phenylindole
dH_2O	distilled water
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dsRNA	double stranded RNA
EDTA	ethylene diamine tetraacetic acid
EtOH	ethanol
FACS	fluorescence activated cell sorting
FITC	fluorescein isothiocyanate
5-FOA	5-fluoroorotic acid
G418	geneticin
GFP	green fluorescent protein
HRP	horseradish peroxidase
kb	kilobase pairs

kD	kilo Dalton
LB	Luria-Bertani
ME	malt extract
miRNA	micro RNA
nmt	no message in thiamine
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PMG	pombe media glutamate
qRT-PCR	quantitative real-time PCR
rDNA	ribosomal DNA
RdRP	RNA-dependant RNA polymerase
RDRC	RNA-directed RNA polymerase complex
RISC	RNA induced silencing complex
RITS	RNA induced initiation of transcriptional silencing
RNA	ribonucleic acid
RNase A	ribonuclease A
RNAi	RNA interference
mRNA	messenger RNA
rRNA	ribosomal RNA
RT-PCR	reverse transcription PCR
SDS	sodium dodecyl sulphate
SET	*Suvar3-9, Enhancer of zeste, Trithorax*

siRNA	small interfering RNA
ssRNA	single stranded RNA
TBE	tris-borate EDTA
TBZ	thiabendazole
TE	tris-EDTA
TSA	trichostatin A
ts	temperature sensitive
Tween 20	polyoxyethylenesorbitan monolaurate
YES	yeast extract supplemented

Standard nomenclature for histone modifications used in this thesis:

Histone – residue (single letter code plus number) – modification (lower case) e.g. H3K9me2

Acetylation	ac
Methylation	me
Ubiquitination	ub

CHAPTER 1

Introduction

The faithful transmission of genetic material from generation to generation is an essential process. The centromere is vital for the correct segregation of sister chromatids during mitosis and meiosis as it is the site of kinetochore formation. The kinetochore is a multiprotein complex which mediates centromere-microtubule interactions during mitosis (Cleveland et al., 2003; Sullivan et al., 2001; Wiens and Sorger, 1998). Understanding the processes which contribute to chromosome segregation and the chromatin structures underlying centromere integrity is important, as the resulting cellular defects both in *S. pombe* and in more complex eukaryotes, can cause genomic instability. Chromosome loss or gain as a consequence of aberrant centromere function causes aneuploidy which can result in tumour formation and ultimately leads to a reduction in organism viability (Hassold and Hunt, 2001; Wassmann and Benezra, 2001).

1.1 Chromatin organisation

Eukaryotic genomes are packaged into higher-order chromatin structures which has implications for cellular processes such as DNA replication, recombination and transcription (Morales et al., 2001). The nucleosome is the basic repeating unit of chromatin, around which 146 bp of DNA is wrapped. The core particle of the nucleosome consists of a protein octamer of a histone H3-H4 tetramer and two H2A-H2B dimers (Luger et al., 1997). Nucleosomes allow the formation of higher-order chromatin structures. At the first level of compaction, DNA is wrapped around the core nucleosome particle which forms a 10 nm fiber. This is often referred to as the 'beads-on-a-string' array, the structure of which has been well characterised (Mohd-Sarip and Verrijzer, 2004). This 10 nm fiber can be further compacted into a 30 nm fibre which is thought to form a solenoid arrangement (Hayes and Hansen, 2001; Kornberg and Lorch, 1999). The 30 nm fiber is anchored to the nuclear periphery to form loops of chromatin of approximately 50-100 kb (Morales et al., 2001). Higher-order chromatin structure is illustrated schematically in

Figure 1.1. Nucleosomes are spaced at approximately 200 bp between which linker histone H1 binds and acts to stabilise the condensed states of chromatin (Kornberg and Lorch, 1999). The core histones are highly conserved proteins whereas the linker histones are less conserved. Nucleosomes are not static entities; rather histones undergo numerous posttranslational modifications on their N-terminal tails which are essential to encrypt different chromatin conformation and gene expression states (Morales et al., 2001; Heit, 2006).

Besides packaging the huge length of chromosomal DNA into the relatively small nucleus, higher-order chromatin structure is essential for many processes, ranging from gene regulation to accurate chromosome segregation during mitosis and meiosis. The organisation of chromatin can be described as two functionally and structurally distinct regions of the genome. These regions are known as euchromatin (or active chromatin) and heterochromatin (or silent chromatin) (Richards and Elgin 2002). Euchromatin is historically associated with regions of transcriptional activity. In contrast, heterochromatin remains condensed throughout the whole cell cycle and was thought to be transcriptionally inactive by virtue of its inaccessibility to transcription factors. This transcriptionally inactive state is also imposed on genes placed within heterochromatic regions. The 'off' or 'silent' state requires specific chromatin modifications which allow its duplication and propagation through mitotic and meiotic divisions (Richards and Elgin, 2002). Heterochromatin has a vital role in maintaining the structural integrity of specific chromosomal regions; it is essential to sustain stable structures at defined regions of repetitive DNA such as centromeres, telomeres and transposable elements. Recombination is repressed across centromeres in general and the silent mating type loci in fission yeast (Nielsen and Egel, 1989; Niwa et al., 1989). It is likely that silent chromatin structures inhibit the potentially detrimental effects of homologous recombination between repetitive elements on different chromosomes.

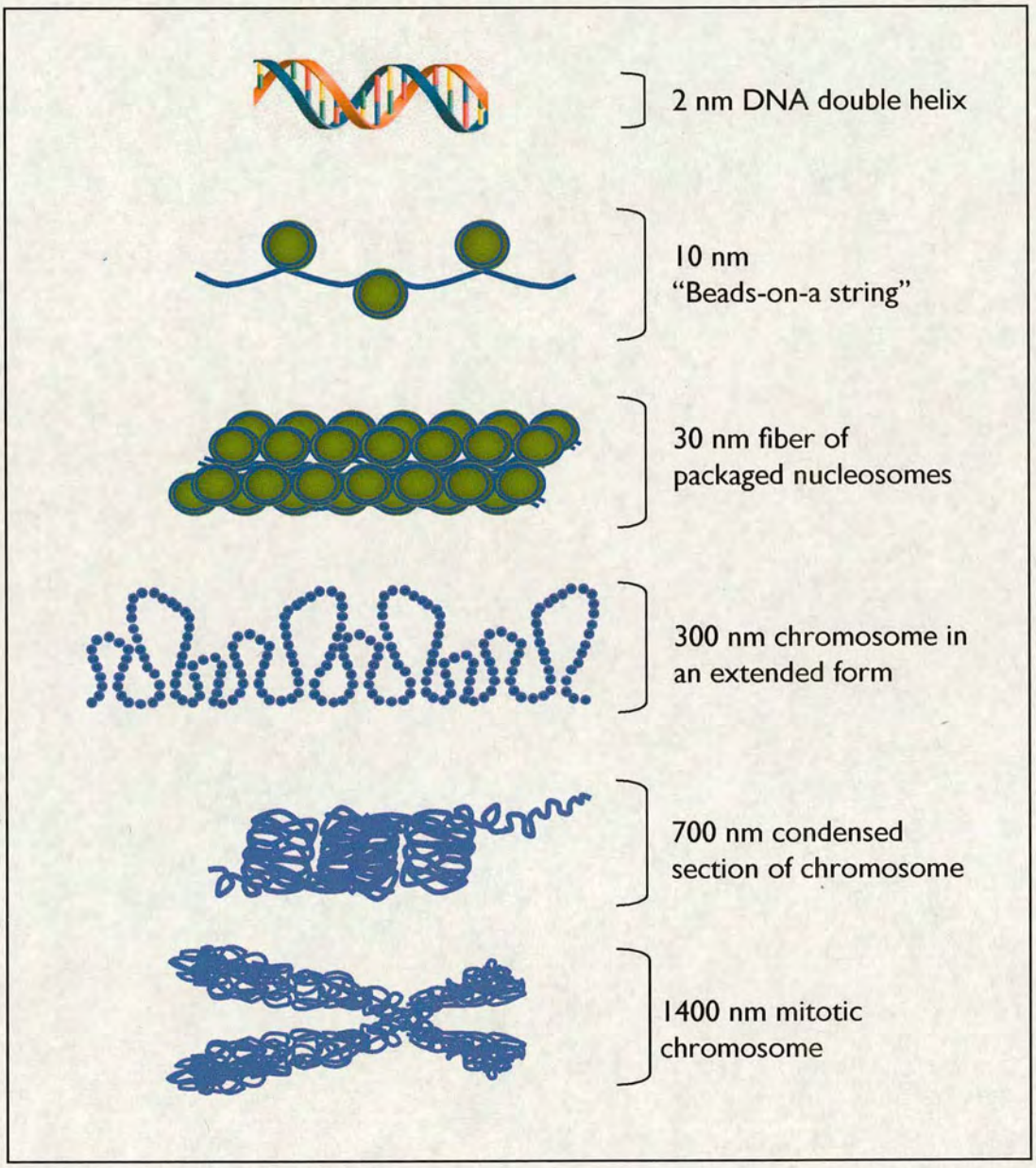


Figure 1.1. DNA is packaged into higher-order chromatin structure. The DNA double helix is wrapped around the core nucleosome particle to form the 10 nm "beads-on-a-string structure". This is further compacted into a 30 nm fibre of 6 nucleosomes per turn. Chromatin is packaged as loops which anchor to the nuclear matrix.

1.2 Histone modifications

Nucleosomes are dynamic entities. Histones are post-translationally modified on their N-terminal tails which provides a dynamic environment for the binding of chromatin associated proteins (Jenuwein and Allis, 2001). These modifications and proteins which bind them define the chromatin state; that is, particular modifications can facilitate or repress transcription by regulating access to the underlying DNA (Jenuwein and Allis, 2001). Histones can be acetylated, methylated, phophorylated, ubiquitinated, sumolyated, biotinylated and ADP-ribosylated (Krebs, 2007; Bernstein, 2006). It is known that specific modifications also define heterochromatin and euchromatin. The enzymes which effect histone modifications are highly specific for particular amino acid residues. For example, general hypoacetylation of histones and phophorylation of serine 10 on histone H3 is required for correct chromosome segregation by regulating chromosome condensation during mitosis (Hendzel et al., 1997). In addition the methylation of lysine 9 by the Suv39 family of histone methyltransferases which creates a binding site for HP1 proteins is required for the formation of heterochromatin (Peters et al., 2001, Bannister, 2001; Rea et al., 2000).

Histone modifications do not act independently of one another. It has been demonstrated that the ubiquitination of H2B causes repression of transcription initiation by inhibiting the di- and tri-methylation of H3K4 in mice (Nakagawa et al., 2008). Ubiquitination of H2A has also been shown to inhibit transcription by preventing elongation by RNA polymerase II (RNAPII) (Zhou et al., 2008). Moreover, Set2 mediated H3K36 methylation which is associated with actively transcribed genes, acts as a mark to recruit histone deacetylases (Lee and Shilatifard, 2007). This is thought to stabilise chromatin and inhibit aberrant transcriptional initiation (Lee and Shilatifard, 2007).

1.3 Epigenetic regulation of centromeres

Several lines of evidence contribute to the view that centromeres are epigenetically regulated. Centromeric DNA is usually found to be enriched for repetitive elements and AT-rich sequences yet the primary sequence is not conserved among different species and indeed may vary within an individual organism (Sullivan et al., 2001). Centromeric DNA is not always sufficient to form a functional centromere and active centromeres can be formed on DNA sequence bearing little resemblance to that found at endogenous centromeres (Karpen and Allshire, 1997; Marshall et al., 2008).

Most organisms are monocentric, that is they possess only one centromere per chromosome. Dicentric chromosomes which have more than one centromere have been recovered and appear to be stably transmitted in both flies and mammals. In some instances this could be due to inactivation of one of the centromeres although exactly how the active and inactive centromeres are defined is unknown. Thus, centromeres can become inactivated with no alteration to the DNA sequence. Usually the presence of more than one centromere per chromosome would result in chromosome loss as the formation of an anaphase bridge would occur and cause each centromere to attach to opposite poles and thus fragment (Karpen and Allshire, 1997; Wiens and Sorger, 1998) (Figure 1.2a).

Centromeres are able to form on noncentromeric DNA. For example, the human marker chromosome mar(del)10, which is a rearrangement of chromosome 10, does not contain any α-satellite DNA yet is mitotically stable (Voullaire et al., 1993). Centromere proteins can be visualised at a site of primary constriction indicating the formation of a neocentromere. The way in which activation of neocentromeres occurs is unclear. Two hypotheses are presented in Figure 1.2b. The neocentromere may become activated after which chromosome breakage occurs, resulting in loss of the endogenous centromere. Alternatively, deletion of the endogenous centromere may occur first and then the neocentromere is activated (Figure

1.2b). Many human marker chromosomes have been studied which lack α-satellite DNA yet are stably transmitted and bind proteins usually found at centromeres (Depinet et al., 1997; Marshall et al., 2008; Sullivan and Schwartz, 1995; Voullaire et al., 1993). In fact, it may be proteins which specify centromere function as CENP-B, which is known to bind α-satellite DNA, can bind both active and inactive centromeres whereas only active centromeres bind CENP-C, CENP-E and CENP-A (Depinet et al., 1997). However, it has been shown that *de novo* centromere formation on artificial chromosomes requires α-satellite DNA containing the 17 bp binding site for CENP-B (Okada et al., 2007). CENP-B also binds α-satellite DNA inserted at ectopic loci where no centromere is formed (Okada et al., 2007). It has been proposed that CENP-B may act to form neocentromeres on acentric chromosomes but may suppress excess centromere formation (Okada et al., 2007). Interestingly, one factor which is conserved at all active centromeres, and neocentromeres, is the histone H3 variant CENP-A (Heit et al., 2006). CENP-A and its homologues have been identified at centromeres in mammals, flies and yeast (Black and Bassett, 2008).

Drosophila are capable of forming neocentromeres after irradiation mutagenesis of a minichromosome (Murphy and Karpen, 1995; Williams et al., 1998). Typically, these minichromosomes are stably transmitted and appear to have normal centromeres. However, chromosome fragments were only able to form neocentromeres if they had previously been adjacent to the centromere of the minichromosome prior to irradiation. This

suggests that formation of a neocentromere may require spreading of some kinetochore/centromere proteins in the absence of any boundary elements.

Evidence from *S. pombe* supports the argument for epigenetic regulation of the centromere. Minichromosomes containing central core and an outer repeat sequence could convert from a nonfunctional centromere to a functional centromere without any alteration to the DNA sequence or plasmid structure (Steiner and Clarke 1994). Once the active state was

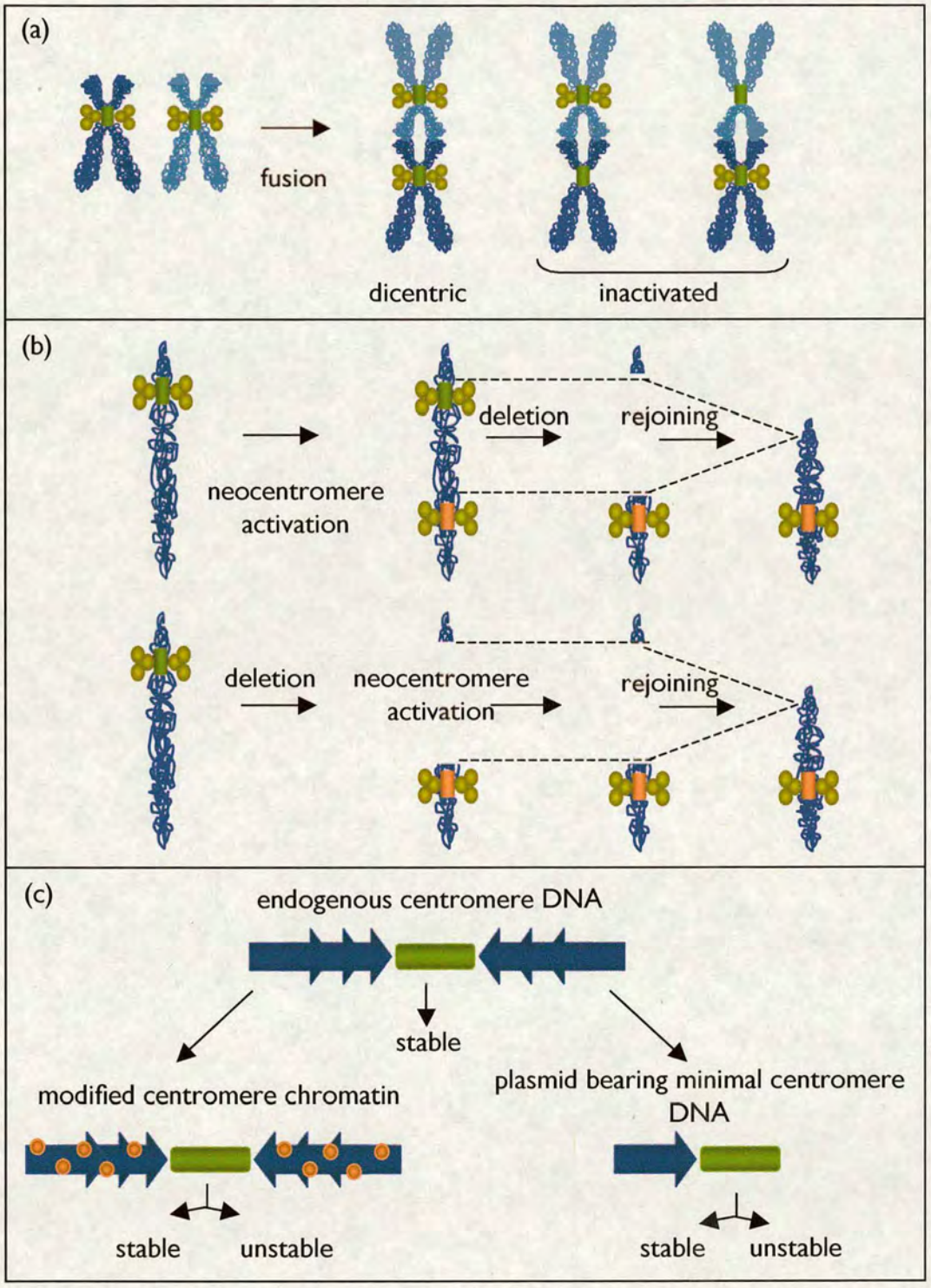


Figure 1.2. Centromeres are epigenetically regulated. **(a)** Chromosome fusions/translocations can cause formation of dicentric chromosomes which can have one or other of the centromeres inactivated. **(b)** Neocentromeres may be formed in two ways. A neocentromere becomes activated, part of the chromosome becomes lost during breakage where the endogenous centromere is deleted and then rejoins. Alternatively, deletion may occur first and then the neocentromere is activated. **(c)** In *S. pombe*, modification of the centromere can induce heritable changes without alteration of the DNA sequence. Chromatin which is chemically treated can form both active and inactive states, as can minichromosomes bearing a minimal centromere.

established it could be propagated for many divisions (Figure 1.2c). This demonstrates that it is possible for the same DNA sequence to have two functionally independent states. In addition, transient trichostatin A (TSA) treatment, which inhibits histone deacetylation, induces a heritable hyperacetylated state which induces functional changes within the centromere. This disruption of centromeric chromatin can be inherited through several generations and is propagated even in the absence of TSA (Ekwall et al., 1997). This demonstrates that the active and inactive states coexist with no alteration to DNA sequence (Figure 1.2c).

Thus, centromeric DNA is not always required or sufficient to form an active kinetochore. The plasticity of the centromere is demonstrated by the fact that the kinetochore can form on sequence other than that found at endogenous centromeres. However, given that the function of the centromere is essential to viability, it is vital that the cell retains some way of regulating centromere identity. This paradox of centromeric plasticity and stability can be explained by the epigenetic nature of the locus. That is, centromeres are epigenetically defined and propagated so that a site previously designated as centromeric will be inherited for many generations.

1.4 CENP-A may define centromere identity

Centromeres are generally composed of two elements: highly repetitive tandemly arranged DNA sequences and a distinct chromatin domain containing a histone H3 variant, CENP-A (Morris and Moazed, 2007). *H. sapiens* CENP-A, and its homologs, *S. cerevisiae* Cse4p, *S. pombe* Cnp1, *Drosophila* CID, is found at all active centromeres and as such is a good candidate for the epigenetic mark which specifies the site of kinetochore assembly (Black and Bassett, 2008; Morris and Moazed, 2007; Pidoux and Allshire, 2000). CENP-A is incorporated into nucleosomes presumably in the place of H3 but how CENP-A becomes deposited specifically at the correct location is unclear (Castillo et al., 2007). It has been suggested that CENP-A is deposited during replication but data from mammals and flies show that

replication is not required for CENP-A incorporation (Mellone and Allshire, 2003). It may be that a specific loading factor, such as Mis6 or Sim4 in fission yeast, is required for CENP-A loading at centromeres. Mutants of Mis6 or Sim4 display reduced CENP-A localisation at centromeres (Pidoux et al., 2003; Takahashi et al., 2000). However, the budding yeast Mis6 homolog, Ctf3, is not required for CENP-A loading (Measday et al., 2002). Many proteins are found to affect CENP-A localization. In *Drosophila*, the histone chaperone RbAp48 has been shown *in vitro* to assemble CENP-A chromatin, but as it also contributes to H3 loading it is unclear how this would facilitate CENP-A localisation *in vivo* (Furuyama et al., 2006).

1.5 Centromere structure and kinetochore assembly

The centromere, and the associated kinetochore complex, is a highly specialised structure on a chromosome which is responsible for the correct segregation of sister chromatids to both daughter cells during mitosis. To this end, the centromere must fulfill several functions. The sister chromatids must be attached to microtubules and this attachment must be such that the chromatids are bi-orientated to opposite poles (Pidoux and Allshire, 2000; Pluta et al., 1995). A surveillance checkpoint, the spindle checkpoint, exists to delay the onset of anaphase when a single kinetochore is incorrectly attached or other spindle damage occurs (Pidoux and Allshire, 2000; Pluta et al., 1995). The centromere is required to maintain cohesion until all of the chromosomes are correctly oriented and only then can segregation occur (Pidoux and Allshire, 2000; Pluta et al., 1995).

1.5.1 Budding yeast centromeres

The best characterised, and perhaps the simplest, centromeres are found in the budding yeast *Saccaromyces cerevisiae*. It has point centromeres of 125-bp that bind a single microtubule (Pluta et al., 1995; Cleveland 2003). Each centromere contains three distinct DNA sequences; CDE-I and CDE-III are conserved at each chromosome whilst CDE-II has no sequence conservation

but length (76-84 bp) and AT-content (90%) are similar (Cleveland et al., 2003; Sullivan et al., 2001). CDE-I is not essential for centromere function but serves as a binding site for the kinetochore protein Cbf1 (Ortiz et al., 1999). CDE-II interacts with the single Cse4 nucleosome which is required for correct chromosome segregation (Keith and Fitzgerald-Hayes, 2000; Stoler et al., 1995). Cse4 is the budding yeast CENP-A homolog. CDE-III is only 25-bp long but the integrity of this sequence is essential as mutations of specific single bases can abolish centromere function (Figure 1.3) (McGrew et al., 1986). CDE-III is bound by the Cbf3 complex which is composed of Ndc10, Cep3, Ctf13 and Skp1 (Sorger et al., 1995). This complex along with Ctf19, Mcm21 and Okp1 is thought to mediate kinetochore-microtubule interactions (Ortiz et al., 1999).

1.5.2 *Drosophila melanogaster* centromeres

In metazoa, centromeres tend to be much larger and more complex. Analysis of the X-derived minichromosome Dp1187 in *Drosophila* has demonstrated that a 420 kb region is required for proper centromere function (Murphy and Karpen, 1995; Sun et al., 1997). This is composed of two distinct simple repeats, AATAT and AAGAG, single interspersed complete transposons and a unique AT-rich region (Figure 1.3) (Murphy and Karpen 1995). The transposable elements can be found at many chromosomal sites and are not present at all centromeres (Sun et al., 1997). In addition, the AATAT and

AAGAG repeats are found scattered thoughout the genome but alone are not sufficient to induce centromere formation (Sun et al., 1997). The *Drosophila* CENP-A homolog, CID, is required for kinetochore assembly and chromosome segregation during mitosis (Blower and Karpen, 2001). The CID protein is localised along extended chromatin fibers interspersed with blocks of H3 which may have functionally distinct properties (Blower et al., 2002). The overexpression of CID causes the formation of ectopic centromeres and multicentric chromosomes resulting in misegregation, anueploidy and growth defects, demonstrating the importance of centromere specification (Heun et al., 2006).

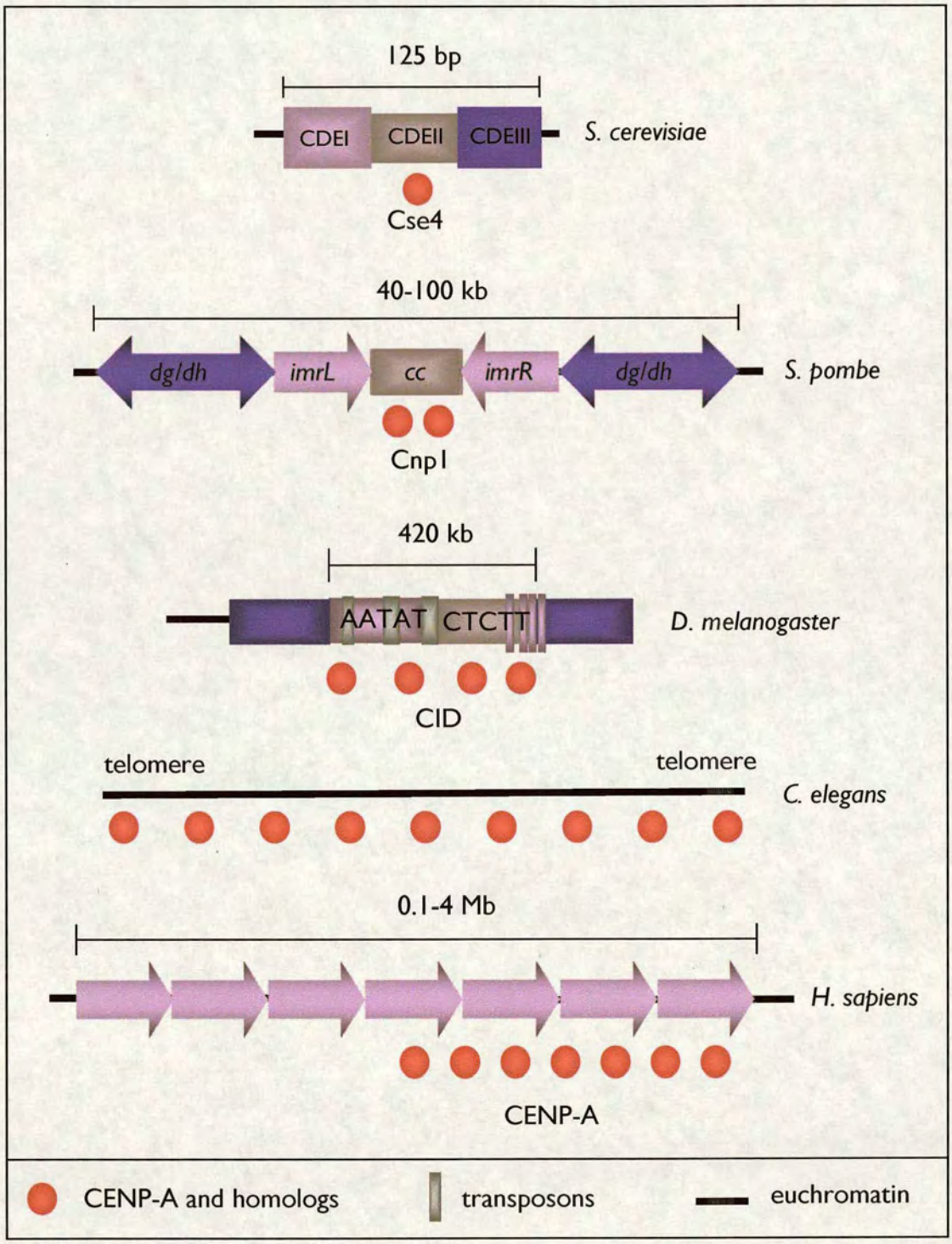


Figure 1.3. Centromere structure varies in different organisms. *S. cerevisiae* centromeres contain 3 distinct DNA sequences. *S. pombe* centromeres consist of a unique central core with flanking inverted repeats. *Drosophila* minichromosome Dp1187 containing a core of 5 bp satellites interspersed with transposons (in light gray) and flanked by repetitive DNA in purple. *C. elegans* forms holocentric centromeres along the entire length of the chromosome. Human centromeres consist of tandem arrays of α-satellite DNA. (Adapted from *Sullivan et al 2001*).

1.5.3 Plant centromeres

Plants show a similar centromere DNA composition to metazoans. *Arabidopsis thaliana* centromeres consist of 180-bp repeats surrounded by complex DNA and retrotransposons and flanked by ribosomal DNA (Copenhaver et al., 1999). Although these centromeres show repression of recombination the flanking regions contain repetitive elements which display normal levels of recombination. CENH3, the *Arabidopsis* CENP-A homolog, is known to associate with centromere repeats and localises at a region of low DNA methylation (Talbert et al., 2002; Zhang et al., 2008).

Interestingly, *Arabidopsis* centromeres contain genes that are thought to be expressed. This is rare in more complex eukaryotes but there are exceptions (Copenhaver et al., 1999). Rice centromeres are composed of two elements: the 155 bp satellite CentO and the retrotransposon CRR (Yan et al., 2006). Transcription has been demonstrated across specific rice centromeres which corresponds to active genes, but also to intergenic regions and repetitive DNA (Yan et al., 2006).

1.5.4 *Caenorhabditis elegans* centromeres

Centromeres in fission yeast, *Drosophila,* plants and mammals are regional centromeres as they assemble at a specific locus on the chromosome. Most organisms contain only one centromere per chromosome and are thus termed monocentric. However, the nematode *Caenorhabditis elegans* forms holocentric centromeres where kineotchore proteins extend ribbon-like along the entire length of the chromosome (Figure 1.3) (Sullivan et al., 2001). This results in the formation of diffuse kinetochores along the chromosome which coincides with CENP-A localisation (Maddox et al., 2004). Holocentric centromeres may provide a way of retaining genetic material after double-strand breaks by allowing the transmission of chromosome fragments.

1.5.5 Mammalian centromeres

Human centromeres primarily consist of tandemly arranged 171-bp monomer repeats called α-satellite DNA. These repeats can extend from 100kb to several megabases (Figure 1.3). Centromere function has been mapped to these α-satellite arrays by deletions and insertions (Brown et al., 1994; Schueler et al., 2001). However, the insertion of α-satellite DNA into ectopic chromosomal sites is not sufficient to form an active centromere (Earnshaw et al., 1989). It has been shown in mammalian cells that α-satellite DNA containing binding sites for the centromere protein CENP-B is sufficient to form *de novo* centromeres (Okada et al, 2007). However, mice lacking CENP-B are viable and do not demonstrate any significant defects in chromosome segregation (Hudson et al., 1998). CENP-A the kinetochore specific H3 variant, is found on α-satellite DNA interspersed with H3 on stretched chromatin fibres (Blower et al., 2002). CENP-A chromatin is predominantly found beneath the kinetochore and thus provides a base for kinetochore assembly (Blower et al., 2002).

1.6 Fission yeast centromeres

S. pombe provides an excellent model organism for the dissection of molecular events involved in chromosome structure and function due to its genetic tractability and comparatively small genome size. *S. pombe* has 4979 protein-coding genes contained within 13.8 Mb. The genome is divided between three chromosomes: chromosome I is 5.7 Mb, chromosome II is 4.6 Mb and chromosome III is 3.5 Mb (Wood et al., 2002). *S. pombe* is a unicellular archiascomycete fungus which shares many biological characteristics with more complex eukaryotes. For this reason it has been used with great success to study many cellular processes including cell-cycle control, DNA repair and recombination as well RNAi-mediated heterochromatin formation and chromosome segregation (Egel, 2004).

1.6.1 Centromere structure

The structure of *S. pombe* centromeres is somewhat similar to that of more complex eukaryotes in that they are relatively large, repetitive and complex structures which occupy 35-110 Kb (Figure 1.3 and Figure 1.4) (Steiner et al., 1993; Takahashi et al., 1992). This is in contrast to the comparatively simple point centromeres of the budding yeast *Saccaromyces cerevisiae* which are only 125 bp (Cleveland et al., 2003; Sullivan et al., 2001). Fission yeast kinetochores bind 2-4 microtubules at mitosis (Ding et al., 1993). This is again more reminiscent of the multiple microtubule association of metazoan kinetochores than the single microtubule attachment observed in budding yeast (Winey et al., 1995). *S. pombe* centromeres are composed of a unique central core (*cc*) of 4-7 Kb which is flanked by the innermost repeats (*imr*L/R) and the outer repeats on which centromeric heterochromatin forms (Allshire, 1995; Cowieson et al., 2000; Steiner et al., 1993; Takahashi et al., 1992). The largest centromere resides on the smallest chromosome and the smallest centromere resides on the largest chromosome (Figure 1.4). Centromere I is the best characterised due to its size and the fact it has the fewest repetitive elements. Together the central core and *imr* repeats make up the central domain and are packaged in a centromere specific form of chromatin containing the histone H3 variant Cnp1 (the CENP-A homolog in fission yeast), which replaces histone H3 (Figure 1.4 and 1.5a) (Castillo et al., 2007; Takahashi et al., 2000). This central domain has an unusual chromatin

structure as partial digestion with micrococcal nuclease produces a smeared pattern rather than the typical ladder pattern (Polizzi and Clarke, 1991; Takahashi et al., 1992). Genes are also silenced when placed in this central domain, and the factors involved affect CENP-A localisation and kinetochore assembly and function, distinct from those that affect heterochromatin formation on the outer repeats (Allshire et al., 1994; Allshire et al., 1995; Ekwall et al., 1996; Partridge et al., 2000; Pidoux et al., 2003). Thus, this central domain is functionally and structurally distinct from the heterochromatic outer repeat regions (Allshire et al., 1995; Partridge et al., 2000). The central core itself is essential for centromere activity but alone it is not sufficient to assemble an active centromere. Studies using

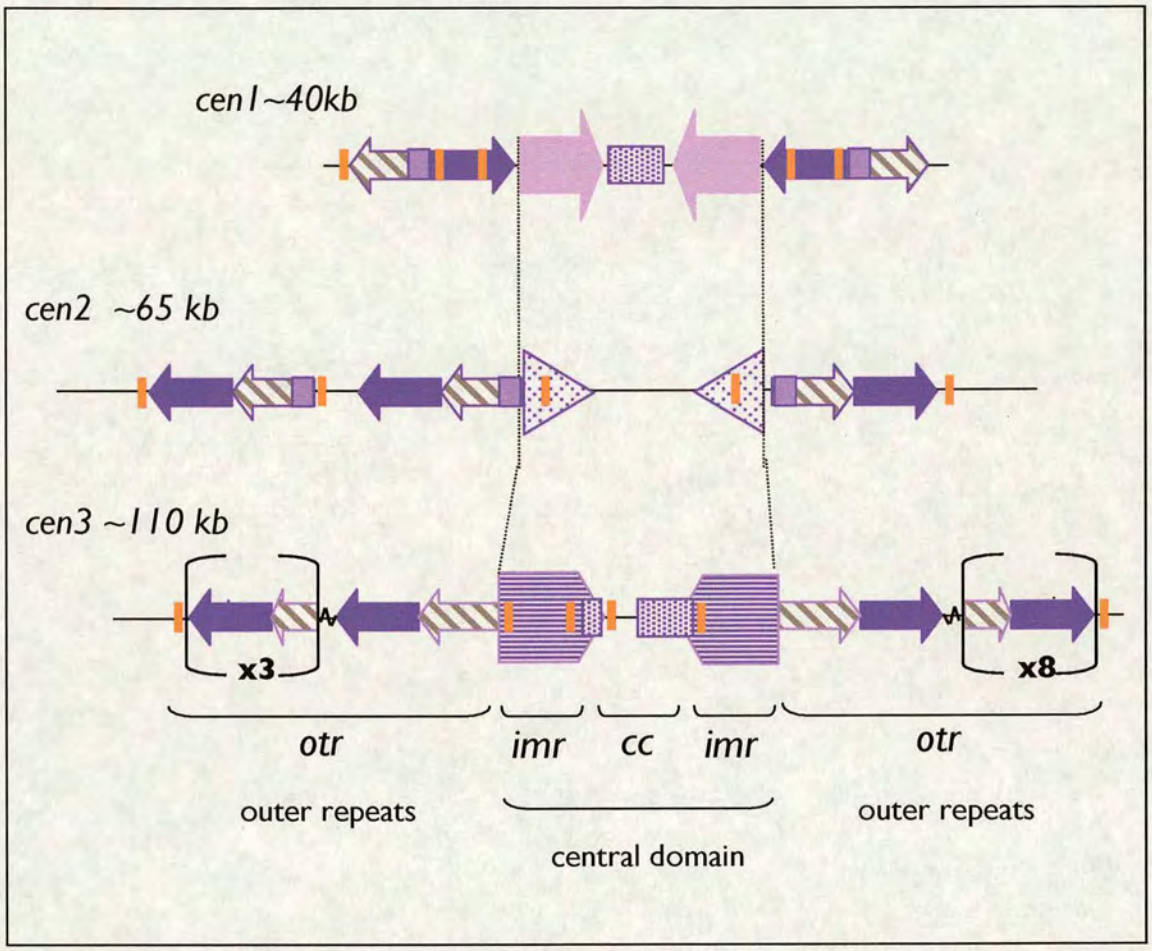


Figure 1.4. Centromere organisation in fission yeast. *S. pombe* has three chromosomes. The outer repeats, *otr*, are composed of *dg*, lilac arrows, and *dh*, cross-hatched arrow. These are arranged differently at each centromere. The central core of centromeres I and III share sequence homology, represented by the spotted box. There is also some sequence similarity across a small region of the central core of chromosome II. The inner repeats, *imrl*, at all three centromeres are distinct. The multiple tRNA genes are represented by orange bars *(from Allshire, 2001)*.

minichromosomes have demonstrated that at least part of the heterochromatic outer repeat, in combination with central domain sequences, is essential to allow the *de novo* formation of active centromeres (Baum et al., 1994; Folco et al., 2008; Ngan and Clarke, 1997; Takahashi et al., 1992).

Large blocks of heterochromatin are prevalent at the centromere regions of many eukaryotes. In metazoa, large arrays of repetitive DNA of up to several megabase pairs are packaged as heterochromatin at centromeres. The outer repeats (*otr*) themselves are composed of two elements, known as the *dh* and *dg* (or K and L) repeats which vary in number and are arranged

differently with respect to each other at each centromere (Figure 1.4 and 1.5a) (Steiner et al., 1993; Takahashi et al., 1992). Because these repeats are packaged into heterochromatin, expression levels of marker genes (*ade6*$^{+}$ and *ura4*$^{+}$ for example) inserted at sites across the outer repeats are subject to variable repression or expression, resulting in phenotypic variegation. This has allowed the development of screens to identify many factors involved in heterochromatin and hence centromere structure and function (Figure 1.5b) (Allshire et al., 1995; Ekwall et al., 1999)

1.6.2 Central core domain and kinetochore proteins

The central core domain, which comprises of the central core and the innermost parts of the *imr* repeats, has a distinct chromatin structure from the heterochromatic outer repeats. The transcriptional silencing found here is less robust than that at the *otr* repeats however, silencing screens like those performed at the *otr* repeats have uncovered numerous kinetochore proteins which alleviate silencing at the central core (Pidoux and Allshire, 2004; Pidoux et al., 2003).

The *sim* (silencing in the middle of the centromere) mutant screen uncovered *sim2* which is an allele of Cnp1, the fission yeast CENP-A homolog, *sim3*

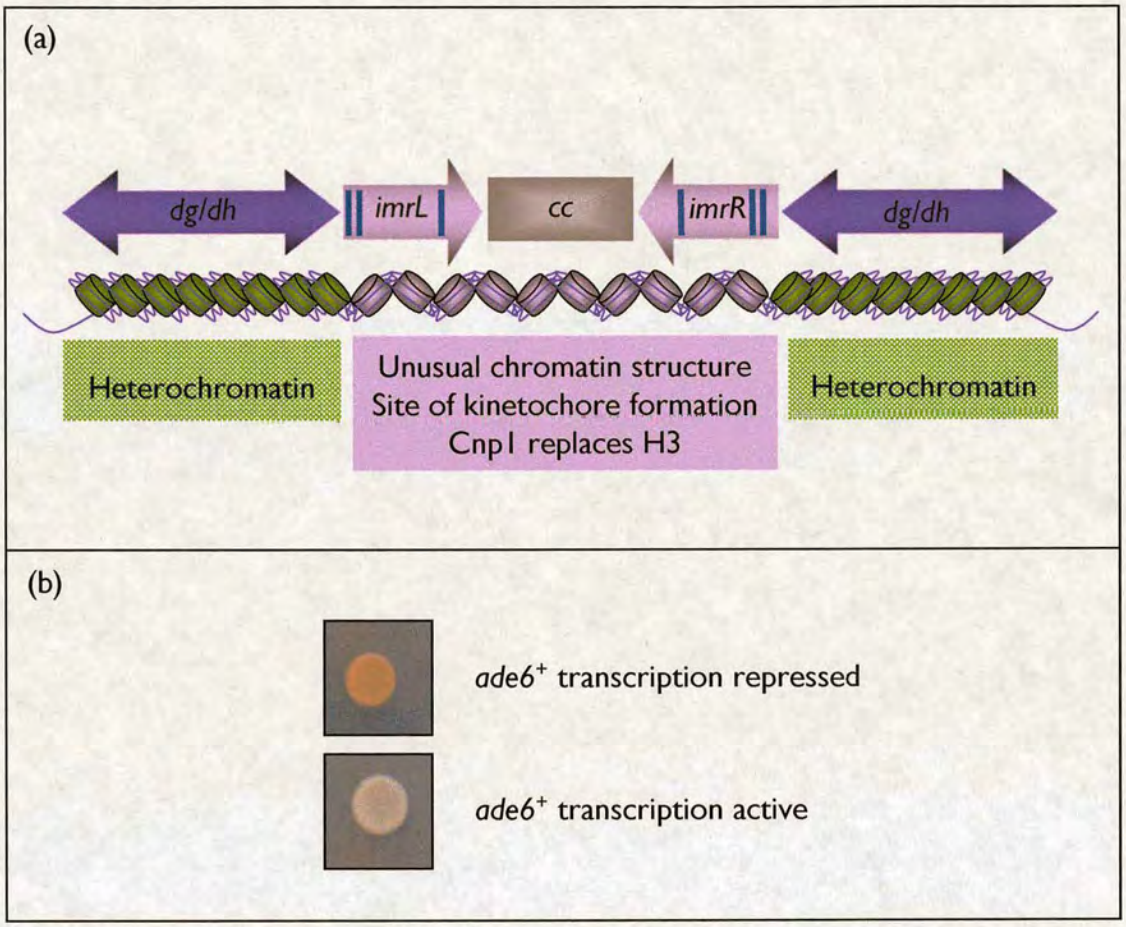


Figure 1.5. Features of the fission yeast centromere. (a) The central core region comprises the innermost repeats and the central core. This is the site of kinetochore formation and is characterised by the replacement of histone H3 by the variant Cnp1. The outer repeats are packaged into heterochromatin. **(b)** Genes packaged into heterochromatin are variably expressed. In this example, the *ade6*$^+$ gene inserted at the outer repeats is repressed under normal conditions which results in phenotypically red colonies. In contrast, when silencing is alleviated the colonies are white. This has allowed the development of several screens for factors required for the maintenance of centromere integrity.

which shares homology the histone binding protein NASP, and *sim4* which shares similarity with CENP-H (Dunleavy et al., 2007; Pidoux et al., 2003). Sim3 is required for Cnp1 deposition at centromeres (Dunleavy et al., 2007). It has been shown that Sim3 can bind both Cnp1 and histone H3 and is required for the incorporation of newly synthesised Cnp1 at centromeres (Dunleavy et al., 2007). Sim3 has been proposed to act as a Cnp1 chaperone to allow its incorporation into centromeric chromatin (Dunleavy et al., 2007).

Sim4 associates with Mis6 and both proteins associate with the central core domain (Pidoux et al., 2003). The *sim4* and *mis6* mutants display reduced Cnp1 association with the central core region and both demonstrate a disrupted micrococcal nuclease digestion pattern (Pidoux et al., 2003; Takahashi et al., 2000). Mis6 is required for the incorporation of newly synthesised Cnp1 at centromeres and as such has been proposed to act as a loading factor for Cnp1, in conjunction with Sim4 (Takahashi et al., 2000).

Sim4 associates with a number of proteins; Mis6, Mis15, Mis17, Mal2, Dad1 and the Fta1-7 proteins (Liu et al., 2005). *mis15* and *mis17* mutants both display reduced Cnp1 association at centromeres and have disrupted centromere chromatin demonstrated by micrococcal nuclease digestion (Hayashi et al., 2004). Mal2 is also required to maintain structural integrity of the central core domain and associates with another central core protein Mis12 (Jin et al., 2002). Fta2, 3 and 4 are novel proteins which are associated with the central core domain (Liu et al., 2005). Fta2 is associated with Mal2 and is required for bipolar chromosome attachment (Kerres et al., 2006).

Mis12 was identified in the same screen as Mis6 and localises to the central core domain (Takahashi et al., 1994). It is associated with Mis13 and Spc7 in a complex similar to the Mtw1 complex in budding yeast (Obuse et al., 2004). Both Mis13 and Spc7 localise to the central core domain (Obuse et al., 2004). Spc7 interacts with the microtubule binding protein Mal3 and may mediate

kinetochore-microtubule interactions (Kerres et al., 2006). The human Mis12 complex associates with HP1 and may provide a bridge between the heterochromatic repeats and central core domain (Obuse et al., 2004). A summary of proteins found at the central core is detailed in Figure 1.6.

1.6.3 Distinct boundaries demarcate the domains within and around centromeres

The transition from outer repeat heterochromatin to central domain CENP-A^{cnp1} chromatin coincides with the presence of two to four tRNA genes (Kuhn et al., 1991; Partridge et al., 2000; Steiner et al., 1993; Takahashi et al., 1992; Takahashi et al., 1991). For example, two tRNA genes are found in the region of transition between the central core domain and the heterochromatic repeats at centromere 1. In addition, tRNA genes are present at five of the six extremities of the three centromeres between the *otr* and surrounding euchromatin, the exception occurring at the right hand side of centromere 1 (Figure 1.4). Strong DNase hypersensitive sites coincide with the tRNA genes in the *imrL/R* of centromere 1 and it had been suggested that these tRNA genes might act to separate outer repeat heterochromatin from the CENP-A^{cnp1} chromatin of the central domain (Partridge et al., 2000; Takahashi et al., 2000; Takahashi et al., 1992). Genome-wide analysis has confirmed that heterochromatin is absent inside of the 2-4 tRNA genes clustered at the *cc/otr* boundary. The transition between outer repeat heterochromatin and adjacent euchromatin also coincides with the presence of tRNA genes, TFIIIC binding sites or other elements which may act as boundaries (Cam et al., 2005; Wallrath and Geyer, 2006). The $tRNA^{Ala}$ found at the transition between the central domain and outer repeats at centromere 1 is transcribed and is required to restrict heterochromatin to its normal location. The barrier activity of this $tRNA^{Ala}$ requires the association of RNA polymerase III and the transcription factor IIIC (Scott et al., 2007). Inactivation of this transcriptionally active tRNA permits heterochromatin to spill into the central domain. However, deletion of the other $tRNA^{Glu}$ gene, only 424 bp away from $tRNA^{Ala}$, had a very weak effect. Attempts to

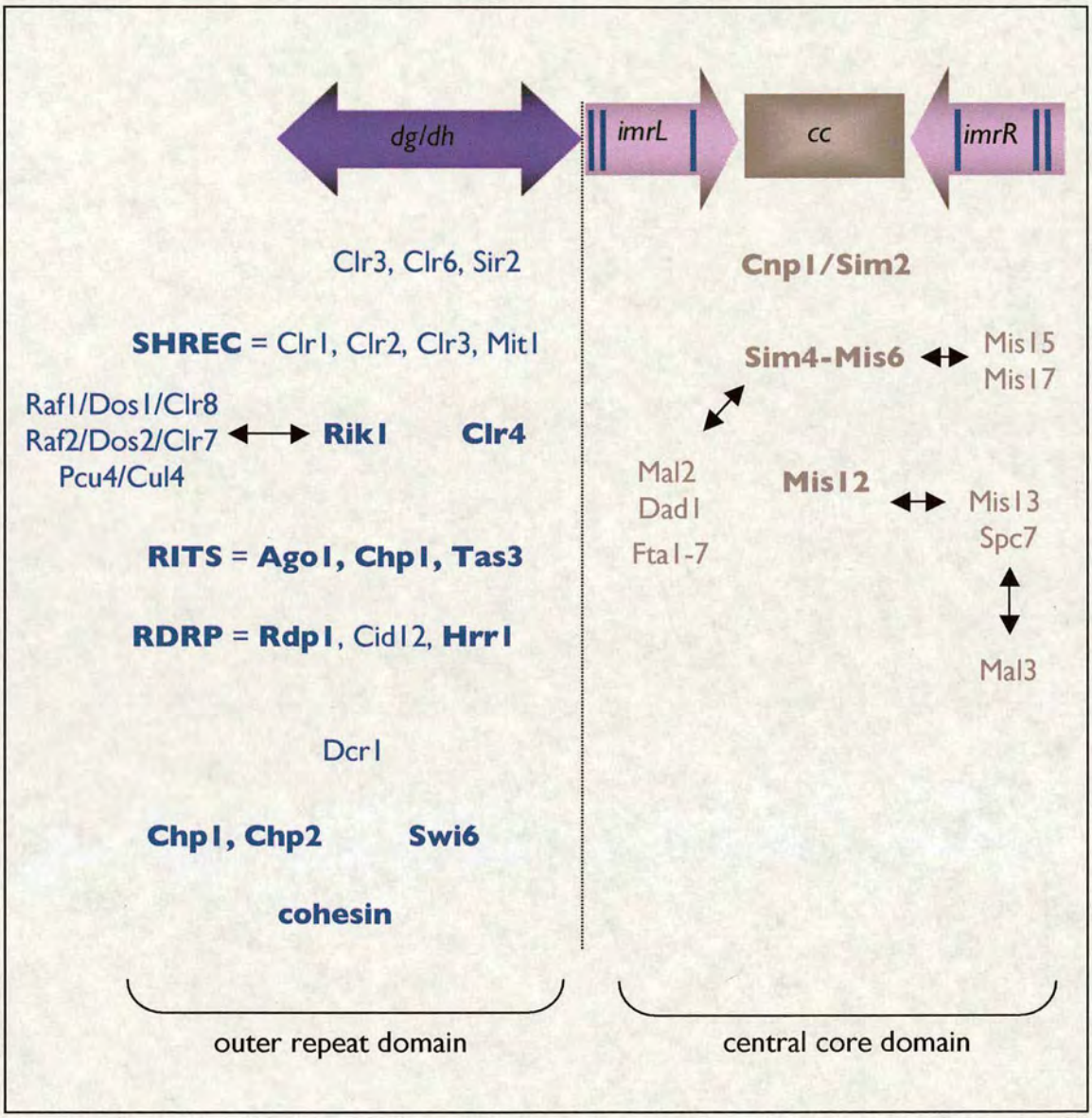


Figure 1.6. Proteins associated with the central domain or outer repeat domain of the fission yeast centromere. Heterochromatic outer repeat proteins are shown in blue on the left and central core domain proteins are shown in grey on the right. Proteins which are physically associated with the centromere are in bold. For simplicity only the left outer repeat is shown - proteins which associate here also associate at the right outer repeat. (Adapted from *Pidoux, 2004*).

simultaneously delete both the $tRNA^{Glu}$ and $tRNA^{Ala}$ failed perhaps indicating that these tRNA genes act together to provide an important function at the centromere (Scott et al., 2006).

1.6.4 Heterochromatin is associated with specific histone modifications and proteins

The definition of heterochromatin is documented as a cytologically visible region of condensed chromatin. More recently it has become possible to analyse heterochromatin at a molecular level and identify the proteins and histone modifications associated with these regions (Richards and Elgin, 2002). It is now commonly accepted that heterochromatin can also be defined as regions which display low levels of histone acetylation and are associated with the methylation of histone H3 on lysine 9 (H3K9me) and binding of chromo domain proteins related to *Drosophila* and mammalian Heterochromatin Protein 1 such as Swi6 in *S. pombe* (Figure 1.6 and Figure 1.7). The specific methylation of histone H3 on lysine 9 creates a binding site for Swi6 allowing it to bind histone H3 via its chromo domain (Bannister et al., 2001). However, *S. cerevisiae*, although it has heterochromatin which mediates transcriptional gene silencing, does not contain homologs of Clr4 or Swi6 and as yet no H3K9 methylation has been detected (Sharp et al., 2003). Swi6, like HP1, dimerises via its chromo shadow domain and this may create an interaction surface for the recruitment of other proteins (Cowieson et al., 2000). Swi6 is required to recruit a high concentration of the cohesin complex over the outer repeats. The cohesin complex is required to maintain tight physical cohesion of sister chromatids until the point of anaphase (Bernard et al., 2001). Methylation of lysine 9 in fission yeast is mediated by the conserved histone methyltransferase Clr4 (Suv39 in *Drosophila* and mammals). Clr4 has been shown to be required for the association of Swi6 with outer repeat heterochromatin at centromeres, the mating type locus and telomeres (Ekwall et al., 1996; Nakayama et al., 2001; Partridge et al., 2000). Strains expressing histone H3 that lack lysine 9 are defective in silencing and Swi6 localisation. This underscores the importance of lysine 9 of H3 and its

methylation by Clr4 in recruiting Swi6 (Mellone et al., 2003).

1.6.5 Histone methylation is required to bind the HP1 homolog, Swi6

Clr4 is the only ortholog of Suv39 in fission yeast. These histone methyltransferases can catalyse mono- di- and tri- methylation of lysine 9 of histone H3. In *S. pombe* most H3 K9 methylation appears to be dimethyl although mono- and tri- methyl states have been detected (Yamada et al., 2005). In the absence of Clr4, all H3 K9 methylation is lost and thus Clr4 is probably the only enzyme responsible for these modifications. Like its Suv39 orthologs, Clr4 contains a chromo and a SET domain. It is the conserved SET domain of Clr4 that is responsible for the H3 K9 methyltransferase activity and mutations in this domain affect the levels of H3 K9 methylation at centromeres and the mating type locus (Nakayama et al., 2001; Rea et al., 2000). Perhaps surprisingly, the genes encoding Clr4 and Swi6 are not essential, thus aiding analyses of these proteins in fission yeast. However, loss of Clr4 or Swi6 function results in defective silent chromatin at centromeres, telomeres and the mating type locus which leads to reduced centromere cohesion and increased chromosome loss (Allshire et al., 1995; Ekwall and Ruusala, 1994; Klar and Bonaduce, 1991; Lorentz et al., 1994; Thon et al., 1994).

1.6.6 Histone deacetylation is required to allow methylation

It is known that histone methyltransferases are unable to methylate target lysine residues that are acetylated and therefore histone deacetylases are required to allow methylation (Rea et al., 2000). Within regions of heterochromatin the lysine residues in the tails of histones H3 and H4 exhibit low acetylation levels and this hypoacetylated state is important for the integrity of heterochromatin. Transient inhibition of histone deacetylases using Trichostatin A (TSA) induced hyperacetylation of histone H3 and H4 on the outer repeats resulting in derepression of marker genes, loss of Swi6 localisation and defective chromosome segregation (Ekwall et al., 1997). This

expressed state was found to be heritable through several generations even in the absence of TSA. It is likely that this forced hyperacetylation blocked methylation of lysine 9 by Clr4, thereby causing loss of H3 lysine 9 methylation and thus propagation of the expressed state. Deacetylation of H3 and H4 is therefore essential for the formation of intact heterochromatin and associated functions.

Several histone deacetylases (HDACs): Sir2, Clr3, and Clr6, are involved in heterochromatin formation. Clr6 is an essential gene, with broad substrate specificity (Bjerling et al., 2002; Nakayama et al., 2003; Wiren et al., 2005), Sir2 specifically deacetylates H3K9 and H4K16 residues and is required for H3K9 methylation (Shankaranarayana et al., 2003; Wiren et al., 2005). Clr3 specifically deacetylates H3K14 and it is required to recruit the histone methyltransferase Clr4 (Bjerling et al., 2002; Nakayama et al., 2001; Wiren et al., 2005). It has been proposed that Clr3 may stabilise histone H3K9 methylation by prohibiting histone modifications associated with active transcription, thereby discouraging RNA polymerase II (RNAPII) association with regions of heterochromatin (Yamada et al., 2005). Furthermore, Clr3 has been identified as a component of the effector complex SHREC (**S**nf2/**H**dac-containing **Re**pressor **C**omplex) which also comprises Clr1, Clr2, and Mit1, a SNF2 chromatin-remodeling factor (Sugiyama et al., 2007). Clr2 alone has been found to associate with the central core, suggesting it may play a role in kinetochore formation (Sugiyama et al., 2007). SHREC is found at all heterochromatic loci and acts to mediate transcriptional gene silencing, presumably due to the activities of Clr3 and Mit1. SHREC binding to heterochromatic loci requires Swi6, however the complex can also localise to euchromatin independently of Swi6 (Figure 1.7). Its recruitment to telomeres is dependent on the telomere binding protein Ccq1 and Taz1. In addition, it has been hypothesised that SHREC acts to regulate nucleosome positioning and thus maintain higher-order chromatin structure as mutations of SHREC components cause pronounced differences in micrococcal nuclease digestion patterns (Sugiyama et al., 2007).

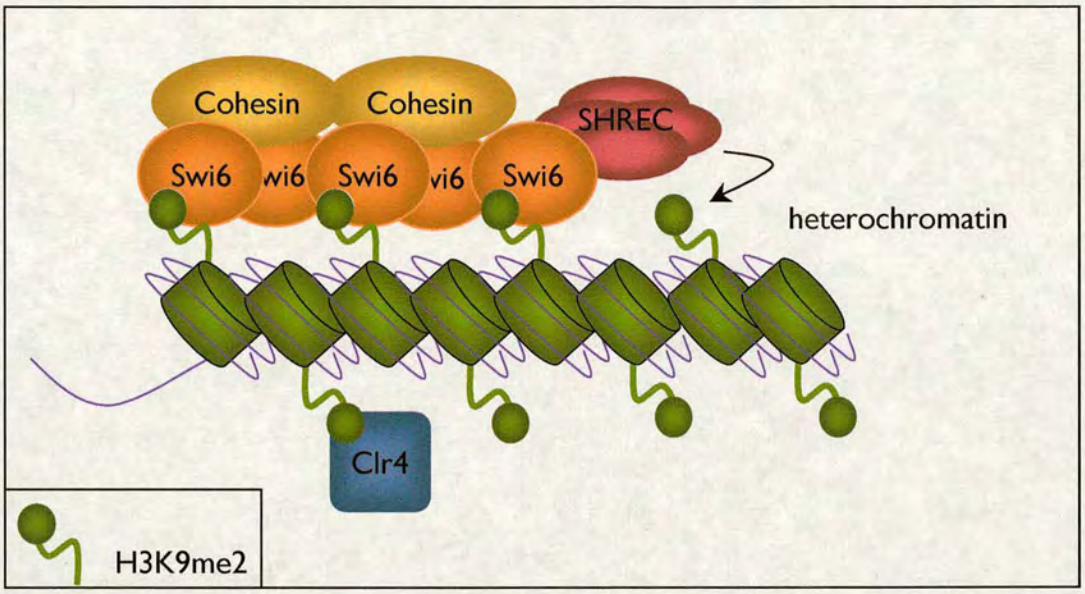


Figure 1.7. Heterochromatin is associated with specific histone modifications and proteins. Histones H3 and H4 within heterochromatic regions are hypoacetylated on their N-terminal tails. In addition, histone H3 is dimethylated on lysine 9 by the action of the histone methyltransferase Clr4. This H3K9me2 is bound by Swi6, which can dimerise. Swi6 is required at centromeres to recruit a high concentration of cohesin to centromeric regions which holds sister chromatids in tight physical cohesion until the onset of anaphase. SHREC association with centromeric heterochromatin is dependant on Swi6. Clr3 alone has been shown to be required for the recruitment of Clr4.

1.7 RNAi components are required for heterochromatin integrity

Heterochromatin forms on related repetitive sequences at fission yeast centromeres, the mating type locus and adjacent to telomeres. Although not fully understood, it initially seemed most likely that the formation of this silent chromatin was driven by this repetitive DNA and specific DNA binding proteins which would attract histone deacetylases and methylases to promote binding of Swi6 and other proteins. However, it is now apparent that the RNAi machinery is required for the assembly and maintenance of heterochromatin in fission yeast. Like Clr4 and Swi6, deletion of RNAi components was found to result in defective heterochromatin formation and chromosome missegregation (Hall et al., 2003; Volpe et al., 2003; Volpe et al., 2002).

It is ironic that despite centromeres having been previously thought of as transcriptionally silent regions, the *dg/dh* repeats themselves were found to produce convergently transcribed non-coding RNA transcripts. These transcripts accumulate in many mutants involved in heterochromatin formation and in mutants lacking RNAi components and are found to be preferentially transcribed during S-phase (Chen et al., 2008; Volpe et al., 2003; Volpe et al., 2002). Non-coding transcripts have also been shown to originate from the mating type locus and sequences adjacent to telomeres (Kanoh et al., 2005; Mandell et al., 2005; Noma et al., 2004). Thus, at these regions transcription itself contributes to the transcriptionally silent state. In wild-type cells these transcripts are made but are continually processed. Moreover, siRNAs identical in sequence to the *dg/dh* region have been identified (Cam et al., 2005; Reinhart and Bartel, 2002).

The discovery of two key complexes, the RNA-induced initiation of transcriptional gene silencing complex (RITS), which appears to be the main RNAi effector complex, and the RNA-directed RNA polymerase complex (RDRC) provided further insights into the mechanisms of RNAi-mediated heterochromatin formation in fission yeast (Motamedi et al., 2004; Noma et

al., 2004; Verdel et al., 2004). Recently Argonaute1 (Ago1), a component of RISC, has been identified as part of a second complex termed the Argonaute siRNA chaperone (ARC). These findings demonstrate that the formation of heterochromatin is much more complex than first imagined.

Many organisms contain several genes encoding Dicer and Argonaute homologues which complicates analyses of the RNAi pathway. Fission yeast has an advantage in that it only possesses a single gene encoding each of the key proteins required for RNAi and these are not essential for cell viability. In several other organisms, the effector complex RISC (RNA-induced silencing complex) containing Argonaute and guide siRNAs is known to target homologous mRNAs and inhibit their expression by either blocking translation or mediating their degradation (Agrawal et al., 2003; Hannon, 2002). In fission yeast, Dicer (Dcr1) is the ribonuclease which cleaves dsRNA into ~22-25 nt double stranded siRNAs and Argonaute (Ago1) is a component of the RITS effector complex which directly binds these siRNA molecules. These siRNAs act to guide RITS to homologous target RNAs and it appears to act only in the nucleus to bring about modification of homologous chromatin and transcriptional silencing. As well as fission yeast, RNA mediated silencing mechanisms exist in mammals, flies and plants (Allshire, 2002). For example, inactivation of the X chromosome in mammalian cells requires Xist RNA which preceeds histone H3K9 methylation, transcriptional inactivation and DNA methylation (Csankovszki, et al., 2001).

A general model of events is now widely accepted whereby non-coding RNA transcripts derived from repetitive DNA sequences during S-phase form a double-stranded RNA (dsRNA) template (Chen et al., 2008). This dsRNA is processed by Dicer into small interfering RNAs (siRNAs) whose production also peaks during S-phase (Chen et al., 2008). These siRNAs are incorporated into the RITS RNAi effector complex to target homologous RNAs and induce heterochromatin assembly (Motamedi et al., 2004; Noma et al., 2004;

Sugiyama et al., 2005). In fission yeast, siRNA production must somehow bring about the recruitment of histone deacetylases and the histone methylase Clr4 to methylate H3 on lysine 9 allowing Swi6 binding and heterochromatin formation on homologous *dg/dh* repeats (Figure 1.8). In plants it has also been shown that the RNAi pathway can feedback onto homologous chromatin to induce modifications such as DNA methylation, another mark of silent chromatin (Mathieu and Bender, 2004; Matzke and Birchler, 2005). However, DNA methylation has not been detected in fission yeast (Wilkinson et al., 1995).

Both RITS and RDRC components can be detected on centromeric outer repeats (Motamedi et al., 2004; Noma et al., 2004; Sugiyama et al., 2005; Verdel et al., 2004). While RITS must utilise siRNAs to somehow home in on homologous sequences, RDRC may play a role in providing the source of dsRNA for siRNA generation by Dcr1. Indeed, Dcr1 has been shown to associate with the RDRC and facilitate the dsRNA synthesis activity of RDRC (Colmenares et al., 2007). However, the exact function of RDRC remains to be resolved given that no activity has as yet been reported for two of its components, the predicted helicase and poly(A) polymerase. Furthermore, recent studies have identified factors involved in ubiquitination, sumoylation, and RNAPII transcription as affecting RNAi mediated heterochromatin formation. The role of these complexes and modifications will be discussed in detail below. A summary of proteins found at the heterochromatic outer repeats is shown in Figure 1.8.

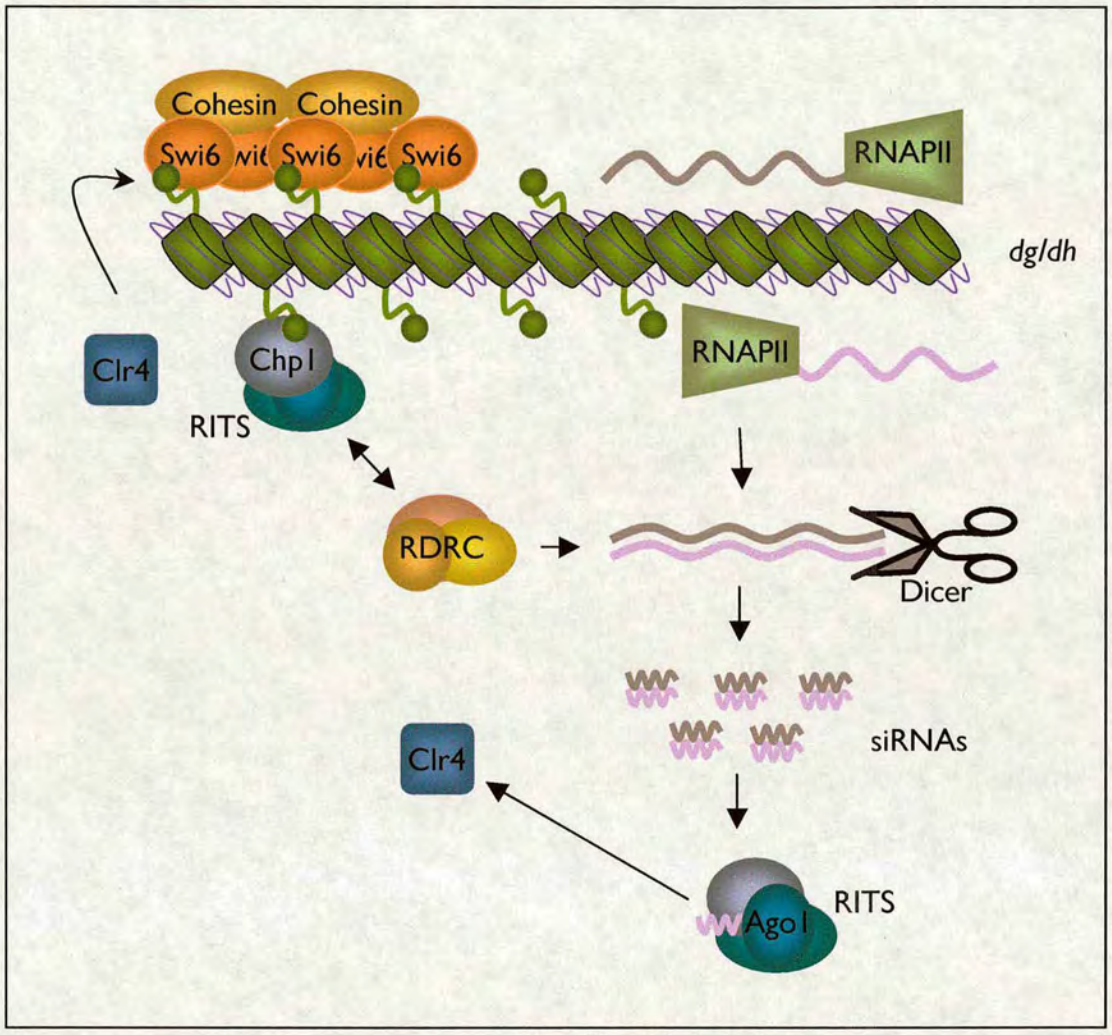


Figure 1.8. An overview of RNAi-induced heterochromatin formation in fission yeast. The outer repeats are transcribed by RNA polymerase II. These non-coding transcripts are assumed to provide a substrate for Dcr1, perhaps by annealing or through the action of Rdp1. These transcripts are a source of siRNAs which are incorporated into the RITS complex. This somehow recruits the histone methyltransferase Clr4 which methylates histone H3 on lysine 9. This, in turn, creates a binding site for Swi6. The exact role of RDRC is unclear but Rdp1 is known to associate with the outer repeats and is thought to be required to recruit RITS to this region.

1.8 Heterochromatin has several roles at independent loci

1.8.1 Centromeres

In *S. pombe*, heterochromatin is mainly found at centromeres, telomeres, the mating-type locus and rDNA (Figure 1.9a). The role of heterochromatin at each of these sites is different. For instance, cells with defective centromeric heterochromatin display increased rates of chromosome loss and an elevated frequency of lagging chromosomes on late anaphase spindles (Allshire et al., 1995; Ekwall et al., 1995; Ekwall et al., 1996). Consequently, mutants are sensitive to microtubule destabilising drugs such as thiabendazole. This indicates that loss of heterochromatin from centromeres affects centromere function. These defects arise because Swi6 is somehow required to recruit a high concentration of the cohesin complex over the outer repeats. The cohesin complex is required for tight physical cohesion of sister chromatids. In the absence of Swi6 (or Clr4), subunits of cohesin (Rad21 and Psc3) dissociate from centromeric outer repeats and cohesion at centromeres, but not chromosome arms, is lost (Bernard and Allshire, 2002; Bernard et al., 2001; Nonaka et al., 2002). Thus, any mutation affecting the formation of heterochromatin at centromeres ultimately leads to defective chromosome segregation. Fission yeast cells that lack centromeric heterochromatin remain viable because cohesion along chromosome arms is unaffected and is sufficient to sustain reasonable levels of chromosome segregation in an organism with just three chromosomes. Consistent with this, cells with a mild lesion in the Rad21 cohesin subunit require Swi6/heterochromatin for viability (Bernard et al., 2001).

1.8.2 Mating-type locus

Heterochromatin also plays an important role in regulating mating type switching. The fission yeast mating type locus contains three mating-type cassettes, *mat1* (either *P* or *M*), *mat2-P* and *mat3-M* over a region of approximately 30 Kb on chromosome 2 (Figure 1.9b) (Egel, 2004; Thon and Klar, 1992). Depending on whether *P* or *M* information is present at *mat1*,

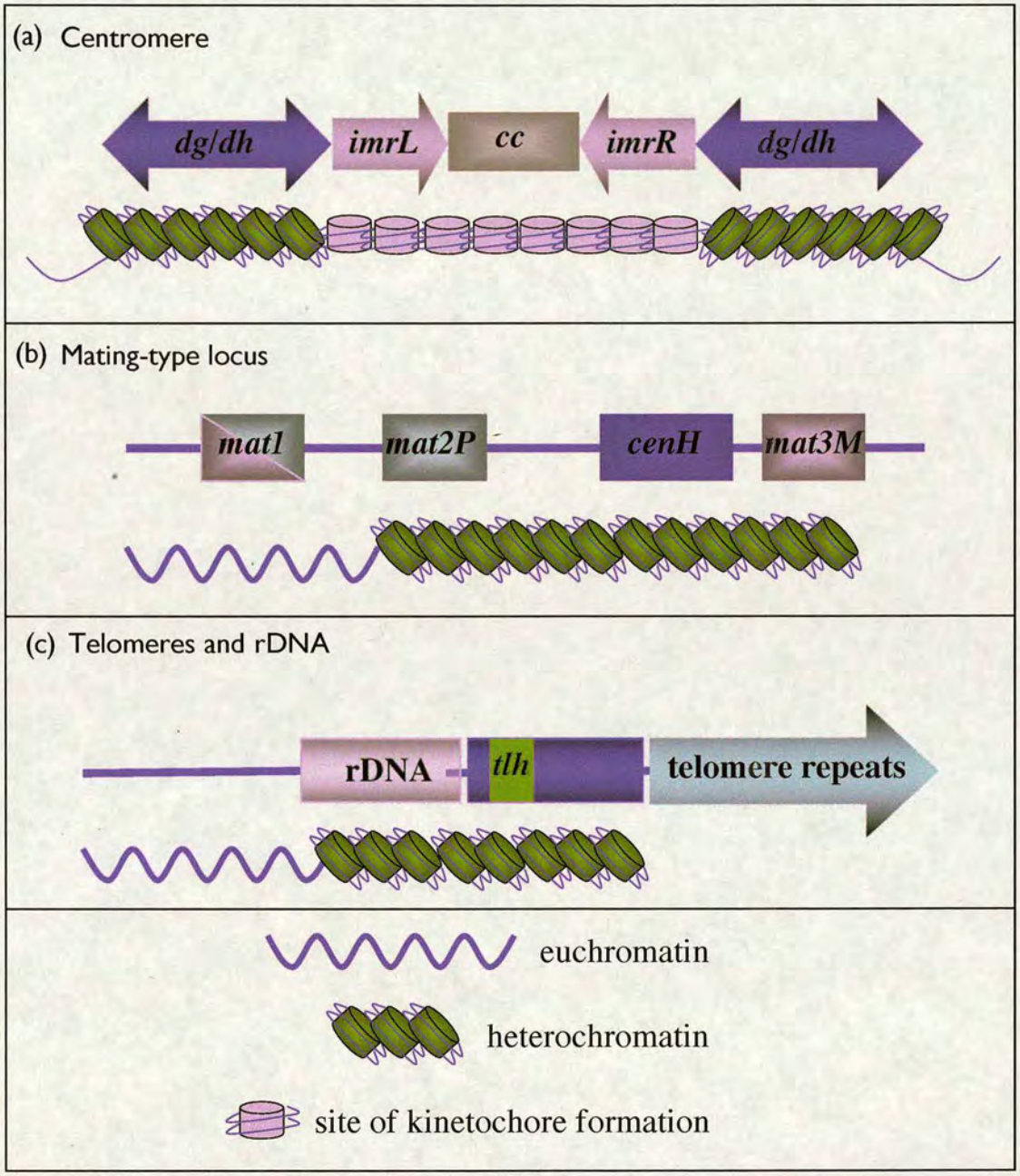


Figure 1.9. Heterochromatin exists at several locations in fission yeast. **(a)**Heterochromatin is found at pericentric regions of fission yeast centromeres. The central core (*cc*) is flanked by the inner inverted repeats (*imr*) and the heterochromatic outer repeats (*otr* or *dg/dh*). **(b)** At the mating type locus, heterochromatin spans around 10 Kb covering *mat2-P, cenH* and *mat3-M*. **(c)** Only telomeres on chromosome 3 contain rDNA repeats. Subtelomeric regions on chromosome 1 and 2 contain telomere-linked helicase genes (*tlh*) with homology to *dg/dh* centromeric repeats. The sequencing of telomeres is not yet complete, presumably due to the highly repetitive nature of these regions

cells preferentially recombine either *mat2-P* (in a *mat1-M* cell) or *mat3-M* (in a *mat1-P* cell) with *mat1* in a process known as switching. *mat1* is transcriptionally active but *mat2-P* and *mat3-M* are maintained in a silent state. The mating type of a haploid cell is determined by the exchange between *P* and *M* information at the *mat1* locus i.e. whether *P* or *M* information is expressed. Heterochromatin is required to maintain the 20 Kb region containing *mat2-P* and *mat3-M* in a silent state as expression of both

causes haploid cells to undergo an aberrant meiosis which is usually lethal when it occurs in haploid cells (Thon et al., 2005). The *cenH* region between *mat2* and *mat3* has 96% homology to *dh* elements at centromeres. *cenH* is required for efficient silencing and switching as replacement of this region by a marker gene causes variegated expression (Grewal and Klar, 1997). As at centromeres, Swi6 also attracts cohesin to *mat2-mat3* and mutations in cohesin subunits lead to defective mating type switching (Nonaka et al., 2002). Further analyses suggest that heterochromatin influences long-range chromatin interactions between *mat1* and the silent mating type cassettes to determine the direction of the switching event (Jia et al., 2004).

At centromeres, it is apparent that the RNAi pathway is necessary for the formation and maintenance of silent chromatin although some features, such as residual H3K9 methylation and Swi6 localisation, remain even after inactivation of RNAi (Sadaie et al., 2004). In contrast, RNAi is required to establish heterochromatin at the mating type locus but is dispensable for its maintenance. Transcription of the *cenH* element residing between *mat2* and *mat3* attracts the RNAi machinery to nucleate heterochromatin formation in a similar fashion to that seen at centromeres (Hall et al., 2002). However, unlike at centromeres the silent state is propagated in the absence of active RNAi. This is due to an alternative pathway involving Atf1 and Pcr1, two members of the stress-activated ATF/CREB protein family, which act in an RNAi-independent manner to recruit heterochromatin components to the mating type locus. When either of the genes encoding Atf1 or Pcr1 is deleted

in combination with RNAi components, heterochromatin is completely abolished. This suggests that the two pathways act in parallel and that Atf1/Pcr1 act to retain specific factors such as Clr4 and hence Swi6 once they have been delivered to the locus by the RNAi machinery (Jia et al., 2004; Kim et al., 2004). Interestingly, both Atf1 and Pcr1 physically interact with Swi6. In addition, Atf1 associates with the Clr6 histone deacetylase, while Clr4 can bind both Atf1 and Pcr1 *in vitro* (Jia et al., 2004; Kim et al., 2004). This supports the idea that these DNA binding proteins act to maintain the silent state at the mating type locus in the absence of RNAi.

1.8.3 Telomeres

Blocks of heterochromatin are found over regions of approximately 40 Kb adjacent to each telomere (Figure 1.9c) (Kanoh et al., 2005; Nimmo et al., 1994; Nimmo et al., 1998). This telomeric heterochromatin is possibly required in some way to prevent end-to-end fusion, to protect chromosome ends from enzymatic degradation or to prevent homologous recombination between telomere repeats at the ends of different chromosomes (Ferreira et al., 2004; Mandell et al., 2005; Sadaie et al., 2003). It is known that telomeres are clustered at the nuclear periphery in mitotically dividing cells (Funabiki et al., 1993) whereas during meiotic prophase they gather together at the spindle pole body to aid pairing of homologous chromosomes and recombination (Chikashige et al., 1994; Cooper et al., 1998; Nimmo et al., 1998). When telomeric heterochromatin is impaired telomere length is unaffected but telomere clustering is disrupted to some extent (Ekwall et al., 1996; Hall et al., 2003; Tuzon et al., 2004). This demonstrates a possible role for telomeric heterochromatin in maintaining proper chromosomal organisation within the nucleus. Disruption of telomeric heterochromatin also causes derepression of genes within the subtelomeric repeats and also of marker genes inserted adjacent to telomeric regions (Allshire et al., 1995; Hansen et al., 2006; Kanoh et al., 2005; Mandell et al., 2005; Nimmo et al., 1998).

Similar to the situation at the mating-type locus, a distinct process also occurs at telomeres where Taz1, a telomere terminal repeat DNA binding protein, is able to establish heterochromatin independently from the RNAi machinery (Allshire, 1995; Kanoh et al., 2005; Nimmo et al., 1998). RNAi components are also required for normal clustering of telomeres at the nuclear periphery in interphase cells (Hall et al., 2003). Telomere length remains normal in cells lacking genes required for RNAi-mediated heterochromatin formation (Ekwall et al., 1996; Hall et al., 2003). Although Clr4 and Rik1 are required for Swi6 localisation and silencing at telomeres, Swi6 localisation and silencing is retained in cells lacking Dcr1, Ago1 or Rdp1 (Allshire et al., 1995; Hall et al., 2003). This RNAi-independent form of silencing at telomeres is due to a redundant pathway where the terminal telomere repeats themselves can recruit Clr4 via Taz1 bound to terminal telomere repeats (Allshire, 1995; Cooper et al., 1997; Kanoh et al., 2005). Loss of Taz1 causes the terminal repeats at telomeres to elongate and leads to loss of silencing, but Swi6 remains localised due to the maintenance of heterochromatin on telomere associated repeats (Cooper et al., 1997; Kanoh et al., 2005; Nimmo et al., 1998).

1.8.4 RNAi acts at rDNA and other loci

In fission yeast, approximately 100 copies of the 5.8S, 18S and 25S ribosomal RNA genes are tandemly arranged as 10.4 Kb repeats occupying ~1000 Kb adjacent to telomeres on chromosome 3 and are transcribed by RNA polymerase I in the nucleolus (Figure 1.9c). When RNAPII transcribed marker genes are placed within rDNA they are transcriptionally silenced in a process that requires Clr4, Chp2, Swi6 and to a lesser extent Chp1 (Thon and Verhein-Hansen, 2000). Genome-wide heterochromatin and euchromatin profiling confirmed that in addition to centromeres, telomeres and the mating-type loci, heterochromatin is also found associated with rDNA and siRNA homologous to rDNA can be detected (Cam et al., 2005). H3K9 methylation and Ago1, but not Rdp1, was found to be associated with particular regions of rDNA repeats. Moreover, H3 K9 methylation, Swi6,

RITS components, and Rdp1 were found to associate with a silenced RNAPII marker gene inserted within rDNA. H3 K9 methylation and Swi6 association with this gene requires Chp1, Dcr1 and Clr4. The rDNA arrays themselves are subject to increased inter-repeat recombination, indicating that this heterochromatin contributes to the mitotic stability of rDNA arrays by suppressing recombination. In other organisms it is known that only a proportion of ribosomal repeats are actively transcribed (Dammann et al., 1993, 1995). It is possible that this RNAi-mediated heterochromatin also acts to regulate the number of active rRNA genes.

Recently, the poly(A) polymerase Cid14 and the exosome component Dis3 have been shown to play a role in maintaining the stability of the rDNA locus indicating that RNAi-independent mechanisms exist here as well as at the mating-type locus and telomeres (Wang et al., 2008).

In genome wide studies, a number of other chromosomal loci were also highlighted as being potential sites of heterochromatin formation by their relatively high levels of H3K9 methylation in mitotically dividing cells. These islands of heterochromatin mainly corresponded to genes which are only expressed in meiosis (Cam et al., 2005). Therefore, heterochromatin may be required to maintain repression of these genes in vegetative cultures but it is not known if RNAi is required to direct H3K9 methylation to these loci. Regardless, it is possible that RNAi is involved in endogenous gene regulation in fission yeast as it is in *Drosophila* and plants (Aravin et al., 2001; Chan et al., 2004).

It is clear that in fission yeast heterochromatin is required to form stable structures at distinct chromosomal loci in order to contribute to the normal function of these regions. As well as the contribution to endogenous loci, heterochromatin can also regulate exogenous sequences. In many organisms the expression of a synthetic double stranded RNA homologous to an endogenous gene can target homologous RNA resulting in degradation of

that RNA and in some cases modification of DNA/chromatin at the homologous locus (Agrawal et al., 2003; Hannon, 2002). In fission yeast the expression of an exogenous dsRNA hairpin with homology to a GFP transgene can induce the production of siRNAs homologous to GFP, thus allowing some silencing of the transgene (Sigova et al., 2004). It is not known if these hairpin derived GFP siRNAs are incorporated into the RITS complex. Transgene silencing was shown to require the presence of Clr4, Rdp1, Dcr1 and Ago1 but not Swi6, Tas3 or Chp1. However, the level of ongoing transcription from the transgene does not appear to be affected indicating that this silencing must be due to post-transcriptional processing of the GFP transcript by RNAi. Further investigation of such silencing is required as in this GFP system, both target GFP transcripts and homologous siRNAs are thought to be very highly expressed compared to the apparent lower levels of naturally occurring centromere transcripts and siRNAs. Strong transcription of the target or too much siRNA could interfere with, rather than promote, RNAi-mediated heterochromatin formation at an artificial locus. Nonetheless, such artificial assays provide a useful tool to further investigate defects in mutants affecting RNAi-mediated heterochromatin formation and offer some clue as to where and how specific proteins may act in the process. Other assays utilising tricks to direct the RNAi machinery to particular loci such as ectopic silencing via repeats placed in euchromatin or tethering components to RNA or DNA at euchromatic loci should also allow further insights into the mechanism of RNAi-mediated heterochromatin assembly (Buhler et al., 2006; Hall et al., 2002; Partridge et al., 2002).

1.9 RNAi is required for heterochromatic gene silencing

1.9.1 Non-coding transcripts and siRNAs are produced from silent loci

Although overlapping non-coding transcripts derived from *dg/dh* repeats at centromeres can be detected, it is not known how the initiating dsRNA that provides the template for siRNA production is formed. It seems reasonable to assume that these centromeric transcripts are the source of a dsRNA substrate that is processed by Dcr1 to produce homologous siRNAs (Figure

1.10). Dcr1 generates siRNAs in an ATP-dependant manner which also requires its conserved helicase domain (Buker et al., 2007). Dcr1 has been shown to interact with RDRC via its C-terminus, an interaction which is required for siRNA synthesis and heterochromatic gene silencing (Buker et al., 2007). The first few siRNAs identified were homologous to the centromeric *dh* element but comprehensive sequence analyses of siRNA associated with RITS identified siRNAs homologous to both the *dh* and *dg* centromeric repeats (Noma et al., 2004; Reinhart and Bartel, 2002). These siRNAs are concentrated in specific regions. This distribution could reflect variation in the density of transcripts from certain regions or in the way the transcripts are converted to dsRNA. This remains to be investigated further as the transcripts arising from heterochromatic regions have not been characterised in detail. However, comprehensive mapping of these transcripts is challenging because the arrangement of repeats at each centromere varies. In addition, the sequence similarity of *dg* and *dh* elements makes it difficult to distinguish repeats from each centromere and other regions of heterochromatin.

RNAs homologous to centromere repeats may be produced by transcription, however, Rdp1 has been shown to be able to synthesise RNA from an RNA template (Motamedi et al., 2004; Sugiyama et al., 2005). This activity of Rdp1 could be required to produce a complementary second strand using primary centromeric non-coding RNA transcripts as a template (Volpe et al., 2002). It has been demonstrated that the dsRNA synthesis activity of the RDRC is strongly stimulated by its interaction with Dcr1 (Buker et al., 2007).

Apart from siRNA derived from the centromeric outer repeat *dg*/*dh* elements siRNAs were also identified which are homologous to unique inverted repeat elements found at the outer boundaries on centromere 1 and 3, the region of centromere homology (*cenH*) at the mating type locus, the sub-telomeric *cenH*-like sequences, rDNA and also a small number from *imr*

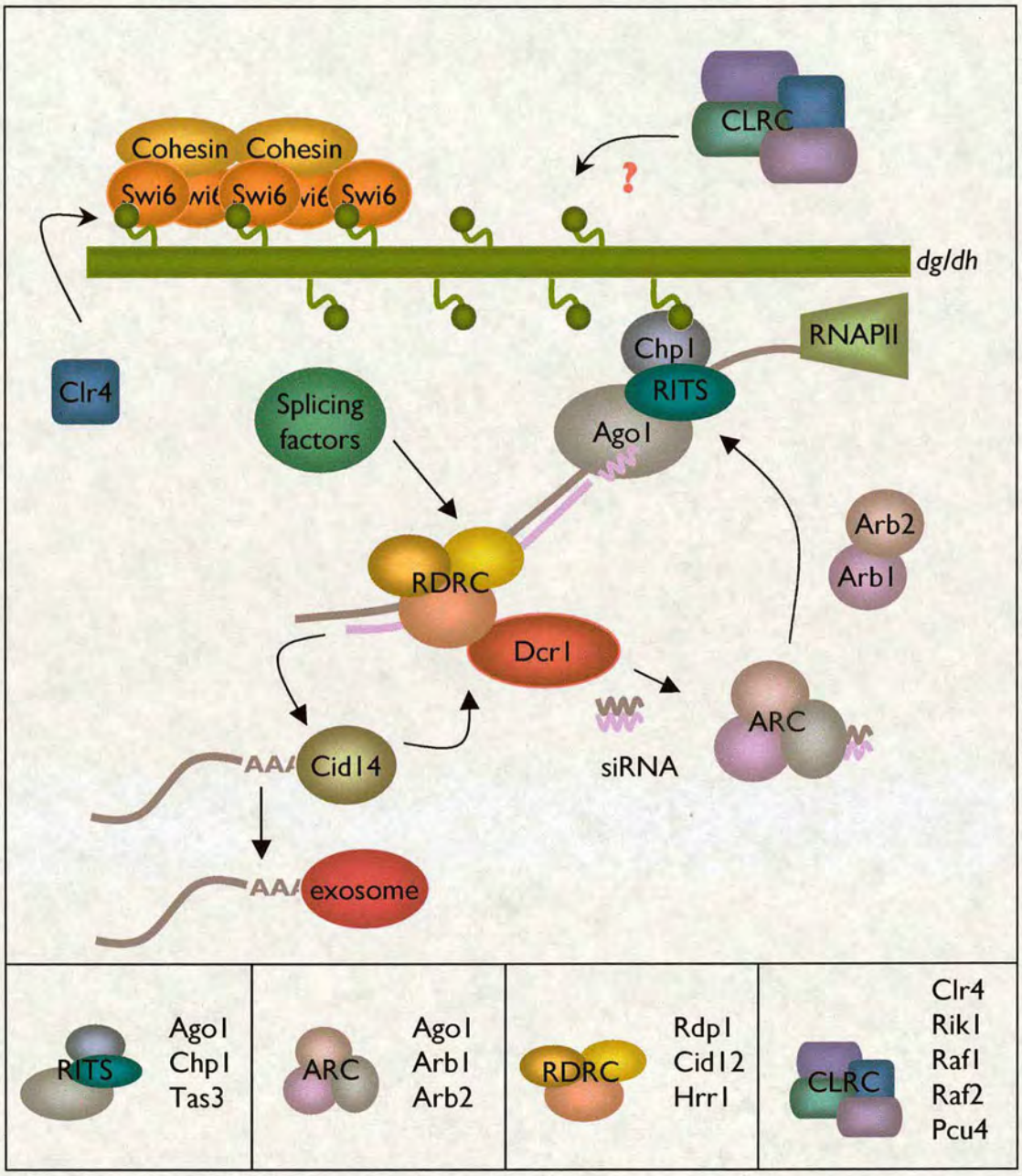


Figure 1.10. Several complexes are involved in heterochromatin formation in fission yeast. RNAPII transcripts may provide a platform to recruit RITS and RDRC. RDRC could be involved in the production of dsRNA which is required for siRNA production, RITS association and subsequent H3K9me2. RNAi, H3K9me2 and formation of intact heterochromatin act in a closed loop where loss of any one component leads to collapse of the pathway. ARC could be the loading complex for RITS and transfer siRNA from Dcr1 to RITS. Loading of siRNAs into RITS allows its targeting to homologous nascent transcripts which recruits Clr4. The CLRC may be responsible for the modification of histones or other factors involved in RNAi-induced heterchromatin formation. Splicing factors could provide a platform for the amplification of siRNAs. Cid14 may target transcripts for degradation via the exosome or the RNAi machinery.

region of centromere 1 (Cam et al., 2005). Since these siRNAs were associated with RITS this suggests that all of these sequences can be targeted for RNAi-induced heterochromatin formation.

1.9.2 RITS: the effector complex

The RNA-induced initiation of Transcriptional gene silencing complex (RITS) comprises three proteins: Ago1, Chp1 and Tas3. The complex also contains siRNA which directly bind Ago1 and presumably guide the complex to homologous target RNAs (Figure 1.10). In other organisms, the effector complex RISC (RNA-induced silencing complex) containing Argonaute and guide siRNAs is known to target homologous mRNAs and inhibit their expression by either binding the mature mRNA and blocking their translation or by inducing their degradation by virtue of the 'slicer' endonuclease activity inherent in some Argonaute proteins (Agrawal et al., 2003; Baumberger and Baulcombe, 2005; Hannon, 2002; Liu et al., 2004; Miyoshi et al., 2005; Rivas et al., 2005). In *S. pombe,* the PIWI domain of Ago1 has been shown to be required for its 'slicing' activity which is thought to cleave and degrade its target mRNA (Buker et al., 2007; Irvine et al., 2006).

The incorporation of siRNA into *Drosophila* or mammalian RISC requires a loading complex containing Dcr1 (Preall and Sontheimer, 2005). siRNAs are loaded as a duplex and one strand is cleaved by Argonaute leaving behind a single 'guide' strand which confers target specificity (Miyoshi et al., 2005; Preall and Sontheimer, 2005). In fission yeast it is not known how siRNAs are loaded into RITS. However, it is possible that ARC acts as the loading complex for RITS as it contains duplex siRNAs which conceivably could be passed directly from Dcr1, similar to interactions observed in *Drosophila* and human (Figure 1.10). As yet, no interaction has yet been identified between Dcr1 and ARC (Buker et al., 2007).

Like Swi6, the Chp1 subunit of RITS contains a chromo domain and this chromo domain has also been shown to bind histone H3 when methylated on lysine 9 (Partridge et al., 2002). However, Chp1 not only binds to H3K9me2, but as part of the RITS complex it is required to target this modification to sequences homologous to the siRNA carried by RITS (Figure 1.10) (Ekwall, 2004; Partridge et al., 2002). The fact that Chp1 also binds target chromatin when methylated on lysine 9 implies a physical link between Chp1/RITS and its chromosomal targets and that binding of RITS to chromatin via RNAi reinforces transcriptional silencing. After the initial unknown events that nucleate a patch of heterochromatin, Chp1 could be required to stabilise the interaction of RITS with heterochromatin. Therefore, the binding of the RITS components themselves may contribute to heterochromatin integrity by being loaded *in cis* with siRNA generated from any RNA synthesised in the vicinity. Consistent with this, each of the individual RITS components is required for complete H3K9 methylation of and Swi6 association with marker gene insertions at centromeres (Verdel et al., 2004). Surprisingly, all RITS components are also required for siRNA generation, again indicating that a feedback mechanism operates between chromatin modification and siRNA generation (Noma et al., 2004). RITS components do not always act together, for example, Ago1 alone is required for the post-transcriptional repression of a transgene via expression of an exogenous dsRNA hairpin but Tas3 and Chp1 are not (Sigova et al., 2004). This makes sense since only the single Argonaute protein in fission yeast can be responsible for the targeting of nascent and mature transcripts. Tas3,like Chp1, is located mainly in the nucleus (Noma et al., 2004). Tas3 acts as an adaptor and links Ago1 to Chp1 (Partridge et al., 2007). However, mutations which abolish the Tas3-Ago1 interaction do not affect heterochromatin formation and RITS is still able to localise to the centromere (Partridge et al., 2007). Tas3 appears to be required for the maintenance of centromeric heterochromatin but cannot mediate *de novo* establishment in cells where H3K9 methylation is depleted (Partridge et al., 2007). However, a Tas3 mutation which abolishes Chp1 binding cannot maintain centromeric heterochromatin demonstrating the importance of Chp1 (Debeauchamp et

al., 2008).

Chp1, and presumably other RNAi components, are required for the establishment of heterochromatin at centromeres, mating-type locus and telomeres (Sadaie et al., 2004). In the absence of RNAi, H3K9 methylation is reduced but it is not completely abolished from repetitive sequences at these locations. Swi6 and a related chromo domain protein, Chp2, are required to maintain this residual H3 K9 methylation at centromeres and the mating type locus in the absence of Chp1. Thus, it appears that chromo domain proteins contribute in several ways to heterochromatin formation in fission yeast. Chp1 appears to be a key player since it is required for full methylation of histone H3 K9, it associates with chromatin only when it is methylated on lysine 9 and it is required for the production of the siRNA that allow it and H3K9me to be targeted to homologous chromatin. Because of these inherent interdependencies, the order of events that trigger RNAi—mediated heterochromatin formation are difficult to determine.

1.9.3 Heterochromatin formation and RNAi are closely coupled

This interdependency is further highlighted by the fact that all components of RITS associate with regions of heterochromatin and that this is dependent also on Clr4 and Dcr1 (Noma et al., 2004). As with Chp1, the production of siRNAs and their incorporation into RITS is required for the association of Ago1 and Tas3 with centromeric heterochromatin. However, all RITS components remain associated with the mating type locus in cells lacking Dcr1 and thus siRNA (Jia et al., 2004; Noma et al., 2004). In addition, in the absence of Ago1, Tas3 and Chp1 can still interact and both proteins still associate with the mating type locus and telomeres but not centromeres (Petrie et al., 2005). This may indicate an RNAi-independent role for these proteins at these regions or could simply reflect the ability of Chp1 to bind methylated H3K9 after targeting. This also suggests that RITS is required for the maintenance of heterochromatin at centromeres but not at other loci.

Exactly how the RITS complex loaded with siRNAs recognises homologous targets to induce the specific chromatin modifications that lead to heterochromatin formation at these locations is not known and requires further scrutiny. It is possible that siRNAs recognise homologous chromatin by targeting homologous nascent transcripts still associated with chromatin templates in an RNA-RNA mediated interaction. Equally, RITS associated siRNAs could somehow bind or interact with homologous DNA sequences to induce the modification of nearby chromatin. Evidence to date points towards an RNA-RNA interaction as the RITS complex has been shown to associate with non-coding centromere RNA transcripts but only when Dcr1 is present in the cell (Motamedi et al., 2004). Consistent with this, tethering Tas3, and consequently RITS, to a normal euchromatic transcript (*ura4*) allows production of *ura4* homologous siRNAs, lysine 9 methylation of H3 on the *ura4*$^{+}$ gene and silencing (Buhler et al., 2006). This suggests that nascent transcripts can be converted to dsRNA at their site of production, allowing Dcr1 to act *in cis* to form siRNAs which are directly loaded into RITS to allow chromatin modification. This artificial RNA-tethered RITS version of heterochromatin requires Dcr1, all RITS and RDRC components, and Clr4. It is also possible that mature centromere and other transcripts are exported to the cytoplasm for processing to siRNA where these are then loaded into Ago1 to form RITS on their journey back to the nucleus.

1.9.4 ARC: the siRNA chaperone complex

The **A**rgonaute si**R**NA **c**haperone complex (ARC) is composed of Ago1 and two previously uncharacterised proteins Arb1 and Arb2 (Figure 1.10). Arb1 is conserved in fungi and contains a C-terminal domain which shares homology with organellar maturases which facilitate intron self-splicing (Buker et al., 2007). Arb2 is conserved from yeast to humans but has no obvious domains. All of the ARC components were found to be required for centromeric heterochromatin assembly. Unlike the RITS complex components, Arb1 and Arb2 do not localise to centromeric heterochromatin

and can be observed both in the nucleus and the cytoplasm by immunostaining (Buker et al., 2007). Furthermore, ARC contains predominantly double-stranded siRNA whereas RITS contains mainly single-stranded siRNA. This may account for the lack of ARC at centromeres as it is widely assumed that targeting of RITS occurs through base pairing of siRNAs to either centromeric DNA or nascent RNA transcripts. From this data, it has been proposed that ARC acts to inhibit release of the siRNA passenger strand from Ago1. This is confirmed by the inhibition of Ago1 slicer activity by Arb1 and by the fact that catalytically dead Ago1 only copurifies with double-stranded siRNA (Figure 1.10). It may also be the case that ARC functions to transfer duplex siRNA from Dcr1 to Ago1 although no interaction has yet been identified between Dcr1 and ARC. It is thought that ARC may regulate the slicer activity of Ago1 and prevent the cleavage of nascent centromere transcripts which act as an assembly platform for RITS and thus heterochromatin formation (Buker et al., 2007).

1.9.5 RDRC: RNA-directed RNA polymerase complex

The RITS complex has been shown to physically interact with the RNA-directed RNA polymerase complex (RDRC). The components of RDRC are also required for the integrity of silent chromatin. RDRC is composed of Rdp1, Hrr1, and Cid12. Rdp1 is an RNA-dependent RNA polymerase, Hrr1 is a putative RNA helicase, and Cid12 is a putative poly(A) polymerase (Figure 1.10) (Motamedi et al., 2004). As with the RITS complex each of the components of RDRC are required for siRNA generation, complete H3 K9 methylation of, and Swi6 association with heterochromatic loci (Motamedi et al., 2004; Sugiyama et al., 2005). The association of RDRC components with RITS subunits is also dependent on Dcr1 and Clr4 and the catalytic activity of Rdp1 itself (Motamedi et al., 2004; Sugiyama et al., 2005). Thus, both RDRC and RITS appear to be mutually dependent on one another for their association with heterochromatic loci and the formation of silent chromatin. The dependency of RITS on RDRC holds steadfast even when silent chromatin is induced by tethering Tas3/RITS to euchromatic *ura4* transcripts

(Buhler et al., 2006). Thus, the generation of dsRNA substrate, the processing of dsRNA to siRNA, loading of siRNAs into RISC and subsequent targeting of chromatin are all intimately linked. This also suggests that Rdp1 is part of a self-enforcing RNAi feedback loop that couples siRNA production and heterochromatin formation (Buhler et al., 2006; Noma et al., 2004; Sugiyama et al., 2005).

In vitro analyses indicate that Rdp1 can act as an RNA-dependent RNA polymerase in that it can synthesise RNA from a single stranded RNA substrate in the presence or absence of a complementary primer. Mutations which destroy this activity cause phenotypes equivalent to those observed in cells lacking RNAi (Motamedi et al., 2004; Sugiyama et al., 2005). Hence the ability of Rdp1 to synthesise complementary RNA is essential for the production of centromeric siRNA and heterochromatin formation. It remains unclear why Rdp1 is so important for RNAi-mediated chromatin modification in fission yeast as other eukaryotes such as *Drosophila* and mammals do not encode an RNA-dependent RNA polymerase but still have an active RNAi pathway. In plants RNA dependent RNA polymerase is required for transgene silencing, but not for silencing mediated by viruses. This suggests that exogenous viruses are capable of synthesizing sufficient dsRNA to bypass the need for RdRP activity (Dalmay et al., 2000). In the filamentous fungi *Neurospora crassa* and *Aspergillus nidulans* the requirement of RdRP for robust RNA dependent silencing are variable (Catalanotto et al., 2002; Hammond and Keller, 2005). Fission yeast seems to be extremely dependent on Rdp1 for RNAi-mediated silencing since in its absence, although centromeric transcripts are still produced, no siRNAs are detectable. The observation that Rdp1 associates with centromeric chromatin and transcripts is compatible with a model where Rdp1 acts on nascent transcripts to synthesise dsRNA leading to the production of the initial siRNAs (Motamedi et al., 2004; Sugiyama et al., 2005; Volpe et al., 2002). Alternatively, Rdp1 may utilise pre-existing rare primary siRNAs, formed by Dcr1 mediated cleavage of annealed centromere transcripts, to prime synthesis of additional dsRNA and amplify the signal. However, Rdp1 is

also required for a form of PTGS in *S. pombe* triggered by the expression of an exogenous hairpin RNA (Sigova et al., 2004). In this case siRNAs are presumably not limiting, as with plant viruses, so it is not entirely clear why Rdp1 is required.

The role of Hrr1 is not known but it has significant similarity to DEAD box helicases such as Smg2 in *C. elegans,* which acts in the nonsense mediate decay pathway as well as RNAi, and Sde3 in plants which is required for RNAi-mediated transgene silencing (Dalmay et al., 2001; Domeier et al., 2000). It is conceivable that Hrr1 is required to unwind siRNA duplexes prior to loading into RISC or it might act upon dsRNA providing single stranded RNA templates for Rdp1 (Motamedi et al., 2004). The role of Cid12 is also not known, it is possible that it binds the 3′ end of transcripts producing a poly(A) tract that somehow primes RNA synthesis by Rdp1. In *C. elegans* a related putative poly(A) polymerase, RDE-3 has also been shown to be required for efficient RNAi and siRNA production (Chen et al., 2005). Polyadenylation by proteins such as Cid12 might also play a role in RNA degradation since the addition of short poly(A) tracts is known to attract the exosome and degrade RNAs (Anderson, 2005). Cid12 may be required for the specific degradation of non-coding transcripts originating from regions of heterochromatin either for regulation or to somehow aid the provision of a template for Rdp1. The details of how these activities act on endogenous transcripts to execute efficient siRNA production and silencing remains to be determined.

1.9.6 Transcription of centromere repeats and silencing requires RNAPII

Transcripts from the mating type locus and from the centromere are polyadenylated (Djupedal et al., 2005). This is a well known hallmark of mature transcripts produced by RNAPII (Birse et al., 1997). However, it is possible that this polyadenylation is due to the putative activity of Cid12 rather than that normally associated with termination of RNAPII transcription. RNAPII itself is enriched at heterochromatic loci which

reinforces the idea that it is responsible for the transcription of these regions (Figure 1.10) (Cam et al., 2005; Kato et al., 2005). Consistent with this, a specific mutation in the second largest subunit of RNAPII, Rpb2, causes loss of silent chromatin from outer repeat regions of centromeres regulating the expression of normally silent marker genes. Interestingly mutation of Rpb2 causes a reduction in H3K9me2, accumulation of centromere transcripts and loss of siRNA homologous to centromere repeats. General transcription does not appear to be affected in Rpb2 mutant cells, so the loss of heterochromatin is probably due to a defect in processing non-coding centromere transcripts to siRNAs (Kato et al., 2005). A specific role for RNAPII in the production of non-coding centromeric transcripts is also supported by the finding that a mutation in the small RNAPII subunit Rpb7 results in loss of centromeric siRNA defective heterochromatin formation at centromeres (Djupedal et al., 2005). However, Rpb7 mutation causes decreased transcription of the centromeric repeats, indicating that Rpb7 has a specific role in promoting transcription of centromere repeats under conditions where general transcription of euchromatic genes appears normal. Thus, in Rpb7 mutant cells, no RNA substrate is made therefore no siRNAs are formed. It is not clear how the Rpb2 mutant affects heterochromatin formation. One possibility is that when the RITS complex and RDRC engage a nascent transcript associated with RNAPII on its template there is an interaction between RITS/RDRC and RNAPII subunits. Once stabilized, such interactions might promote RNA production by Rdp1 and the recruitment of chromatin modifying activities. Such interactions between RNAi components and RNAPII may be disrupted in the Rpb2 mutant.

1.9.7 Clr4 affects siRNA production and associates with Rik1

The RNAi pathway is required for full methylation of H3 K9 on homologous chromatin and thus Clr4 would be expected to act downstream of the RNAi components. Clr4 is essential to create the H3K9me2 binding site for the chromo domain proteins Chp1, Chp2, Swi6 and possibly Clr4 itself (Bannister et al., 2001; Partridge et al., 2002; Sadaie et al., 2004). Surprisingly,

Clr4 is also required to produce centromeric siRNAs which accounts for why RITS and RDRC are delocalised in its absence (Noma et al., 2004) (Sugiyama et al., 2005). The complete role of Clr4 in the RNAi pathway is difficult to understand mainly due to the inherent feedback in the process and thus our inability to decipher the initiating events that lead to heterochromatin formation. However, it is clear that Clr4 plays a central role since the methylation of H3K9 is required to allow binding of key components.

In most cases loss of any component involved in heterochromatin formation results in, if not complete loss, then at least a significant reduction of H3K9 methylation. However, it is still not known how Clr4 itself is recruited via RNAi to form heterochromatic loci. It had been demonstrated that Clr4 interacts with Rik1 (Sadaie et al., 2004). As with other components Rik1 is known to be required for silencing, H3K9 methylation, Swi6 association/localisation and production of centromeric siRNAs (Allshire et al., 1995; Ekwall et al., 1996; Ekwall and Ruusala, 1994; Hong et al., 2005; Horn et al., 2005; Jia et al., 2005; Li et al., 2005; Partridge et al., 2000). The Rik1 protein contains a ß-propeller domain with similarity to a cleavage specificity and polyadenylation factor (CSPF-A) which may be involved in RNA binding (Neuwald and Poleksic, 2000). It has been proposed that Rik1 could act to guide Clr4 to its target regions (Jia et al., 2005; Li et al., 2005).

1.9.8 Rik1 and Clr4 interact in a complex which has E3 ubiquitin ligase activity

Rik1 is related to DDB1 (DNA damage binding protein 1), a component of an E3 ligase complex in plants (Yanagawa et al., 2004). Recent analyses have demonstrated that Rik1 copurifies and associates with several other proteins; Raf1 (also known as Dos1, Cmc1 and Clr8), Raf2 (or Dos2/Cmc2/Clr7), the E3 ubiquitin ligase subunits Pcu4 and Pip1, the small ubiquitin like protein Nedd8 and the histones H2B and H4, as well as Clr4 (Figure 1.10). This has been called the Clr4-Rik1 complex (CLRC). Deletion of the genes encoding Raf1, Raf2 or Pcu4 perturbs heterochromatin formation at centromeres,

telomeres and at the mating type locus (Hong et al., 2005; Horn et al., 2005; Jia et al., 2005; Li et al., 2005; Thon et al., 2005). Levels of H3K9 methylation are substantially reduced at centromeres and at the mating type locus while a modification normally associated with expressed genes, methylation of H3K4, increases. As with other mutants affecting H3K9 methylation and RNAi the generation of centromeric siRNAs is abolished and chromosome segregation is defective (Hong et al., 2005; Horn et al., 2005; Jia et al., 2005; Li et al., 2005; Thon et al., 2005).

Ubiquitin is a small regulatory protein that can be covalently attached to substrate proteins and is another post translational modification which, like acetylation and methylation, occurs on lysine residues (Hershko and Ciechanover, 1998). Polyubiquitination (the addition of chains of ubiquitin) is a multi step pathway that ultimately targets proteins for degradation via the proteasome (Hershko and Ciechanover, 1998). Monoubiquitination, the addition of a single ubiquitin molecule to a substrate is involved in protein regulation. Histone H2A and H2B are known to be monoubiquitinated and in *S. cerevisiae* ubiquitination of H2B K120 is required to regulate the methylation of H3 on K4 and K79 (Osley, 2004). Rik1, Raf2 and Clr4 purifications were demonstrated to have E3 ubiquitin ligase activity *in vitro* (Horn et al., 2005). The *in vivo* substrates for this ubiquitination are not known, however, the fact that H2B and H4 copurify with Clr4 and Rik1 may indicate that ubiquitination of histones is involved in heterochromatin formation (Hong et al., 2005; Horn et al., 2005). A related complex from human cells (Cul4-DDB1-Roc1) has recently been shown to ubiquitinate histones H3 and H4 on several lysines *in vivo* and *in vitro* (Wang et al., 2006). Given that Pcu4 and Rik1, fission yeast homologs of Cul4 and DDB1, are required for methylation of H3 on K9 and associate with the H3K9 methyltransferase Clr4 it is conceivable that ubiquitination of histones by the Rik1 complex promotes H3K9 methylation. Ubiquitination of H3/H4 might destabilise nucleosomes and force exchange with new H3 which is then methylated on H3 K9 by Clr4 during the replacement process. Alternatively, ubiquitination of H3/H4 may induce conformational changes in

nucleosomes presenting the H3 tail and lysine 9 to Clr4 for methylation.

1.9.9 Sumoylation is required for heterochromatin integrity

SUMO (small ubiquitin-related modifier) is a small peptide that is also conjugated to specific target lysine residues in a manner similar to ubiquitin. SUMO may act to prevent other modification on lysines such as acetylation, methylation and ubiquitination. Many regulators of transcription are known to be sumoylated and in general this promotes transcriptional repression by interactions with histone deacetylases. Intriguingly all four histones have also been shown to be sumoylated in *S. cerevisiae* and this appears to act to oppose ubiquitination and acetylation and inhibit transcription (Nathan et al., 2006). Sumoylation is also involved in maintaining heterochromatin stability in fission yeast (Shin et al., 2005). Deletion of the gene encoding SUMO (Pmt3) causes defective silencing at centromeres and at the mating-type locus but had no effect at telomeres. In addition, a SUMO-conjugating enzyme has been shown to interact with Chp2 and also to be associated with regions of heterochromatin perhaps through interactions with Swi6 or Clr4. Swi6, Clr4 and Chp2 are sumoylated *in vivo* and defective sumoylation of either Swi6 or Chp2 impairs silencing (Shin et al., 2005).

The involvement of ubiquitination and sumoylation in heterochromatin formation in fission yeast are relatively new discoveries. These modifications may act to promote or inhibit specific protein-protein interactions and/or other modification in a variety of ways. Apart from promoting repressive modification of histones it is perhaps possible that RNAPII is ubiquitinated and/or sumoylated in response to RNAi, allowing RNAPII and its nascent transcript to be efficiently engaged by RNAi components. RITS, RDRC, Clr4 and Swi6 might also be regulated by post-translational modification during transcription and cell cycles.

1.10 General RNA processing factors contribute to silent chromatin formation

It has been demonstrated that mutations in several specific splicing factors disrupt heterochromatic gene silencing. Defects in centromeric silencing coincide with an increase in accumulation of non-coding centromere transcripts and reduced levels of siRNAs (pers. com. Elizabeth Bayne). Despite this, splicing mutants can maintain a relatively high level of H3K9me2 and Swi6 at centromeres (pers. com. Elizabeth Bayne). This suggests that low levels of siRNAs are sufficient to establish heterochromatin. The effect of these splicing factors on centromere silencing appears to be direct as defective splicing does not interfere with the RNAi mediated gene silencing or transcription of centromeric non-coding RNA (pers. com. Elizabeth Bayne). In addition, splicing factors are known to associate with the Cid12 component of RDRC and with Ago1 (Motamedi et al., 2004 and pers. com. Elizabeth Bayne). It has been proposed that interactions between the RNAi pathway and splicing factors may provide a platform to promote siRNA amplification to ensure robust heterochromatin assembly.

The phenotypes observed in splicing factors which display defective heterochromatin gene silencing is reminiscent of cells lacking the poly(A) polymerase Cid14. Mutations in Cid14 cause decreased levels of siRNA production but do not affect the structural integrity of heterochromatin (Buhler et al., 2007). Cid14 is found in a protein complex that resembles the budding yeast TRAMP complex which promotes degradation of aberrant transcripts via the exosome (Buhler et al., 2007). It has been hypothesised that Cid14 may polyadenylate non-coding centromere transcripts and mark them for degradation via the exosome or via the RNAi machinery (Figure 1.10) (Buhler et al., 2007). However, centromere transcripts from *cid14* mutants have been demonstrated to be polyadenylated (Wang et al., 2008). In addition, Cid14 is not enriched at centromeres and is instead found in the nucleolus, indicating that it acts away from chromatin (Wang et al., 2008).

However, mutations in exosome components which have been shown to affect heterochromatin silencing do not interfere with siRNA production, suggesting that Cid14 plays a more complex role than simply recruiting the exosome (Buhler et al., 2007). Cid14 may act to mediate degradation of transcripts from other heterochromatic loci such as the telomeres and mating-type locus as transcripts from these regions accumulate in Cid14 mutants (Buhler et al., 2007). Cid14 has also been shown to be required to maintain the genomic integrity of rDNA. It would appear that Cid14 is involved in several RNA turnover processes which affects diverse functions within the cell (Wang et al., 2008).

In any case, heterochromatic gene silencing and RNA processing appear to be closely coupled events although exactly how all of these pathways converge to mediate the formation of higher-order chromatin structures such as at centromeres seems destined to become yet more complex.

1.11 The centromere; suppressor of position effect (*csp*) mutants affect heterochromatic gene silencing

Several years ago, a genetic screen was carried out to identify genes encoding proteins involved in heterochromatin formation. The screen was devised to identify factors specifically involved in silencing at centromeres and not at other regions so as to eliminate the possibility of re-isolation of genes such as *swi6*$^+$ and *clr4*$^+$ (Ekwall et al., 1999). A tester strain containing the *ade6*$^+$ gene inserted at the *SphI* site on the right hand side of centromere 1 (*cen1*), *otr1R(SphI):ade6*, or the *ura4*$^+$ gene inserted 15 kb away at the *NcoI* site on the left hand side, *imr1L(NcoI):ura4*, was used as a reporter for mutations affecting centromere silencing. In a wild-type strain, the *ade6*$^+$ gene is transcriptionally repressed resulting in red colonies on plates containing low supplementing adenine (Allshire et al., 1994; Allshire et al., 1995). The tester strain was mutagenised using ethyl methanesulfonate (EMS) which causes point mutations, preferentially G/C to A/T mutations.

The *csp* (*c*entromere: *s*uppressor of *p*osition effect) mutants were isolated as mutations which specifically alleviate the silencing of marker genes inserted into the heterochromatic repeats of centromeres (Ekwall et al., 1999). Two classes of mutants were recovered by this strategy, the temperature-sensitive (ts) and non-temperature sensitive (non-ts) mutants. In-depth analysis and cloning of *csps1* to *6* was undertaken by a former PhD student, Manuela Portoso (Portoso, 2005). The *csp3*$^{+}$ and *csp4*$^{+}$ genes were identified by Karl Ekwall (Djupedal et al., 2005). The affected genes in the *csp* mutants identified so far are all involved in fundamental pathways of RNA metabolism and processing. It is therefore unsurprising that these genes are essential. *csp3* is an allele of Rbp7 which is a conserved subunit of RNA polymerase II required to promote pre-siRNA transcription and RNAi-mediated chromatin silencing (Djupedal et al., 2005). *csp4* is an allele of Cwf10 which is orthologous to the *S. cerevisiae* splicing factor Snu114. Snu114 is a GTP-binding component of the U5 snRNP which is involved in U4/U6 unwinding during spliceosome activation. *csp5* is an allele of Prp39 which is a U1-associated protein involved in pre-mRNA splicing. The phenotypes of these splicing factors have analysed by Elizabeth Bayne and are described above. The affected genes in the *csp1, 2* and *csp6* mutants remain to be identified although the phenotype of *csp6* was found to be suppressed by the overexpression of several Hsp70 heat-shock proteins (Portoso, 2005). The identification of these ts mutants provides a link between general RNA processing, transcription and chromatin structure.

1.12 Summary

Although much is known about RNAi-mediated heterochromatin assembly in fission yeast, many questions remain regarding specific aspects of the pathway. In this thesis, I present the analysis of several mutants known to affect heterochromatic gene silencing, the *csp7* to *13* mutants, in order to identify the affected genes and their impact on centromere function. The genes I have identified are found to encode components of the RNAi pathway so it is unsurprising that the mutants affect heterochromatin formation. In addition, I present in-depth analysis of the putative poly(A) polymerase, Cid12 which was found to be encoded by *csp10*.

2.1 *S. pombe* culture and media

2.1.1 Growth

S. pombe cultures and colonies were incubated at temperatures between 18°C and 36°C for between overnight and 3 days as indicated for each experiment. Haploid strains will grow with the following generation times:

Medium	Temperature °C	Generation Time
YE	25	3 hours
	32	2 hours 10 minutes
	36	2 hours
PMG minimal	25	4 hours
	32	2 hours 30 minutes
	36	2 hours 20 minutes

The generation time of mutant strains may vary. The time required to double the population of cells can be accurately calculated using the following formula: $T = \log(2^{t2-t1})/\log(x/y)$, where T is the generation time, x is cells per ml at $_t1$ and y is cells per ml at $_t2$.

2.1.2 Growth Media

All solutions were made up to a final volume in dH_2O and autoclaved unless otherwise stated.

PMG agar in 900ml:

Pthallic acid	3g
di-sodium orthophosphate	2.2g
glutamic acid	3.75g
D-glucose anhydrous	20g
vitamins 1000x	1ml
minerals 10,000x	0.1ml
salts 50x	20ml
agar (OXOID)	20g

PMG liquid in 900ml:

Pthallic acid	3g
di-sodium orthophosphate	2.2g
glutamic acid	3.75g
D-glucose anhydrous	20g
vitamins 1000x	1ml
minerals 10,000x	0.1ml
salts 50x	20ml

YES agar (-ade):

Yeast extract (DIFCO)	5g
D-glucose anhydrous	30g
Arginine (Sigma)	0.2g
Lysine (Sigma)	0.2g
Histidine (Sigma)	0.2g
Uracil (Sigma)	0.2g
Leucine (Sigma)	0.2g
Agar (OXOID)	20g

YES liquid:

Yeast extract (DIFCO)	5g
D-glucose anhydrous	30g
Arginine (Sigma)	0.2g
Lysine (Sigma)	0.2g
Histidine (Sigma)	0.2g
Uracil (Sigma)	0.2g
Leucine (Sigma)	0.2g

4 x YES liquid: As above all reagents x 4.

ME plates (1L):

Malt extract (OXOID) 30g/L

Adenine (Sigma)	250g/L
Arginine (Sigma)	250g/L
Histidine (Sigma)	250g/L
Uracil (Sigma)	250g/L
Leucine (Sigma)	250g/L

Vitamins 1000x (100ml):

Pantothenic acid	0.5g
Nicotinic acid	1g
Inositol	1g
Biotin	1mg
Filter sterilised	

Minerals 10,000x (100ml):

Boric acid	5g
$MnSO_4$	4g
$ZnSO_4$	4g
$FeCl_26H_2O$	2g
Molybdic acid	1.6g
$CuSO_4$ $5H_2O$	0.4g
Citric acid	10g
Filter sterilised	

Salts 50x:

Magnesium chloride	53.5g
Calcium chloride	1g
Potassium chloride	50g
di-sodium sulphate	2g

Supplement stocks:

Adenine 50x (Sigma)	5g/L
Arginine 100x (Sigma)	10g/L
Histidine 100x (Sigma)	10g/L

Uracil 20x (Sigma)	2g/L
Leucine 100x (Sigma)	10g/L

Additional supplements:

Fluoroorotic acid (FOA) (Melford Laboratories) was added to media at a concentration of 0.5g/500ml (1x) or 1g/500ml (2x).

Thiabendazole (TBZ) (Sigma) was added to media at concentrations of 10μg/ml or 20μg/ml in DMSO.

Nourseothricin (cloNAT) (Werner BioAgents) was added to media at a concentration of 2000x

Geneticin (G418) (Gibco) was added to media at a concentration of 0.1mg/ml.

6-azauracil (6AU) (Sigma) was added to media at a concentration of 2mg/ml.

2.1.3 Cell counting

Coulter counter

Cell number was estimated using a Beckman Z2 Particle Count and Size Analyzer. 100μl of cells were mixed with 10ml Isoton II (Beckman Coulter) solution and counted according to manufacturers instructions.

Haemocytometer

Cells were also counted using a haemocytometer. A haemocytometer is a special microscope slide which has a grid etched onto the glass. This grid is on a region of the slide which is 0.1mm lower than the rest of the slide. The grid consists of 25 large squares which are subdivided into 16 smaller squares. Once the coverslip has been applied to the slide, 10μl of cell culture is pipetted underneath. This creates a known volume of $0.1mm^3$. The number of cells/ml can be calculated by multiplying the number of cells in the 25 large squares by 1×10^4.

2.1.4 Cell culture

For physiological experiments cells are required to be in mid-exponential growth which is between 2×10^6 and 1×10^7 cells/ml. To generate such cultures a loopful of freshly growing yeast was inoculated into 10mls liquid media and incubated generally overnight at 32°C (or for 1-2 days at 25°C) until cells reach

early stationary phase. This pre-culture was then diluted in an appropriate volume for a suitable amount of time to reach the desired concentration. Flask size is an important consideration and should be taken into account according to the volume of culture required: 25ml flask for up to 10ml culture, 100ml flask for up to 50ml culture, 250ml flask for up to 125ml culture and 500ml flask for up to 250ml culture.

2.1.5 Auxotrophy

The most commonly used auxotrophic markers in *S. pombe* are uracil, leucine, arginine, histidine and adenine. These amino acids are used at a concentration of 100μg/ml. To test auxotrophy, cells are grown as single colonies on non-selective media and then replica plated to minimal media lacking the appropriate supplement. The plates are then incubated for 2-3 days and examined for growth.

2.2. Yeast Molecular Genetics

2.2.1 Mating and random spore analysis

Crosses were carried out on ME medium in order to nitrogen starve the cells and induce sporulation. A similar amount of cells from two strains of opposite mating types (h^+/h^-) were mixed together and incubated for 2-3 days at 25°C. The cells were checked for the presence of ascii containing four spores by light microscopy. Cells were resuspended in 500μl of filter sterilised dH_2O containing 5μl of glusulase (NEN) and incubated for between 5 hours and overnight at 37°C. Glusulase digests the acsus wall and vegetative cells so that only the spores remain. The spores were plated on selective media at dilutions of 1:100 and 1:1000 and grown at 32°C, or appropriate temperature until colonies are formed.

2.2.2 *S. pombe* transformations

Electroporation

A 50ml culture of cells in log phase ($5x10^6$ to $1x10^7$ cells/ml) was harvested at 3000rpm for 3 minutes in a Sorvall Legend RT benchtop centrifuge. The pellet was washed once in 20ml ice-cold 1.2M sorbitol (Sigma) and then three times in

10ml 1.2M ice-cold sorbitol. The pellet was resuspended in 1.2M ice-cold sorbitol to a concentration of $1x10^9$ cells/ml. 200µl of cells were mixed with between 100ng (plasmids) and 10µg (linear fragments) of DNA in an ice-cold cuvette. Cells were pulsed using a Bio-Rad Gene Pulser II at a setting of 2.25kV, 200Ω and 25µF. Immediately following pulsing, 500µl of 1.2M ice-cold sorbitol was added. Cells were spread at various dilutions onto selective media using sterile glass beads and incubated at 32°C until colonies appeared.

Lithium acetate transformation

A 50ml culture of log phase cells was harvested as before. Cells were washed in 10ml 0.1M lithium acetate pH4.95 (Sigma), resuspended in 10ml 0.1M lithium acetate pH4.95 and incubated for between 30 minutes and 1 hour at 32°C. Cells were pelleted and resuspended at a concentration of $1x10^9$/ml in 0.1M lithium acetate pH4.95. 1µg of DNA was added to 150µl of cells, mixed and then 370µl 50% PEG 3350 (Sigma) dissolved in TE was added. Cells were again incubated for between 30 minutes and 1 hour at 32°C, heatshocked for 20 minutes at 42°C then pelleted and resuspended in sterile dH_20. Cells were spread at various dilutions onto selective media using sterile glass beads and incubated at 32°C until colonies appeared. When transforming linear DNA fragments, after heatshocking cells are allowed to recover for a few hours to overnight in liquid non-selective media and then plated on selective plates.

2.2.3 Serial dilution assay

To assay the growth of different *S. pombe* strains on different media cells were taken from a plate and counted using a Beckman Z2 Particle Count and Size Analyzer. Serial dilutions of 1:4 were made in sterile microtitre plates in dH_2O starting with $5x10^6$/ml cells and 5µl of each plated on the appropriate media. Cells were then incubated at the desired temperature until colonies were formed.

2.2.4 Centromere silencing assay

Wild type cells which have the *ade6+* gene inserted into centromeric outer repeats are red when grown under restricted adenine conditions. This is due to transcriptional repression which causes the accumulation of amino-imidazole

ribonucleotide (AIR) (Fisher 1969). Mutants which alleviate silencing at the outer repeats are white due to alleviation of silencing. This assay can also be carried out using the insertion of the *ura4+* gene. In wild type cells, *ura4+* expression is repressed and cells are able to grow well on counter-selective media containing FOA. Mutant cells grow well on media lacking uracil but are unable to grow on media containing FOA (Boeke, LaCroute et al. 1984). Cells are spotted onto appropriate plates as described in 2.2.3.

2.2.5 *S. pombe* expression vectors

ars vectors

S. pombe vectors contain a bacterial origin of replication and selectable marker as well as a yeast selectable marker and an autonomous replication sequence (ars) or equivalent. Budding yeast markers are frequently used such as LEU2 which complements a mutation in the *leu1* gene of *S. pombe.* Plasmids based on the *S. cerevisiae* origin 2μ are mitotically unstable, have a low copy number, are more prone to rearrangements and are more difficult to recover from fission yeast than plasmids containing *S. pombe* ars1. However, the copy number of ars1 vectors may vary as they can produce polymers with variable numbers of repeats.

Inducible vectors

The most commonly used inducible vectors in *S. pombe* are the pREP vectors. These contain the thiamine responsive promoter of the *nmt1*$^{+}$ gene which is repressed in the presence of thiamine and expressed in the absence of thiamine and gives full induction after around 16 hours (Maundrell 1993). The *nmt1* promoter has been mutated to give different levels of expression as the fully induced level is very high. pREP1 and pREP3X have the highest levels of expression, pREP41X has a 15 fold lower level than pREP1 and pREP81X has an 80-fold reduction in expression level than pREP1 (Basi et al., 1993).

2.2.6 Plasmid recovery from *S. pombe*

To recover plasmids from fission yeast transformed with a genomic library, single colonies were grown at 32°C for 3 days in PMG media lacking the appropriate auxotrophic marker to select for colonies retaining the plasmid.

Cells were harvested by centrifugation at 3000rpm for 2 minutes. The pellet was resuspended in SP1 containing 1mg/ml zymolyase 100-T (MP Biomedicals) and incubated for 1 hour at 37°C. Cells were harvested again and resuspended in Buffer P1 from the Qiagen Plasmid Miniprep kit. The Qiagen minprep protocol was then followed as per manufacturers instructions and the DNA eluted in 30µl dH_2O. 10µl of the recovered DNA was transformed into 30µl DH5α competent cells (Invitrogen) and plated on selective LB plates containing 30µg/ml ampicillin. As a low yield of plasmid DNA occurs from this initial rescue, a miniprep was performed on colonies from this first transformation and the DNA was then re-transformed into DH5α competent cells as before.

SP1: 1.2M sorbitol, 50mM sodium citrate, 50mM $Na_2HPO_4.7H2O$, 40mM EDTA, pH 5.6.

2.2.7 *S. cerevisiae* transformation

A 50ml cell culture was grown to exponential phase overnight at 25°C in YP media containing galactose. Cells were harvested by centrifugation at 3000rpm for 5 minutes. The pellet was washed once in dH_2O, transferred to an eppendorf tube and resuspended in 300µl LiAc/TE solution. 50µl of the cell suspension was mixed with 1µg DNA and 5µg salmon sperm DNA and then 300µl of LiAc/PEG/TE solution was added. The cells were heatshocked for 40 minutes at 42°C, then harvested, resuspended in 100µl dH_2O and plated on appropriate media as indicated.

YP: 1% (w/v) yeast extract, 2% (w/v) Bacto-peptone containing either 2% (w/v) glucose or 3% (w/v) glycerol

LiAc/TE: 0.1M LiAc, 1xTE

LiAc/PEG/TE: 0.1M LiAc, 40% PEG 3350, 1xTE

2.2.8 FACS analysis

A 1ml culture of log-phase cells were harvested and washed in PBS. These were then resuspended in 1ml PBS. Fluorescence was analyzed using a FACScan Flow Cytometer (Becton-Dickinson). Constant settings were maintained for all experiments. Data were acquired from 10,000 cells in all experiments and analyzed with Cell Quest (Becton-Dickinson) software.

2.3 DNA protocols

2.3.1 Genomic DNA Isolation

A 5ml stationary phase culture was harvested at 3000rpm for 5 minutes. The pellet was resuspended in 250µl SP1 buffer containing 0.4mg/ml zymolyase 100-T (MP Biomedicals) and incubated for 30 to 60 minutes at 37°C. The cells were then pelleted at 13000rpm in a microfuge for 15 seconds and the pelleted resuspended in 0.5ml TE, 50µl 10% SDS and vortexed. 165µl 5M potassium acetate was then added and the samples incubated on ice for 30 minutes. After centrifugation at 13000rpm at 4°C for 10 minutes, the supernatant was added to 0.75ml iospropanol, incubated on dry ice for 5 minutes and then centrifuged as before. The pellet was resuspended in 0.3ml TE containing 10µg/ml of RNase A (Roche). After 30 minutes at 37°C the sample was then extracted with phenol/chloroform and precipitated by addition of 2-3 volumes of ethanol and 1/10 volume of 3M sodium acetate. The pellet was resuspended in 20µl TE and stored at -20°C.

SP1: 1.2M sorbitol, 50mM sodium citrate, 50mM sodium phosphate, 40mM EDTA, pH 5.6

2.3.2 Rapid DNA isolation for PCR

A small scrape of a single colony of *S. pombe* was suspended in 10µl SPZ buffer and incubated at 37°C for 10 minutes. Crude DNA was used in PCR analysis diluted at 1:10.

Alternatively, a small scrape of cells was placed directly into a PCR tube and microwaved at full power for 1 minute 30 seconds then placed immediately on ice. PCR mix was added directly to the tube and used for PCR analysis.

SPZ: 1.2M sorbital, 100mM sodium phosphate, 2.5mg/ml zymolyase-100T (MP Biomedical)

2.3.3 Agarose gel electrophoresis

Agarose gel electrophoresis was used to analyse the size of DNA fragments. Agarose (Melford) at a final concentration of between 0.8% and 2% was dissolved in 1 x TBE buffer by heating in a microwave. Once cooled, ethidium

bromide (Sigma) was added to a concentration of 0.03μg/ml. DNA samples were loaded in Orange G loading buffer and visualised under a UV transilluminator. Data capture was achieved using Kodak DC software.

10xTBE: 108g Trizma base, 55g boric acid, 9.3g EDTA

Loading buffer: 15% ficoll in TE, Orange G

2.3.4 Polymerase Chain Reaction

PCR reactions were carried out as follows in 0.2μl thin walled PCR tubes: template DNA, 10pM primer, 2.5mM dNTPs , 10 x PCR buffer, 0.5U Taq (Roche), dH2O. When precise amplification was required, in the case of cloning, Platinum *Pfx* taq polymerase from Invitrogen was used as per manufacturers instructions. All reaction were carried out using a PTC-225 thermal cycler (MJ Research). The following programs were used as indicated.

Ura program: 94°C for 4 minutes, (94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute), 29 cycles, 72°C for 5 minute.

Ali3 program: 94°C for 2 minutes, (94°C for 30 seconds, 52°C for 30 seconds, 72°C for 2 1/2 minute), 34 cycles, 72°C for 10 minutes.

Bahlong: 96°C for 5 minutes, (94°C for 1 minute, 55°C for 1 minute, 72°C for 4 minutes), 34 cycles, 72°C for 10 minutes.

Bahvlong: 96°C for 5 minutes, (94°C for 1 minute, 55°C for 1 minute, 68°C for 6 minutes), 34 cycles, 68°C for 10 minutes.

Ade6otr: 94°C for 4 minutes, (94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute 45 seconds), 29 cycles, 72°C for 5 minutes.

2.3.5 Sequencing

Reactions were set up as follows: 2μl ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit v 3.0 (Applied Biosystems), 3.2pmol/μl primer, template DNA as recommend by manufacturers (1-1000ng for PCR products, 200-500ng for dsDNA) and dH_2O up to 20μl. Reactions were run on the following program in 0.2μl thin walled PCR tubes in a PTC-225 thermal cycler (MJ Research): 95°C for 5 minutes, (95°C for 30 seconds, ramp 1°C per second to 55°C, 55°C for 15 seconds, ramp 1°C per second to 64°C, 64°C for 4 minutes),

perform 25 cycles. Samples were then sent to the central sequencing service for analysis.

2.4 RNA protocols

2.4.1 Total RNA isolation

A 50ml culture of cells in log phase was harvested then resuspended in 1ml TE and transferred to an eppendorf tube. Cells were pelleted and resuspended in 600µl extraction buffer, 600µl phenol:chloroform 5:1 (Sigma) and 600µl 425-600 micron acid-washed beads (Sigma). Cells were lysed on a multi-head vortexer at maximum speed for 30 minutes at 4°C. The samples were spun at 13,000rpm at 4°C for 5 minutes and the supernatant transferred to a fresh tube. Samples were extracted with phenol:chloroform and subsequently with chloroform then precipitated by adding 3 volumes of ice-cold 100% ethanol and centrifuging at 13,000rpm for 15 minutes. The pellet was resuspended in 30-50µl 50% formamide (Sigma).

Alternatively, RNA was made using a Qiagen RNeasy Miniprep or Midiprep kit as per manufacturer's instructions. RNA was quantified using a Nanodrop spectrophotometer.

Extraction buffer: 50mM Tris-HCl pH7.5, 10mM EDTA pH8, 100mM NaCl,1% SDS

2.4.2 Small RNA isolation

A 50ml culture of cells in log phase was harvested then resuspended in 1ml TE and transferred to an eppendorf tube. Cells were pelleted and resuspended in 600µl extraction buffer, 600µl phenol:chloroform 5:1 (Sigma) and 600µl 425-600 micron acid-washed beads (Sigma). Cells were lysed on a multi-head vortexer at maximum speed for 30 minutes at 4°C. The samples were spun at 13,000rpm at 4°C for 5 minutes and the supernatant transferred to a fresh tube. Samples were extracted with phenol:chloroform and subsequently with chloroform then precipitated by adding 3 volumes of ice-cold 100% ethanol and centrifuging at 13,000rpm for 15 minutes. The pellet was resuspended in 400µl dH_2O. Large rRNA, mRNA and genomic DNA were removed by precipitation with 10% polyethylene glycol 8000 and 0.5M sodium chloride. The samples were

incubated on ice for 30 minutes then spun at 13,000rpm for 20 minutes. The pellet was dissolved in 25µl of 50% formamide. The supernatant containing the siRNAs was then precipitated by the addition of 3 volumes of 100% ethanol and 1/10 volume sodium acetate and incubating at -20°C for between 3 hours and overnight. Pellets were then washed in 95% ethanol, spun for 10 minutes and resuspended in 50µl 50% deionised formamide. The samples were stored at -80°C. RNA was quantified using a Nanodrop spectrophotometer.

Extraction buffer: 50mM Tris-HCl pH7.5, 10mM EDTA pH8, 100mM NaCl,1% SDS

2.4.3. Poly(A) RNA isolation

A cell culture of 50ml was grown to log phase (1 x 10^8 cells/ml) in 4 x YES media and a total of 1.6 x 10^9cells/ml were used to make total RNA. Poly(A) RNA was isolated from 500µg total RNA prepared using a Qiagen Midiprep Kit. Poly(A) RNA was isolated using PolyATract mRNA Isolation System IV (Promega). RNA was quantified using a Nanodrop spectrophotometer.

2.4.4 Denaturing Polyacrylamide Gel Electrophoresis

siRNA samples were diluted in FDE sample buffer (deionised formamide, 0.5M EDTA pH8, 10mg xylene cyanol,10mg bromophenol blue), denatured for 15 minutes at 65°C then stored on ice prior to loading. ^{32}P end-labelled RNA Decade ladder (Ambion) was run as a size marker.

siRNA samples were run out on an 8% 16 x 18cm polyacrylamide gel using the Sequagel system (National Diagnostics). Gels were run in the Hoefer SE600 Ruby apparatus first at 150V to pre-run and then at 300V once the samples were added. The gel was run until the bromophenol blue dye front was approximately 2 cm from the bottom. The gel was cut above the xylene cyanol dye band and stained with ethidium bromide to check loading. The rest of the gel was soaked in 10mM sodium phosphate pH7 for 10 minutes and then in 20 x SSC for 10 minutes then transferred to a membrane by northern blotting.

2.4.5 Northern Blotting

Gels were blotted by capillary transfer for at least 16 hours in 20 x SSC using Hybond-NX (Amersham). Transfer was checked by monitoring for the ^{32}P-labelled Decade markers using a Geiger counter. The membrane was crosslinked twice at 1200 joules in a UV crosslinker (Stratagene).

Hybridisation and probe preparation

Membranes were pre-hybridised for a minimum of 1 hour at 42°C in PerfectHyb buffer (Sigma) or in standard sodium phosphate buffer. During this time DNA probes were labelled using the High Prime (Roche) random labelling kit. Briefly, 25ng of purified PCR product was mixed with 1µl each of 0.5mM dATP, dTTP and dGTP, 4µl High Prime reaction mixture containing 200µl random primer mixture and 1U/µl Klenow polymerase, and 50µCi [α^{32}P]dCTP. The reaction was incubated for 1 hour at 37°C and unincorporated radionucleotide was removed using a Microspin S-200 HR column (Amersham) or a NucAway Spin column (Ambion). The probe was denatured for 5 minutes at 95°C and added directly to 20ml fresh hybridisation buffer. Membranes were hybridised overnight at 42°C and subsequently washed twice for 30 minutes at 50°C in 2xSSC/0.2%SDS. The membranes were sealed in a bag and signals were visualised after between 4 hours and overnight using a phosphoscreen. Data was captured on a Storm phosphoimager with ImageQuant TL v 2005 (Amersham).

RNA probes were labelled with [α^{32}P]UTP using a MAXIscript *in vitro* transcription kit (Ambion) as per manufacturer's instructions.

Hybridisation buffer: 0.5M sodium phosphate pH 7.2, 1mM EDTA, 7% SDS

2.4.6 RT-PCR

1µg of total RNA was aliquoted into an eppendorf tube. 1µl Turbo DNAse (Ambion) and 1µl 10 x Turbo DNAse buffer was added and samples made up to a final volume of 10µl with dH_2O. Samples were incubated for 30 minutes at 37°C. For straightforward RT-PCR, 1µg oligo dT_{18} was added. For strand-specific RT-PCR 1µg of either cenF, cenR or actR was added and the samples made up to 25µl with dH_2O. Samples were incubated at 70°C for 10 minutes and then placed on ice. For first strand synthesis, the following reagents were

added; 8µl 1st strand buffer (Invitrogen), 4 µl 0.1M DTT (Invitrogen) and 2µl 2.5 mM dNTPs (Roche). Samples were then mixed and split into two aliquots. 1µl Superscript II Reverse Transcriptase (Invitrogen) was added to one of each pair of tubes only. Samples were then incubated at 42°C for 50 minutes and 70 °C for 15 minutes and then placed on ice. 1µl of this cDNA was used as a template in a 20µl PCR reaction.

2.4.7 RNA preparation for microarray analysis (K.Ekwall)

100ml of cell culture was grown overnight at 32°C to mid-exponential phase (5 x 10^6 – 1 x 10^7 cells/ml) and harvested at 3000rpm for 2 minutes at room temperature. Cells were resuspended in 2ml TES and 500µl of this was added to a pre-prepared tube containing 500µl phenol:chloroform 5:1 (Sigma). The tubes were vortexed vigorously for 10 seconds and then incubated for 45 minutes at 65°C in a heated vortex. Samples were then placed on ice for 5 minutes and then centrifuged at 15,000rpm at 4°C for 5 minutes. Samples were phenol and chloroform extracted and precipitated with 3M sodium acetate pH 5.3 and 100% ethanol on dry ice. RNA was pelleted, washed in ice-cold 70% ethanol and resuspended in 100µl dH_2O. The RNA was cleaned up using an RNAeasy Mini Protocol (Qiagen) as per manufacturer's instructions.

TES: 10mM Tris-HCl pH7.5, 10mM EDTA, 0.5% SDS

2.5 Microscopy

2.5.1 Live cell imaging

Cells were grown to log phase, harvested and resuspended in a small volume of culture medium so a cloudy suspension was formed. 1% low melting point agarose (Gibco) in culture medium was prepared and cooled to 37°C. 4µl of cell suspension and 6µl of agarose were mixed on a glass slide and a coverslip applied. Cells were visualised with a Carl Zeiss Microimaging, Inc Axioplan 2 IE fluorescence microscope with Chroma 83000 and 86000 filter sets, a Prior ProScan filter wheel (Prior Scientific), and Photometrics CoolSnapHQ CCD camera (Roper Scientific). Image capture was achieved using MetaMorph software (Universal Imaging Corp).

2.5.2 Immunostaining

α-tubulin staining

A 10ml of cells was grown to log phase and fixed by adding 3.7% formaldehyde and 0.0625% glutaraldehyde final concentration. Cells were fixed for 10 minutes at room temperature. Cells were then pelleted by centrifugation at 3000rpm for 2 minutes and washed twice in 5ml PEM. Cells were resuspended in 1ml PEMS, transferred to 1.5ml eppendorf tubes and pelleted. Pellets were resuspended at $1x10^8$/ml in PEMS containing 1mg/ml zymolyase and incubated for 2 hours 30 minutes at 37°C. After centrifugation, cells were washed in 0.5ml PEMS then incubated for 1 minute in 1ml PEMS containing 1% Triton-X100 (Sigma). Cells were again washed in 0.5ml PEMS and then incubated twice for 10 minutes in 2mg/ml sodium borohydride in PEM. After two further washes in PEM the cells were resuspended in PEMBAL and incubated at room temperature for 1 hour. Cells were then pelleted and resuspended in 100µl PEMBAL containing the appropriate dilution of primary antibody. Antibody incubations were carried out at 4°C overnight with rotation. Cells were washed three times in PEMBAL for 30 minutes at room temperature. Pellets were resuspended in Alexa green or Alexa red secondary conjugated antibody at 1:1000 in 100µl PEMBAL. Incubations were carried out at 4°C between 4 hours and overnight with rotation. Cells were washed once in 0.5ml PEMBAL for 30 minutes then incubated in PEM + 0.1% sodium azide and 1:500 DAPI for 5 minutes. Cells were washed in PEM with 0.1% sodium azide for 30 minutes and then resuspended in a small amount of PEM with azide to make a cloudy suspension. 2µl of this suspension was applied to poly-L-lysine coated slides (Fisher) and spread with a pipette tip. Once cells were dry a small drop of Vectashield (Vector Laboratories Inc.) was added and a coverslip was sealed on top. Cells were visualised with a Carl Zeiss Microimaging, Inc Axioplan 2 IE fluorescence microscope with Chroma 83000 and 86000 filter sets, a Prior ProScan filter wheel (Prior Scientific), and Photometrics CoolSnapHQ CCD camera (Roper Scientific). Image capture was achieved using MetaMorph software (Universal Imaging Corp).

Swi6 and Cnp1 co-staining

Cells were stained as detailed above with the following adjustments. Ten minutes prior to fixation, 10ml 2.4M sorbitol was added to 10ml cell culture and incubated at room temperature. Cells were then fixed in 3.7% paraformaldehyde for 5 minutes and no sodium borohydride step was required.

PEM: 100mM PIPES pH7, 1mM $MgCl_2$, 1mM EDTA

PEMS: PEM, 1.2M sorbitol

PEMBAL: 1% BSA, 100mM lysine hydrochloride, 0.1% sodium azide

2.6 Protein Protocols

2.6.1 *S. pombe* protein extraction

A 50ml culture was grown to log phase, harvested at 3000rpm at 4°C and washed once in dH_2O. The sample was transferred to a screw-capped eppendorf tube and frozen in liquid nitrogen. Cells were then resuspended in 500µl ice-cold lysis buffer with protease inhibitors (Sigma) and 1mM PMSF. After the addition of 500µl sample buffer and 500µl acid-washed glass beads (Sigma), the cells were lysed by bead-beating for 3 minutes. Samples were boiled for 5 minutes at 95°C then spun in a microfuge for 30 seconds to remove beads and cell debris. Samples were then either immediately loaded on an SDS-PAGE gel or stored at -20°C until required.

Lysis buffer: 50mM HEPES pH7.6, 75mM potassium chloride, 1mM magnesium chloride, 1mM EGTA, 0.1% triton-X100

2x Sample buffer: 2% SDS, 50mM Tris-HCl pH6.8, 2mM EDTA, 10% glycerol, 0.03% bromophenol blue, 2% β-mercaptoethanol

2.6.2 *S. cerevisiae* protein extraction

A 10ml cell culture was grown to log phase and 2 x 10^8 cells in total were used. Cells were harvested at 3000rpm for 5 minutes at 4°C and resuspended in 100µl of dH_2O. 15µl of 2M sodium hydroxide and 80mM DTT were added to the cell suspension and incubated on ice for 10 minutes. 15µl of 50% trichloroacetic acid was added and the samples incubated for a further 10 minutes on ice. Samples were then centrifuged at 15,000rpm for 2 minutes at 4°C and the pellets washed

with 1ml of acetone which had been stored at -20°C. The pellet was dried briefly and resuspended in sample buffer with 100mM Tris pH 8.8.

Sample buffer: 100mM Tris pH6.8, 4%SDS, 0.2% bromophenol blue, 20% glycerol, 200mM DTT.

2.6.3 SDS-PAGE

Proteins were separated on 1mm thick SDS polyacrylamide gels using the Hoefer minigel apparatus. Resolving gel was poured at concentrations varying between 6 and 12% depending on the size of the protein. Stacking gel (5%) was poured on top and gels were run at 200V for around 40 minutes in 1 x running buffer. After running gels were either transferred to nylon membrane for Western blotting or stained with Coomassie Brilliant Blue (Sigma) and dried.

Polyacrylamide gel 10ml: 2.5ml 1.5M Tris-HCl pH8.8, 100µl 10% SDS, 100µl 10% ammonium persulphate, 10µl TEMED, 30% acrylamide/bis mix (Sigma) between 2ml and 4ml depending on concentration, up to 10ml with dH_2O.

Stacking gel 100ml: 17ml 30% acrylamide/bis, 12.5ml 1M Tris-HCl pH6.8, 1ml 10% SDS, 69.5ml dH_2O.

5xRunning Buffer: 30g Tris Base, 144g glycine, 5g SDS.

2.6.4 Western analysis

Proteins were transferred onto Protran nitrocellulose membrane (Schleicher and Schuell) using a Hoefer semi-dry electroblotter. Before use, the membrane was floated on top of dH_2O. The blotting apparatus was assembled with 6 pieces of 3MM paper soaked in blotting buffer, the membrane stacked on top, then the gel, then another 6 pieces of 3MM paper. Any bubbles were removed by rolling over the top of the stack with a glass test-tube. Transfer was carried out for 1-2hours at 65mA per gel. After transfer, the membrane was washed briefly in dH_2O and stained with Ponceau S (Sigma) solution to confirm transfer. The membrane was washed in PBS and placed in blocking buffer for 1 hour at room temperature. Primary antibody was then added at appropriate concentration in PBS + 0.1% Tween 20 and incubated for 1 hour at room temperature. Membranes were then washed twice in PBS + 0.1% Tween 20 for 15 minutes each and then the secondary HRP-conjugated antibody was added in blocking

buffer. The secondary antibody was also incubated for 1 hour at room temperature. The membrane was washed again as before and then rinsed briefly in PBS. Proteins were detected using an Enhanced Chemi-Luminescence kit (Amersham) as per manufacturer's instructions. The blot was exposed to Bio-Max Light film (Kodak) for between 10 seconds and 1 hour.

Blotting buffer: 20ml 5 x SDS running buffer, 20ml methanol, 20ml dH_2O

Blocking buffer: 5% Marvel dried nonfat milk, PBS+0.1% Tween 20.

2.6.5 Chromatin Immunoprecipitation (ChIP)

A 50ml culture was grown in YES to between $5x10^6$ cells/ml and $1x10^7$ cells/ml. Cultures were shifted to 18°C/RT for ~2 hours on a shaking incubator $1/10^{th}$ volume of 30% or 10% formaldehyde was added to each culture and mixed. Cells were fixed in the fume hood for between 15 and 30 minutes. Fixation was stopped by addition of $1/20^{th}$ volume of 2.5M glycine. Cells were transferred to a falcon tube and centrifuged for 2 minutes at 4°C in a Sorvall Legend RT benchtop centrifuge. The cells were then washed twice in 20 to 50ml ice-cold PBS. Cells were resuspended at $1x10^8$/ml in PEMS containing 0.4mg/ml zymolyase-100T and incubated for 20-30mins at 37°C and then washed twice in 10ml PEMS.

Cells were resuspended in 1ml PEMS, transferred to an eppendorf tube and pelleted. Cells were resuspended so as to obtain 2.5 x 10^8 cells/ml per ChIP and frozen in 1ml aliquots. Frozen pellets were resuspended in 300ul ice-cold lysis buffer containing 3ul protease inhibitors (1:100) and 2mM PMSF. Samples were then sonicated on ice using a BioRuptor water bath for a total of between 15 and 20 minutes. Samples were then pelleted in a microfuge at 13,000rpm for 5mins at 4°C and the supernatant transferred to a fresh tube. Samples were spun again for 15mins at 4°C and the supernatant transferred to a fresh tube. While samples are centrifuging, Protein A or Protein G agarose (Roche) as appropriate was washed with lysis buffer 3 times and finally resuspended as a 50:50 beads:lysis buffer slurry. The crude lysates were pre-cleared using 25ul Protein A or Protein G as appropriate for 1 hour at 4°C. Beads were pelleted at 13,000rpm for 2 minutes in a microfuge. The lysate was transferred to a fresh tube with 40ul retained as the crude control and frozen at -20°C. The

appropriate concentration of antibody and 40ul Protein A or Protein G beads were added to the cell lysate and incubated at 4°C for between 4 hours and overnight depending on antibody. Following the antibody incubation, beads were centrifuged at 13000rpm for 2minutes in a microfuge. Beads were then washed in 1ml each of the following ice-cold buffers for 5 minutes at room temperature : lysis buffer, lysis buffer + 0.5 M NaCl, wash buffer, TE pH8. 250ul of TES was added to the beads and 210ul TES added to the crude extract. All samples were incubated at 65°C for between 6 hours and overnight to reverse the crosslinks. Samples were centrifuged for 1 minute and the supernatant removed to a fresh tube, discarding the beads. 250ul TE and 25ul 10mg/ml Proteinase K were added to each sample and incubated for 2 hours at 37°C. Samples were then extracted with phenol:chloroform and precipitated with 3M sodium acetate pH5.5 and 100% ethanol. The samples were incubated for 30 minutes on dry ice and then centrifuged at 13000rpm for 30 minutes at 4°C. The supernatant was removed and pellets were dried under a hood then ChIP samples resuspended in 30µl TE and crude samples in 300µl TE. Samples were then analysed by PCR. For all ChIP PCR reactions the 'Ura' program was used.

Lysis buffer: 50mM HEPES-KOH pH7.5, 140mM NaCl, 1mM EDTA, 1% Triton-X100, 0.1% sodium deoxycholate

Wash buffer: 10mM Tris-HCl pH8, 0.25M lithium chloride, 0.5% NP-40, 1mM EDTA, 0.5% sodium deoxycholate

TE: 10mM Tris-HCl pH8, 1mM EDTA

TES: 50mM Tris-HCl pH8, 10mM EDTA, 1%SDS

2.6.6 Recombinant protein expression

Plasmids were transformed into BL-21star (Invitrogen) cells and grown overnight at 37°C in 10mls LB liquid. Cultures were diluted 1:500 and then grown to an OD_{600} of 0.4. Protein expression was induced by adding an appropriate concentration of IPTG and incubating for a further 3 hours. Cells were harvested by centrifugation at 10,000rpm for 20 minutes in a Beckman Avanti J-25. Pellets were frozen and stored at -20°C.

2.6.7 Recombinant protein purification

Cell pellets were thawed on ice and resuspended in 2ml lysis buffer per gram. Lysozyme (Sigma) was added to 1mg/ml and the sample incubated on ice for 30 minutes. Samples were sonicated six times for 10 seconds at a high setting with 10 seconds cooling in between. Lysates were centrifuged at 20,000rpm for 30 minutes at 4°C and then passed through a 0.45μM filter disc. His-tagged proteins were purified under native conditions using Ni-NTA agarose (Qiagen) as per manufacturer's instructions. Purified protein was then dialysed into ice-cold PBS containing 10% glycerol.

PBS (1L): 10g sodium chloride, 0.25g potassium chloride, 1.43g Na_2HPO_4, 0.25g KH_2PO_4

2.6.8 ATPase assay

This ATPase assay takes advantage of the fact that ATP hydrolysis is coupled to NADH oxidation which causes a decrease in absorbance at 340nm. The assay was performed at 37°C with all components pre-warmed before starting. Two mixes were made as follows. Mix K (600μl); 1M KCl, 1M imidazole, 1M $MgCl_2$, 100mM ATP, 1U/μl lactic dehydrogenase, 2.5U pyruvate kinase, 2.5mM phosphoenolpyruvate, 2.5U NADH, dH2O. Mix A (150μl); 500mM PIPES pH7, 100mM EGTA, 1M $MgCl_2$, 1.25mM DTT and 5-50μg of test protein and dH2O. The two mixes were pre-warmed separately and then mixed together in a cuvette (Pidoux *et al*, Mol. Cell Biol. 1996). The optical density was measured using a Ultrospec 2100 *pro* (Amersham) in kinetics mode. ATP hydrolysis was calculated using the following formula;

$$\text{ATPase rate } [\text{min}^{-1}] = -\, dA340/dt[OD/min] \times K_{path}^{-1} \times moles^{-1}\ ATPase$$

where K_{path} is the molar absorption coeffiecient for NADH for a given optical path length. The assay was carried out in the absence and presence of an RNA substrate as indicated.

2.6.9 Poly(A) polymerase assay

Initially, standard 20μl reactions contained 20 mM Tris·HCl, pH 7.5, 50 mM KCl, 0.7 mM $MnCl_2$, 15 mM $MgCl_2$, 0.2 mM EDTA, 100 μg/ml acetylated BSA, 10% glycerol, 1.5μg of RNA and between 100ng and 1μg of protein. Reactions

were incubated at 37°C for 1 hour. RNA was phenol/chloroform extracted and EtOH precipitated before loading on 6% denaturing polyacrylamide gel containing 8M urea. Subsequently, assay conditions were modified and carried out with varying concentrations of $MgCl_2$ and MnCl as indicated. Potassium glutamate was substituted for KCl in order to minimise chloride ion concentration and more closely mimic the intracellular environment. Gels were exposed to a phosphoscreen and images captured using a Storm phosphoimager with ImageQuant TL v 2005 (Amersham).

2.7 Bacterial Protocols

2.7.1 Media

LB medium per litre:	Bacto tryptone	10g
	Bacto yeast extract	5g
	Sodium chloride	10g
LB agar per litre:	Bacto tryptone	10g
	Bacto yeast extract	5g
	Sodium chloride	10g
	Bacto agar	15g

Antibiotics:
Kanamycin 50mg/ml
Ampicillin 100mg/ml
Carbenicillin 50mg/ml
Chloramphenicol 20mg/ml

2.7.2 Transformation

BL-21Star and DH5α (Invitrogen) cells were transformed as per manufacturer's instructions with between 1 and 5μg plasmid DNA. In brief, between 30-100μl of cells were mixed with DNA, incubated on ice for 30 minutes, heatshocked at 42°C for 45 seconds and returned to ice for 1 minute. Cells were then grown at 37°C for 1 hour with the addition of between 100-400μl of SOC medium. Cells

were plated on media or grown in liquid supplemented with the appropriate antibiotic.

SOC 1L: Bacto tryptone 20g, bacto yeast extract 5g, sodium chloride 20g, 10ml 250mM potassium chloride, 5ml 2M magnesium chloride, 20ml 1M glucose pH 7

2.7.3 Plasmid construction

PCRs for cloning were carried out using either HiFi Taq (Roche) or Platinum *Pfx* Taq (Invitrogen). Restriction enzymes were obtained from Roche or New England Biolabs. DNA fragments were recovered and purified using a Gel Extraction kit (Qiagen). Ligations were performed using T4 DNA ligase (Promega) and incubated at 4°C overnight with insert:vector ratios of 1:1 and 3:1. Ligations were transformed into DH5α (Invitrogen) cells and plated on media containing appropriate antibiotic.

2.7.4 Plasmid miniprep

Single bacterial colonies were grown in 5ml LB plus appropriate supplement overnight at 37°C. Cells were harvested and plasmid prep performed using a QIAGEN miniprep kit according to manufacturer's instructions.

2.8 Antibodies

2.8.1 Chromatin Immunoprecipitation

mouse anti-diMeH3K9 1:300 (m5.1.1., gifted from Takeshi Urano lab)

sheep anti-Cnp1 1:30

2.8.2 Immunofluorescence

rabbit anti-Swi6 1:300

sheep anti-Cnp1 1:2000

anti-alpha-tubulin 1:15

Alexa 594 anti rabbit 1:1000

Alexa 477 anti sheep 1:1000

Anti GFP 1:2000 (Molecular Probes)

2.8.3 Western Analysis

PAP-HRP 1:1000 (Sigma)

M2 FLAG-HRP 1:1000 (Sigma)

2.9 Primers

Name	Sequence 5′-3′	Description
pALKS-for	GTA AAA CGA CGG CCA GT	Forward primer to sequence Shimoda library insert
pALKS-rev	AAC AGC TAT GAC CAT GA	Reverse primer to sequence Shimoda library insert
cenF	GAA AAC ACA TCG TTG TCT TCA GAG	Strand-specific RT-PCR and probe for nothern
cenR	CGT CTT GTA GCT GCA TGT GAA	Strand-specific RT-PCR
otrA	CAC ATC ATC GTC GTA CTA CAT	*dg* for ChIP
otrB	GAT ATC ATC TAT ATT TAA TGA CTA CT	*dg* for ChIP
TM-A	AAC AAT AAA CAC GAA TGC CTC	*cc* for ChIP
TM-B	ATA GTA CCA TGC GAT TGT CTG	*cc* for ChIP
fbp-a	AAT GAC AAT TCC CCA CTA GCC	euchromatin control for ChIP
fbp-b	ACT TCA GCT AGG ATT CAC CTG G	euchromatin control for ChIP
actinF	GGC TC ACA CTT TCT ACA ACG	actin RT-PCR control
actinR	GAG TCC AAG ACG ATA CCA GTG	actin RT-PCR control
U6sn	ATG TCG CAG TGT CAT CCT TG	siRNA northern loading control
siRNAH	TAC TGT CAT TAG GAT ATG CTC A	*dh* to make northern probe
Ing-F	GGC TAC TCT TCT CGA TGA TCC TG	*dh* to make northern probe
Ing-R	GGG TAG TAC GAC GAT GAT GTG TTT TC	*dh* to make northern probe
IngT7-F	TAA TAC GAC TCA CTA TAG GGC TAC TCT TCT CGA TGA TCC TG	*dh* to make strand-specific northern probe
IngT7-R	TAA TAC GAC TCA CTA TAG GGG TAG TAC GAC GAT GAT GTG TTT TC	*dh* to make strand-specific northern probe
adh1-f	GTG TCT GCC ACA CCG ATT TAC	loading control for northern
adh1-r	GTG AGG GCA GAT GGT CTC CT	loading control for northern
Rdp-1	CTG ATA CTG CAT AAA GGC GC	Rdp1 sequencing
Rdp-2	CTC CAA GTA CTA CTT CTT TG	Rdp1 sequencing
Rdp-3	TGA GTC ACC CTG GGA AAG AC	Rdp1 sequencing
Rdp-4	TTC CCA GAA TGG AAC TTT GG	Rdp1 sequencing
Rdp-5	CCC CTT CAT GAT TAC GGT AC	Rdp1 sequencing
Rdp-6	GCA TCA AAG AGA ACA CAG AGC	Rdp1 sequencing
Rdp-7	TGT CCC ACT GTT TGG TGT TG	Rdp1 sequencing
Rdp-8	ATA GGA CTA GCA GTC GTT GG	Rdp1 sequencing
Rdp-9	GTT CAA ACT TTC TGG ACT GC	Rdp1 sequencing
Rdp-10	GGC AAA TCA ATT TCT GAT AA	Rdp1 sequencing
Rdp-11	CAT CGC TGT AGT CTA ATA AA	Rdp1 sequencing
Rdp-12	GCA AAG TCC TTA AAA TTT C	Rdp1 sequencing

Rdp-13	GTT ACA CCT ACT ACT CTT CG	Rdp1 sequencing
Rdp-14	TCC TTT GTC GTT GAA AAG C	Rdp1 sequencing
Rdp-15	GGG TGA CTT TAG TGA AAT C	Rdp1 sequencing
Rdp-16	TTT ATC TTG ACT ACG GCG TG	Rdp1 sequencing
Rdp-17	CAT GAT ATG TTT CCC TCT GC	Rdp1 sequencing
Rdp-18	AAG CAT CAT CTC TTT CAA GG	Rdp1 sequencing
Rdp-19	TTT AAT GTT ACG GGA CAA CG	Rdp1 sequencing
Rdp-20	GCA TAA GCT CAT CGC ATC TG	Rdp1 sequencing
Rdp-21	GTT GGA TTG TGT ATT GTT GC	Rdp1 sequencing
Rdp-22	ATG CCT TCC AAG CAT TTG AA	Rdp1 sequencing
Rdp-23	AAA GCC ACT TAT CGA TTC GG	Rdp1 sequencing
Rdp-24	TAA ACA GTA TTC ATT ATC GG	Rdp1 sequencing
Rdp-25	CAG ATA CGC TGC ACG ATT CC	Rdp1 sequencing
Rdp-26	CAG CAA CCG CAC TGT TTA TC	Rdp1 sequencing
Rdp-27	GCG CTT TAT CAA ATC AAT CG	Rdp1 sequencing
Rdp-28	CTT AAC TCT AGC TAC CTG TT	Rdp1 sequencing
Dcr1-af	AAA ACC GAA TCA TTC TAG C	Dcr1 sequencing
Dcr1-ar	GAA ATA CCA AAG GGA CTT TG	Dcr1 sequencing
Dcr1-bf	AAA TTT CTC GTC AAT TGA ATG	Dcr1 sequencing
Dcr1-br	GAA ACC ACG TGT GCT TTA G	Dcr1 sequencing
Dcr1-cf	ATG AGC GAG CAG TTG TTG AC	Dcr1 sequencing
Dcr1-cr	TCA CTT TTG CGG TAA GTA GC	Dcr1 sequencing
Dcr1-df	ATT CTA TTA TCA AGA AAT GC	Dcr1 sequencing
Dcr1-dr	GAA ACT TTG AAG CCA TTG C	Dcr1 sequencing
Dcr1-ef	TGA AAG AAA AGC TAC GGC G	Dcr1 sequencing
Dcr1-er	AGC ATT CTG TTT GGC TTT CC	Dcr1 sequencing
Dcr1-ff	GTT AAT TCA TGA ACG CAT TC	Dcr1 sequencing
Dcr1-fr	TAA CAA CTC AAG CAG ATG AG	Dcr1 sequencing
Dcr1-gf	ACA TAT GAG CGC TAT GTA C	Dcr1 sequencing
Dcr1-gr	AAT GTC TAG AGA CTG GGA G	Dcr1 sequencing
Dcr1-hf	CCG GTT CTT AGA AAA TCT G	Dcr1 sequencing
Dcr1-hr	CAC CGA TAC TAG CTT CGA C	Dcr1 sequencing
Dcr1-if	AAT TGG GTG CTT CTA TTA CAG	Dcr1 sequencing
Dcr1-ir	AGT CTC CCT GAA CGC TTC	Dcr1 sequencing
Dcr1-jf	AAG AAA CTA CTC CAT TTG	Dcr1 sequencing
Dcr1-jr	AAG TAA GTT AGA CTT ATC	Dcr1 sequencing
Dcr1-kf	CGG AAC AAA GTG TGT CAT CG	Dcr1 sequencing
Dcr1-kr	AAG TTT GCA AAG ACG ATG GG	Dcr1 sequencing
Arb1-1	CCA CAC TGA TAA ACT AGT	Arb1 sequencing
Arb1-2	TGT AGG GTC GCT CCA TTT TC	Arb1 sequencing
Arb1-3	CAA GAA CCC GAA CTG ACA GC	Arb1 sequencing
Arb1-4	GGT TTG AGC GGA AAC TTC TC	Arb1 sequencing
Arb1-5	TTT CTA CGG GCC AAA AAC AG	Arb1 sequencing
Arb1-6	TTC AGT TCT GCT TGC TCT GC	Arb1 sequencing
Arb1-7	TCC AGA GA CTC TTG GTC CAC	Arb1 sequencing
Arb1-8	AAG GTG GGT AGC ACA GCA AG	Arb1 sequencing
Arb1-9	TGG GTT GCT GGA GTA TTT CTG	Arb1 sequencing
Arb1-10	TTG CGA TTT GTC CCA TAA GC	Arb1 sequencing
Arb1-11	AAC TTC CCG GTA GAT TGC AG	Arb1 sequencing
Arb1-12	TGG ATC CCA AAC GAT AAA CAC	Arb1 sequencing

NDEcid12f	GGA ATT CCA TAT GAT GGG TAA AGT CCT GTT A	Cid12 cloning and sequencing
BAMcid12r	GCG GGA TCC TTA TCC GCC AGC TTG TAA	Cid12 cloning and sequencing
seqcid12f	ATT GCT GAT GCT ATT GAA GC	Cid12 sequencing
seqcid12r	TCA AGC CAA CCA ATT AGC	Cid12 sequencing
cidtest-for	TTA GCA CCT TTG CAA TTA CGG	Cid12 cloning and sequencing
cid12_3'down	AAC AGC CGA ACC TTG CCT AAG	Cid12 cloning and sequencing
K1546	TGA TGA GTC CTA ATC TAG GG	Ago1 sequencing
L1086	CAG GAAGAT CCA AAA TAC TG	Ago1 sequencing
L1076	GTT CTG TTG TTT ACT TTG CC	Ago1 sequencing
L1085	GAC AGG TCA CCT TTA GAA AC	Ago1 sequencing
L1077	CTG AGA CTT TGT TTG GCT TC	Ago1 sequencing
L1084	CAA CGT CCA CTA ACG GGA CG	Ago1 sequencing
L1078	CGA TGC TTC CTA TTG AAT TC	Ago1 sequencing
L1079	GTT GAA GAA CTA TGT ATA AC	Ago1 sequencing
L1083	GAG CAA TTA ATT CCT TCC AAC	Ago1 sequencing
L1080	CAA CGT ATT ATC TAT TCC G	Ago1 sequencing
L1082	CTT TTC GTA CAA TGA GTG GG	Ago1 sequencing
L1081	CTT TGT TAT GTT TAT GCA AG	Ago1 sequencing
K1547	AGG AAG TAA AAG TTG TGG GC	Ago1 sequencing
pFARdptagF	TTG ATT TCA TTT CCA TAT TTA TTT TCT AGT CGT CTT TGT CAG CTT TCA CGA AAA GCC ATG CTT ACT GCT AAT AAT TTT CGG ATC CCC GGG TTA ATT AA	forward primer to tag Rdp1
pFARdptagR	GTA AAA ATT ATG ACA ACT CTT TTC CTT CAG CAG AAA CAT ACA ACA GTC TTG TTA AAT CTT AAC TCT AGC TAC CTG TTT TGG AAT TCG AGC TCG TTT AAA C	reverse primer to tag Rdp1
pFARdptruncF	CTT TTG CAG GCT TGC CTT TCT AAA AAC TTA TTA TCA GAA ATT GAT TTG CCC ATT ATA TTG GCG AAT TTA AAG AAA TTA CGG ATC CCC GGG TTA ATT AA	forward primer to tag *csp7*^{Rdp1}
cid12-for	TTA CAT ATA ATT ACA AGG CAC TCG CAC GAC CTC GTT ATG TGC GAG GAG CCA TGA AAT TGA ATC CAT TGA TAT TAA AAT TAA TGG GTA AAG TCC TGT TAG A	forward primer to integrate Cid12^{dada} into the genome
cid12-rev	ACC ACA TGC GGC AAG ACA ACT TAG GAA TTG AAA AAC AAA TGT TTA TTT AAA CAG CGA GCA TTA TTT TTT AAA TGC ATT AAT TAT CCG CCA GCT TGT AAT T	reverse primer to integrate Cid12^{dada} into the genome
cid12ctermtagF	GAA AAT GTT ATA AAT ACT AAA CGA AGT TTG GTG GAG GGA TAT GAT AGT GAT ACG GAA TCA GAT GAA TTA CAA GCT GGC GGA CGG ATC CCC GGG TTA ATT AA	forward primer to tag Cid12

cid12ctermtagR	CGA ACT GTA GTG AAA CAT TAC CAC ATG CGG CAA GAC AAC TTA GGA ATT GAA AAA CAA ATG TTT ATT TAA ACA GCG AGC ATT GAA TTC GAG CTC GTT TAA AC	reverse primer to tag Cid12
cid12chk170-f	GAT TAA AGC TGC ATT TGT TGC	forward primer to check Cid12 tagging
PFA6A-insseq_r	CTC AAG AAT AAG AAT TTT CG	reverse primer to check Cid12 tagging
HA-R	CTG AGC AGC GTA ATC TGG	Reverse HA to check tagging
TAP-rev	GTG CTT TGG CTT GGG TCG TC	Reverse TAP to check tagging
siRNAF-S	UCCAACUCCUCUUAUCUC	RNA oligo poly(A) assay substrate
siRNAF-A	AUAAGCAGGAGUUGCGCACU	RNA oligo poly(A) assay substrate

2.10 Strains

Strain Number	Genotype	Source
707	hA clr4-s5 ade6-210 leu1-32 ura4-DS/E otr1R dg-glu (BamHI-Spe1) Sph1::ura4	
972	x	
1034	hA swi6::his1 ade6-210 his1-102 leu1-32 ura4-DS/E otr1R (dg-glu BamHI-Spe1 fragment) Sph1::ura4	
1180	h+ ade6-210 leu1-32 ura4-D18 otr1R (dg-glu) Sph1::ade6	
1181	h- ade6-210 leu1-32 ura4-D18 otr1R (dg-glu) Sph1::ade6	
1193	h+ ade6-210 his1-102 leu1-32 ura4-DS/E otr1R (dg-glu) Sph1::ade6 imr1L (Nco1)::ura4	
1384	h- csp7-473 ade6-210 ura4-DS/E or D18 otr1R-Sph1::ade6+	
1385	h+ csp7-473 ade6-210 ura4-DS/E or D18 otr1R-Sph1::ade6+	
1386	h- csp8-432 ade6-210 ura4-DS/E or D18 otr1R-Sph1::ade6+	
1387	h+ csp8-432 ade6-210 ura4-DS/E or D18 otr1R-Sph1::ade6+	
1388	h+ csp9-434 ade6-210 ura4-DS/E otr1R-Sph1::ade6+	
1389	h- csp9-434 ade6-210 ura4-DS/E otr1R-Sph1::ade6+	
1390	h+ csp10-439 ade6-210 ura4-DS/E or D18 otr1R-Sph1::ade6+	
1391	h+ csp10-439 ade6-210 ura4-DS/E or D18 otr1R-Sph1::ade6+	
1392	h+ csp11-476 ade6-210 ura4-DS/E or D18 otr1R-Sph1::ade6+	
1393	h- csp11-476 ade6-210 ura4-DS/E or D18 otr1R-Sph1::ade6+	
1394	h- csp12-505 ade6-210 ura4-DS/E or D18 otr1R-Sph1::ade6+	
1395	h+ csp12-505 ade6-210 ura4-DS/E or D18 otr1R-Sph1::ade6+	
1396	h- csp13-446 ade6-210 ura4-DS/E or D18 otr1R-Sph1::ade6+	
1397	h+ csp13-446 ade6-210 ura4-DS/E or D18 otr1R-Sph1::ade6+	
1484	h+ csp7 ade6-210 his1-102 ura4-DS/E or D18 otr1R-Sph1::ura4+ [Ch16 LEU2+ ade6-216]	
1485	h- csp7 ade6-210 his1-102 ura4-DS/E or D18 otr1R-Sph1::ura4+ [Ch16 LEU2+ ade6-216]	
1486	h+ csp8 ade6-210 his1-102 ura4-DS/E or D18 otr1R-Sph1::ura4+ [Ch16 LEU2+ ade6-216]	
1487	h+ csp8 ade6-210 his1-102 ura4-DS/E or D18 otr1R-Sph1::ura4+ [Ch16 LEU2+ ade6-216]	

1488	h+ csp9 ade6-210 his1-102 ura4-DS/E otr1R-Sph1::ura4+ [Ch16 LEU2+ ade6-216]	
1489	h- csp9ade6-210 his1-102 ura4-DS/E or D18 otr1R-Sph1::ura4+ [Ch16 LEU2+ ade6-216]	
1490	h+ csp10 ade6-210 his1-102 ura4-DS/E or D18 otr1R-Sph1::ura4+ [Ch16 LEU2+ ade6-216]	
1491	h- csp10 ade6-210 his1-102 ura4-DS/E otr1R-Sph1::ura4+ [Ch16 LEU2+ ade6-216]	
1492	h+ csp11 ade6-210 his1-102 ura4-DS/E or D18 otr1R-Sph1::ura4+ [Ch16 LEU2+ ade6-216]	
1493	h- csp11 ade6-210 his1-102 ura4-DS/E otr1R-Sph1::ura4+ [Ch16 LEU2+ ade6-216]	
1494	h+ csp1 2ade6-210 his1-102 ura4-DS/E or D18 otr1R-Sph1::ura4+ [Ch16 LEU2+ ade6-216]	
1495	h- csp12 ade6-210 his1-102 ura4-DS/E otr1R-Sph1::ura4+ [Ch16 LEU2+ ade6-216]	
1496	h- csp13 ade6-210 his1-102 ura4-DS/E or D18 otr1R-Sph1::ura4+ [Ch16 LEU2+ ade6-216]	
2009	h90 (Xba1-Spe1)clr4::LEU2 ade6-210 leu1-32 ura4-D18 otr1R Sph1::ade6	
2010	h90 (Xba1-Spe1)clr4::LEU2 ade6-210 leu1-32 ura4-D18 otr1R Sph1::ade6	
2221	h- TM1(NcoI)::arg3 ade6-210/D1 arg3-D4 his3-D1 leu1-32 ura4-D18	
3043	h+ ade6-210 arg3-D3 his3-D1 leu1-32 ura4-D18/DS-E TM1::arg3+	
4837	h- TM-ura4 leu1-32 ura4DSE his3D1 arg3D4 ade6-210 #1	
4838	h+ TM-ura4 leu1-32 ura4DSE his3D1 arg3D4 ade6-210 #1	
4551	h- sim3-205 TM1::arg TM3::ade6 otr2::ura4 his3tel1L ade6-210 leu1-32 ura4D18 arg3D4 his3D1	
5805	h? argonaut (ago) cen1-ade6+, G418res, ura4-, his-, leu-	
5806	h? argonaut (ago) cen1-ade6+, G418res, ura4-, his-, leu-	
5807	h? argonaut (ago) cen1-ade6+, G418res, ura4-, his-, leu-	
5808	h? dicer (dcr) cen1-ade6+, G418res, ura4-, his+, leu-	
6222	Rdp G418R ura4DS/E	
6399	h? D Ago G418R ade6-210 otr1R(dg-glu) Sph1::ade6	
6642	h- TFIIS::leu2 leu1-32 ura4D18 ade6-M216 can1-1	
6856	h- cid12-GFP-KAN leu1-32	
6857	h- cid12-HA-KAN leu1-32	
6858	h- cid12-TAP-KAN leu1-32	
6892	h- cid12::KAN leu1-32 ura4-D18	Shao-Win Wang
6893	h- cid12::ura leu1-32 ura4-D18	Shao-Win Wang

7005	h+ DDcr1 (NATR) (KANs) cen1-ade6+ leu? his? ade6-210 ura4-D18	
7051	h- Rdp-TAP-CloNAT leu1-32 ura4-D18 otr1R sph1:: ade6 arg+ his+	this thesis
7052	h- Rdp-TAP-CloNAT leu1-32 ura4-D18 otr1R sph1:: ade6 arg+ his+	this thesis
7053	h- "csp7"-TAP-CloNAT leu1-32 ura4-D18 otr1R sph1:: ade6 arg+ his+	this thesis
7054	h- "csp7"-TAP-CloNAT leu1-32 ura4-D18 otr1R sph1:: ade6 arg+ his+	this thesis
7055	h- \"csp7\"-TAP-CloNAT leu1-32 ura4-D18 otr1R sph1:: ade6 arg+ his+	this thesis
7056	h- "csp7"-TAP-CloNAT leu1-32 ura4-D18 otr1R sph1:: ade6 arg+ his+	this thesis
7344	h? cid12::ura ade6::Kan otr1RSph1:ade6 lys1::NAT leu+ ura4DSE/D18	
7366	h? Ago::Kan (G418R) ade6-210 ura4DS/E otr1RSph1:ura4	
7516	h+ leu1-32 ura4DS/E ade6-210 otr1R::ura4+ rdp1(D/A)HA::KAN	Shiv Grewal
8096	h- cid13::KANMX leu1-32 ura4-D18	Paul Russell
8129	h? ago1::KAN adh1:GFP-KANMX ura4-D18 ade6-210 leu? his? arg?	this thesis
8130	h? rdp1::KAN adh1:GFP-KANMX ura? ade? leu? his? arg?	this thesis
8134	h- cid1::ura4 leu1-32 ura4-D18	Chris Norbury
8135	h+ cid1::leu2 leu1-32 ura4-D18	Chris Norbury
8136	h- cid11::ura4 leu1-32 ura4-D18	Chris Norbury
8207	h+ hrr1::TAP-KANR ura4-D18 leu1-32 ade6-216 imr1R(Nco1)::ura4+ori1	Danesh Moazed
8209	h+ hrr1::KANR ura4-D18 leu1-32 ade6-216 imr1R(Nco1)::ura4+ori1	Danesh Moazed
8284	h? rdp1-TAP-NAT cid12-HA-KAN leu1-32	this thesis
8285	h? rdp1-TAP-NAT cid12-HA-KAN leu1-32	this thesis
8286	h? rdp1-TAP-NAT cid12-HA-KAN leu1-32	this thesis
8287	h? rdp1-TAP-NAT cid12-HA-KAN leu1-32	this thesis
8339	h? cid12::KAN ura4-D18 adh1::GFP-KAN leu1-32	this thesis
8476	h- cid1::ura4 leu1-32 ade6-210 ade6::Sph1 otr1R	this thesis
8477	h- cid11::ura4 leu1-32 ade6-210 ade6::Sph1 otr1R	this thesis
8478	h- cid13::KAN leu1-32 ade6-210 ade6::Sph1 otr1R	this thesis
8533	h- cid14::ura4 ura4-D18	Shao-Win Wang
8535	h- cid14::LEU2 leu1-32 ura4-D18	Shao-Win Wang
8589	h? dcr1::cloNAT arg3::ura4-GFP	this thesis
8590	h? cid12::KAN arg3::ura4-GFP	this thesis
8591	h? csp7 arg3::ura4-GFP	this thesis
8592	h? csp10 arg3::ura4-GFP	this thesis

8593	h? csp11 arg3::ura4-GFP	this thesis
8594	h? csp12 arg3::ura4-GFP	this thesis
8595	h? csp13 arg3::ura4-GFP	this thesis
8760	h- cid14::ura4 ura4-D18 ade6-210	this thesis
8787	h- cid12DADA-HA-KAN leu1-32 ade6-210 ade6otr:sph1	this thesis
8788	h- cid12DADA-HA-KAN leu1-32 ade6-210 ade6otr:sph1	this thesis
8865	h? cid14::ura4 ura4-D18 ade6-210 ade6::(Sph1)otr1R	this thesis
8866	h? rdp1-TAP-NAT cid12::KAN leu1-32 ura4D-18	this thesis
8867	h? rdp1-TAP-NAT cid12::KAN leu1-32 ura4D-18	this thesis
8868	h? rdp1-TAP-NAT cid12::KAN leu1-32 ura4D-18	this thesis
9614	h? ago1::KAN arg3::ura4-GFP leu1-32	this thesis
9616	h? csp9 arg3::ura4-GFP leu1-32	this thesis
9768	h+ Cid12-3FLAG(NATMX6) ura4DS/E leu1-32 ade6-210 otrSph1::ade6	
9845	h? cid12dada leu1-32 ura4D-18	this thesis
9846	h? cid12dada leu1-32 ura4D-18	this thesis
9947	h? cid12dada-3FLAG (NATMX6) leu1-32 ura4D18	this thesis
9948	h? cid12dada-3FLAG (NATMX6) leu1-32 ura4D18	this thesis
9949	h? cid12dada-3FLAG (NATMX6) leu1-32 ura4D18	this thesis
9950	h? cid12dada-3FLAG (NATMX6) leu1-32 ura4D18	this thesis
9951	h? cid12dada-3FLAG (NATMX6) leu1-32 ura4D18	this thesis
10381	h? dcr1::KAN TM1:ura4 leu1-32 his3D1 ura4DS/E	this thesis
10382	h? hrr1::KAN TM1:ura4 leu1-32 his3D1 ura4DS/E	this thesis
10383	h? rdp1::KAN TM1:ura4 leu1-32 his3D1 ura4DS/E	this thesis
10384	h? cid12::KAN TM1:ura4 leu1-32 his3D1 ura4DS/E	this thesis
10385	h? csp10 TM1:ura4 leu1-32 his3D1 ade6-210 ade6:(Sph1)otr1R	this thesis
10386	h? dcr1::KAN TM1:arg3	this thesis
10387	h? hrr1::KAN TM1:arg3	this thesis
10388	h? rdp1::KAN TM1:arg3 leu1-32	this thesis
10389	h? cid12::KAN TM1:arg3 leu1-32	this thesis
10390	h? csp10 TM1:arg3 leu1-32 ade6-210 ade6:(Sph1)otr1R	this thesis
10143	h- arb1::KAN ade6-210 leu1-32 ura4-D18 arg3D	Bioneer
10584	h- cid12::KAN	this thesis

CHAPTER 3
The effect of *csp* mutants on centromeric heterochromatin formation

3.1 Introduction

3.1.1 Properties of the fission yeast centromere

Large blocks of heterochromatin are prevalent at the centromere regions of many eukaryotes. In metazoa large arrays of repetitive DNA are packaged as heterochromatin at centromeres (Sullivan et al., 2001). The structure of *S. pombe* centromeres is somewhat similar to that of more complex eukaryotes in that they are also relatively large, repetitive and complex structures (Steiner et al., 1993; Takahashi et al., 1992). Fission yeast centromeres are composed of a central core region on which the kinetochore forms and flanking outer repeat sequences, known as *dg* and *dh* (Clarke and Baum, 1990; Takahashi et al., 1992). Additionally, fission yeast centromeres share characteristics with more complex eukaryotes in that these outer repeats are packaged as heterochromatin. Insertion of marker genes within these regions of heterochromatin causes variable transcriptional repression also known as position effect variegation (PEV) or transcriptional silencing (Allshire et al., 1994; Allshire et al., 1995). This property has allowed the development of genetic screens to identify factors which disrupt heterochromatin and hence affect centromere structure and function.

At a molecular level, heterochromatic regions display low levels of histone acetylation and are associated with di-methylation of the lysine 9 residue on histone H3 (H3K9me2). The specific H3K9me2 modification creates a binding site for Swi6, allowing it to bind histone H3 via its chromodomain (Bannister et al., 2001; Lachner et al., 2001). Another chromodomain protein which is involved in RNAi-directed heterochromatin formation, Chp1, is known to bind H3K9me2 although its exact role is unclear (Partridge et al., 2002). H3K9me2 is mediated by a conserved histone methyltransferase, Clr4, and has been shown to be required for the association of Swi6 with outer repeat heterochromatin at centromeres, the mating type locus and telomeres

(Ekwall et al., 1996; Nakayama et al., 2001; Partridge et al., 2000). Clr4 contains both a chromodomain and a SET domain and it is this SET domain which is responsible for the methylation activity of Clr4 (Sadaie et al., 2004). Mutations in this SET domain have been shown to affect the levels of H3K9 methylation at both centromeres and the mating-type locus (Nakayama et al., 2001; Rea et al., 2000). In addition, strains expressing histone H3 with lysine 9 mutated to either arginine or alanine are defective in silencing and Swi6 localisation. This underscores the importance of lysine 9 of H3 and its methylation by Clr4 in recruiting Swi6 (Mellone et al., 2003).

The *dg/dh* repeats are transcribed by RNAPII and this transcription is required for heterochromatin assembly at centromeres (Djupedal et al., 2005; Kato et al., 2005). In addition, an active RNAi pathway is required to ensure the establishment and maintenance of centromeric heterochromatin. In brief, the RNAi pathway processes dsRNA originating from the *dg/dh* repeats into siRNAs which are then targeted to homologous sequences via the RITS complex. Somehow this targeting brings about the recruitment of chromatin modifying enzymes such as histone deacetylases and the histone methyltransferase Clr4 which in turn allows Swi6 binding and heterochromatin formation on homologous *dg/dh* repeats (Verdel and Moazed, 2005).

Several genes involved in heterochromatin formation alleviate centromere silencing. Insertions of the *ura4*$^+$ gene at several sites across the centromere display variable expression (Allshire et al., 1995). Deletion of *clr4*$^+$, *swi6*$^+$, *chp1*$^+$ or *rik1*$^+$ all display alleviation of silencing at centromeric outer repeats (Allshire et al., 1995; Partridge et al., 2000). Rik1 is a protein containing a ß-propeller domain and a **c**leavage **s**pecificity and **p**olyadenylation **f**actor domain (CSPF-A) which may be involved in RNA binding (Allshire et al., 1995). Rik1 is also related to DNA damage binding protein 1, DDB1, a component of an E3 ubiquitin ligase in plants (Yanagawa et al., 2004).

Indeed, Rik1 has been found to associate with several other factors involved in ubiquitination as well as Clr4, however the targets and the consequences of this ubiquitination have not yet been identified (Hong et al., 2005; Horn et al., 2005; Jia et al., 2005; Li et al., 2005; Thon et al., 2005). It should be noted that silencing of marker genes is not a function of heterochromatin although it does provide a way to assay heterochromatin structure and thus centromere stability. Nonetheless, it is possible that similar related mechanisms could regulate the expression of endogenous genes. Swi6 and Chp1 localise specifically to the outer repeats at centromeres; very little is found across the central core region (Partridge et al., 2000). Both Rik1 and Clr4 are required for the localisation of Swi6 and Chp1 at centromeres, however Chp1 localisation is independent of Swi6 (Partridge et al., 2000). Clr4, Swi6, Chp1 and Rik1 are also required to silence marker genes inserted at the mating-type locus and at sites adjacent to telomeres (Allshire et al., 1995; Sadaie et al., 2004). Deletion of any of these genes results in increased rates of chromosome loss and chromosome missegregation, in particular a high frequency of lagging chromosomes on late anaphase spindles (Allshire et al., 1995; Ekwall et al., 1995; Partridge et al., 2000; Partridge et al., 2002; Sadaie et al., 2004).

3.1.2 A screen to identify additional factors involved in centromeric heterochromatin formation

A genetic screen was carried out to identify genes encoding proteins involved in heterochromatin formation. Previously identified mutations affecting heterochromatin formation were shown to affect all three sites where heterochromatin is known to form; centromeres, mating-type locus and telomeres. A screen was devised to identify factors specifically involved in silencing at centromeres and not at other regions so as to eliminate the possibility of re-isolation of genes such as *swi6*$^+$ and *clr4*$^+$ (Ekwall et al., 1999). A tester strain containing the *ade6*$^+$ gene inserted at the *SphI* site on the right hand side of centromere 1 (*cen1*), *otr1R(SphI):ade6*$^+$, or the *ura4*$^+$ gene inserted 15 kb away at the *NcoI* site on the left hand side, *imr1L(NcoI):ura4*$^+$, was used

as a reporter for mutations affecting centromere silencing (Figure 3.1a). In a wild-type strain, the *ade6*$^+$ gene is transcriptionally repressed resulting in red colonies on plates containing low supplementing adenine (Allshire et al., 1994). Defects in the *ade6*$^+$ gene causes accumulation of an intermediate, phosphoribosylaminoimidazole (AIR) (Fisher, 1969). The AIR intermediate is uncoloured however and subsequent events are required for the red colour to appear. Mutations which cause the alleviation of *ade6*$^+$ expression result in white colonies. Similarly, wild-type cells repressing *ura4*$^+$ gene expression fail to grow on plates lacking uracil but grow strongly on plates containing the counter-selective 5-fluoro-orotic acid (FOA). Mutants alleviating *ura4*$^+$ repression will grow strongly on media lacking uracil but fail to grow well on FOA (Boeke et al., 1984). The tester strain was mutagenised using ethyl methanesulfonate (EMS) which causes point mutations, preferentially G/C to A/T mutations. White colonies were selected for further analysis (Ekwall et al., 1999).

The *csp* (*c*entromere: *s*uppressor of *p*osition effect) mutants were isolated as mutations which specifically alleviate the silencing of marker genes inserted into the heterochromatic repeats of centromeres (Ekwall et al., 1999). Two classes of mutants were recovered by this strategy, the temperature-sensitive (ts) and non-temperature sensitive (non-ts) mutants. In total, 2200 white *ade6*$^+$ colonies were selected and tested for growth at 36°C. Forty-eight of these colonies were found to be ts. This indicates that these *csp* mutations may be in genes which are required for other essential functions than that at centromeres, unlike previously identified heterochromatin factors which are non-essential. Backcrossing the ts colonies identified 7 ts mutants which grew well at 25°C but are inviable at 36°C. There was one allele each of *csp1* to *5* and two alleles of *csp6*.

Of the remaining 2152 non-ts mutant colonies, 387 were screened for defective mating-type silencing. All of the *csp* mutants were screened

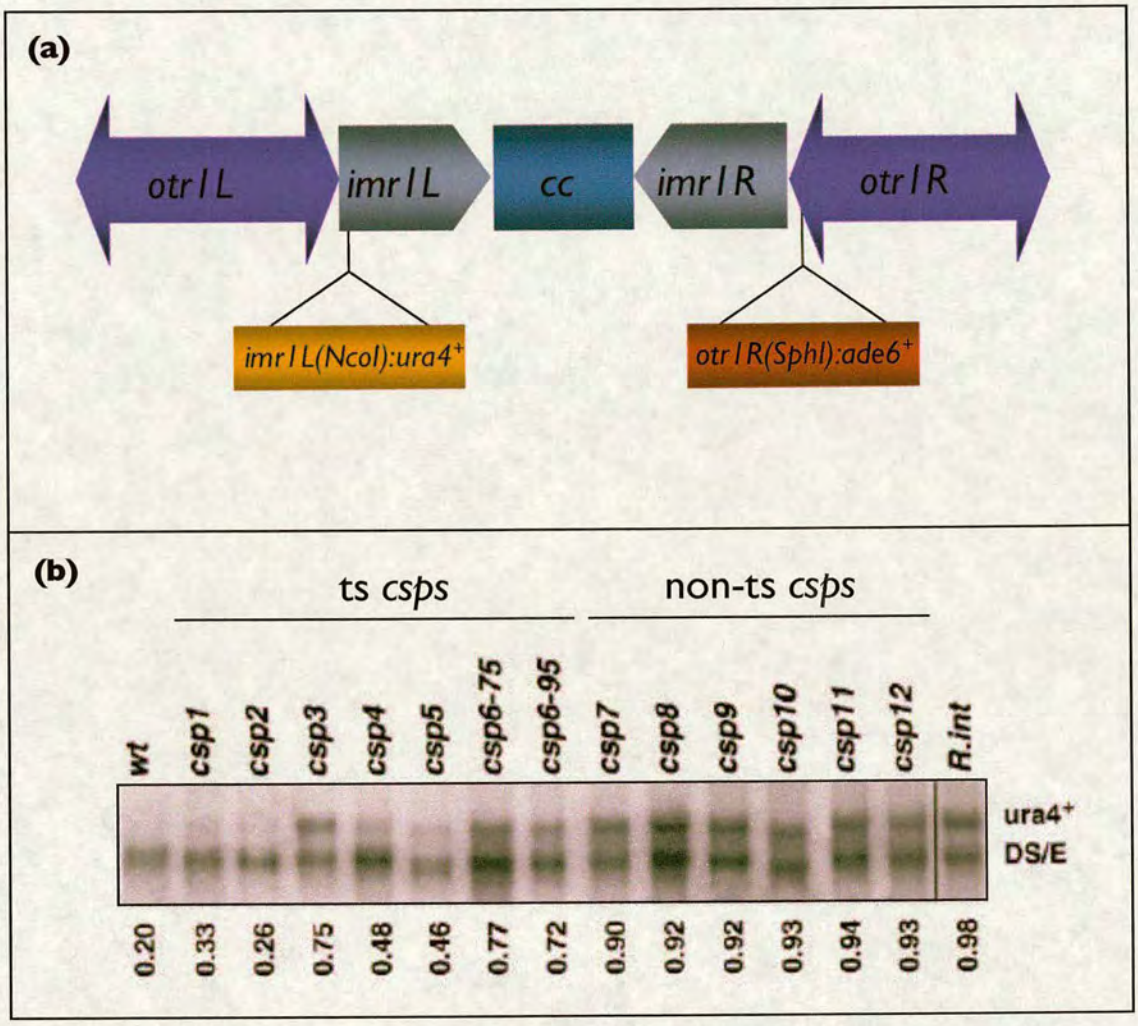


Figure 3.1. *csp* mutants are defective in silencing at the centromeric outer repeats.

(a) A schematic representation of centromere I (*cen1*) showing the marker gene insertions *otr1R(Sph1):ade6+* and *imrlL(Nco1):ura4+*. **(b)** Northern analysis showing a comparison of the centromeric silencing defects of ts and non-ts *csp* mutants using the *imr1L(Nco1):ura4* marker gene insertion as a readout of transcription levels. The wild-type has undetectable levels of *ura4+* gene expression whereas the *csp* mutants show alleviation of *ura4+* gene repression, with expression levels varying greatly between mutants. All of the non-ts *csp* mutants show a strong alleviation of *ura4+* silencing but of the ts mutants only *csp6* and *csp3* show strong expression of *ura4+* *(from Ekwall, 1999).*

against mating-type silencing so as to avoid the re-isolation of mutants such as *swi6* and *clr4*. From this secondary screening of the 387 non-ts mutants, 241 were found to have intact mating-type silencing and these were then further screened for alleviation of silencing of the *imr1L(NcoI):ura4* marker gene inserted at *cen1*. Finally, seven non-ts mutants, named *csp7* to *13*, were isolated. Crossing the mutants in all pair-wise combinations allowed them to be placed in complementation groups; *csp8* and *csp10* were subsequently shown to be allelic.

Although in the first instance only white, *ade6*$^{+}$ colonies were selected, upon further plating some pink and red colonies appear. This suggests that there is a variable degree of marker gene expression in the *csp* mutants although this is much more apparent in the ts mutants than in the non-ts mutants. Northern analysis of *imr1L(NcoI):ura4* was used to quantify expression compared to a euchromatic locus. This demonstrates that the silencing defect is much weaker in the ts csp mutants than in the non-ts mutants (Figure 3.1b) (Ekwall et al., 1999).

3.1.3 Known phenotypes of the *csp* mutants

The *csp* mutants were identified as factors alleviating silencing of marker genes inserted at centromeric heterochromatin (Ekwall et al., 1999). It has also been shown that they are sensitive to the microtubule destabilising drug, thiabendazole (TBZ) and have high rates of chromosome loss (Ekwall et al., 1999). The non-ts mutants, *csp7* to *13*, also display a high incidence of lagging chromosomes on late anaphase spindles which may be caused by failure to establish a correct bipolar orientation of sister kinetochores to spindle microtubules. Initial characterisation has demonstrated that *csp7* to *12* display between 11 and 58% of cells with lagging chromosomes although this analysis was limited to around 20 cells per mutant (Ekwall et al., 1999). *csp13* was also found to display lagging chromosomes but this has not been quantified. In addition, the number of cells with short spindles and the

number of cells in late anaphase was slightly increased in *csp7* to *12*. This may indicate defects in spindle elongation and/or activation of the spindle checkpoint (Ekwall et al., 1999).

However, despite the fact that the *csp* mutants show defective chromosome segregation, markers of heterochromatin appear to be, if not fully intact, at least partially present. Immunofluorescence data has shown that the *csp* mutants retain normal Swi6 localisation within the nucleus, implying that H3K9me2 is present at centromeres (Ekwall et al., 1999). Thus, H3K9me2 and Swi6 may be present at centromeres in the *csp* mutants at amounts sufficient to show normal localisation but may be insufficient to recruit a high enough level of cohesin to allow correct chromosome segregation. Conversely, Swi6, H3K9me2 and cohesin association on a marker gene inserted at heterochromatic repeats, *otr1R(SphI)::ura4*$^{+}$, were shown to be lost by chromatin immunoprecipitation (ChIP) analysis in all of the *csp* mutants (Volpe et al., 2003). This may indicate differences in sensitivity of the assays or may imply that Swi6 is less tightly bound at centromeres, perhaps due to reduced H3K9me2 levels, and therefore cannot be observed by ChIP.

In order to clarify these issues and to investigate the role of *csp* genes in silent chromatin formation, further analysis of the *csp* mutants is presented here. At the time of the *csp* screen, many of the molecular techniques which we now take for granted had not been fully developed and therefore phenotypic analysis was limited. Furthermore, the role of the RNAi pathway in heterochromatin assembly had not yet been uncovered. It is now known that the centromeric outer repeats are transcribed and that these transcripts accumulate in RNAi mutants (Volpe et al., 2002). In addition, RNAi mutants are unable to produce siRNAs homologous to the centromere and are thus unable to correctly form centromeric heterochromatin (Motamedi et al., 2004; Verdel et al., 2004).

The initial aims of this project were to characterise and identify the genes affected in the *csp* mutants and to further investigate the role of the affected proteins in heterochromatin formation and centromere integrity. In this chapter, I analyse the potential role of the non-ts *csp* mutants, *csp7* to *13*, in RNAi-mediated silent chromatin formation and examine further their phenotypes.

3.2 Results

3.2.1 *csp* mutants alleviate silencing at centromeric outer repeats

The *csp* mutants were isolated as factors which specifically alleviate silencing at the centromeric outer repeats (Ekwall et al., 1999). Previous analysis has revealed that all of the *csp* mutants display derepression of both *ade6*$^+$ and *ura4*$^+$ genes at the centromeric outer repeats with *csp1* to *6* showing a markedly lesser effect than *csp7* to *13*. The silencing defect of *csp7* to *13* using *otr1R(SphI):ade6*$^+$as a reporter is shown in Figure 3.2. Although in this assay all of the *csp* mutants look white, when streaked for singles colonies

they do show some variegation of *ade6*$^+$ expression, evident in the appearance of pink and red colonies (not shown). This demonstrates that the effect of the *csp*$^+$ genes on silent chromatin formation at centromeres is variable.

In addition, all of the mutants had been previously tested for effects on silencing at the central core, mating-type locus and telomeres (Ekwall et al., 1999). The central core domain is the region of the centromere where the kinetochore forms and whilst it has an unusual chromatin structure, it is not packaged into heterochromatin (Polizzi and Clarke, 1991; Takahashi et al., 1992). The *ura4*$^+$ gene was inserted at the central core of centromere 2 and at the mating-type locus, *CC2(SphI):ura4* and *mat3M::ura4*, to check for alleviation of silencing at these loci. *csp2*, *6* and all of the non-ts mutants, *csp7* to *13*, which contain *CC2(SphI):ura4* show some growth on plates lacking

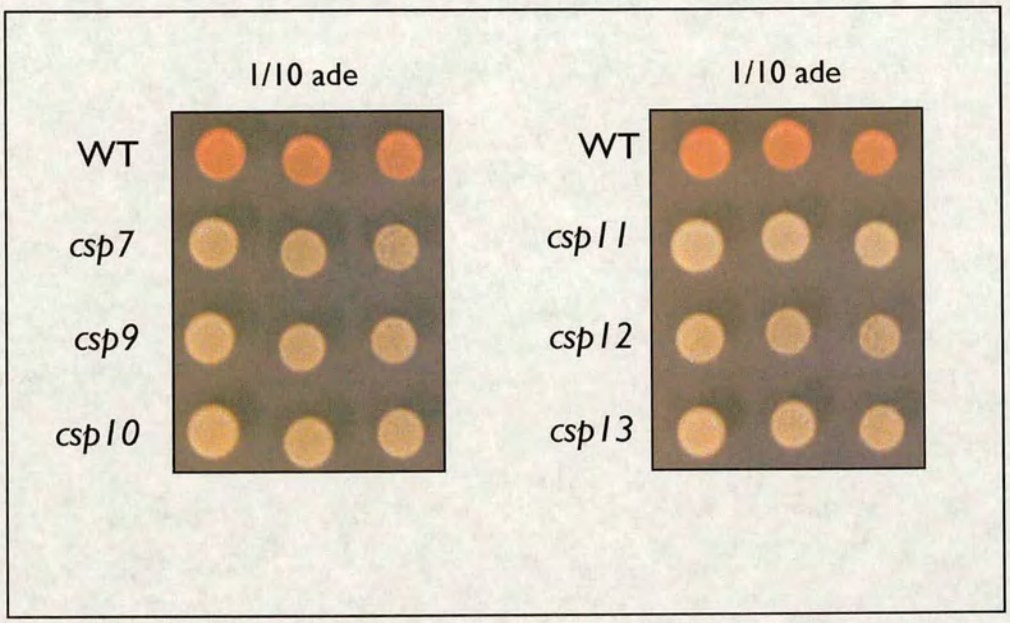


Figure 3.2. *csp* mutants alleviate silencing at centromere 1. Serial dilution assay showing the effect of non-ts *csp* mutants on *otr1R(SphI):ade6*$^{+}$ expression at centromere 1. On low adenine plates a wild-type strain appears red due to silencing of the marker gene while mutants alleviating centromere silencing are white.

uracil and do not grow at all on plates containing FOA, indicating that they have some effect on central core silencing (Ekwall et al., 1999). However, RT-PCR analysis failed to detect any increase in *ura4*$^+$ expression compared to a euchromatic control. As the growth of these mutants on plates lacking uracil is fairly poor it can be taken that these mutants retain silencing at the central core (Ekwall et al., 1999). This is similar to the effect seen in other heterochromatin mutants such as *clr4* and *swi6* (Allshire et al., 1995).

Only *csp2* displays alleviation of silencing at the mating-type locus, all other *csp* mutants behave like wild type cells and show no alleviation of silencing at this locus (Ekwall et al., 1999). Telomere silencing was assayed in a similar manner by insertion of a *his3*$^+$ gene placed adjacent to the left telomere on chromosome 1. Wild type cells bearing this marker are unable to grow on plates lacking histidine. Assaying all of the *csp* mutants containing this marker gene revealed that only *csp4* displays alleviation at telomeres (Ekwall et al., 1999).

3.2.2 Swi6 localises to centromeres in *csp7* to *13* mutants

The phenotypes described in the non-ts *csp* mutants are reminiscent of those seen in heterochromatin mutants such as *swi6* and *clr4*. Clr4 is the histone methyltransferase required to methylate histone H3 on lysine 9 and thus create a binding site for Swi6 (Bannister et al., 2001; Cowieson et al., 2000; Nakayama et al., 2001; Rea et al., 2000). Swi6 is required to promote heterochromatin spreading and recruit a high concentration of cohesin to the centromere (Bernard et al., 2001; Nonaka et al., 2002). In wild type cells, Swi6 is seen as several punctate spots (between two and six) within the nucleus (Ekwall et al., 1995; Ekwall et al., 1996). These punctate foci are known to be heterochromatic loci and have been shown to be clustered centromeres and telomeres and the mating type locus (Ekwall et al., 1995).

csp7 to *13* were tested to examine the nuclear localisation of Swi6 by immunofluoresence. *csp7* to *13* have all previously been shown to retain normal Swi6 localisation (Ekwall et al., 1999). In contrast, it has also been shown that *csp9* to *13* lose Swi6 association with *otr1R(SphI)::ura4*$^+$ by ChIP analysis (Volpe et al., 2003). This may reflect differences in *csp* behaviour at endogenous centromeric sequence as opposed to inserted transgene sequence. However, it may simply be that in *csp* mutants Swi6 is not so tightly associated with centromeric sequence and thus is not detectable by ChIP but can be seen as spots in the nucleus by immunostaining.

In order to ensure that Swi6 is indeed still localised to centromeric heterochromatin and not coincidentally associating with other nuclear loci in a similar pattern, co-immunostaining was carried out using polyclonal anti-Swi6 and anti-Cnp1 antibodies. Cnp1 is the histone H3 variant found only at the central core region of fission yeast centromeres (Takahashi et al., 2000). Cnp1 is seen as a single spot in the cell nucleus due to the clustering of the three centromeres (Takahashi et al., 2000). Figure 3.3 shows that all of the *csp* mutants have a Swi6 localisation pattern of two to four nuclear spots, comparable to wild type cells. The intensity of staining is also similar to that observed in wild type cells. Furthermore, in all of the mutants examined, Swi6 is found to maintain its centromeric localisation as it colocalises with Cnp1.

3.2.3 H3K9 methylation in *csp7* to *13*

Di-methylation of histone H3 on lysine 9 (H3K9me2) is a feature of heterochromatin in fission yeast. This modification has been shown to create a binding site for Swi6 (Bannister et al., 2001). It has previously been demonstrated that *csp9* to *13* display loss of H3K9me2 and hence Swi6 from *otr1R(SphI):ura4*$^+$ by ChIP (Volpe et al., 2003). However, as shown above, *csp* mutants appear to retain Swi6 localisation at centromeres by immunostaining indicating that at least some H3K9me2 must remain. ChIP

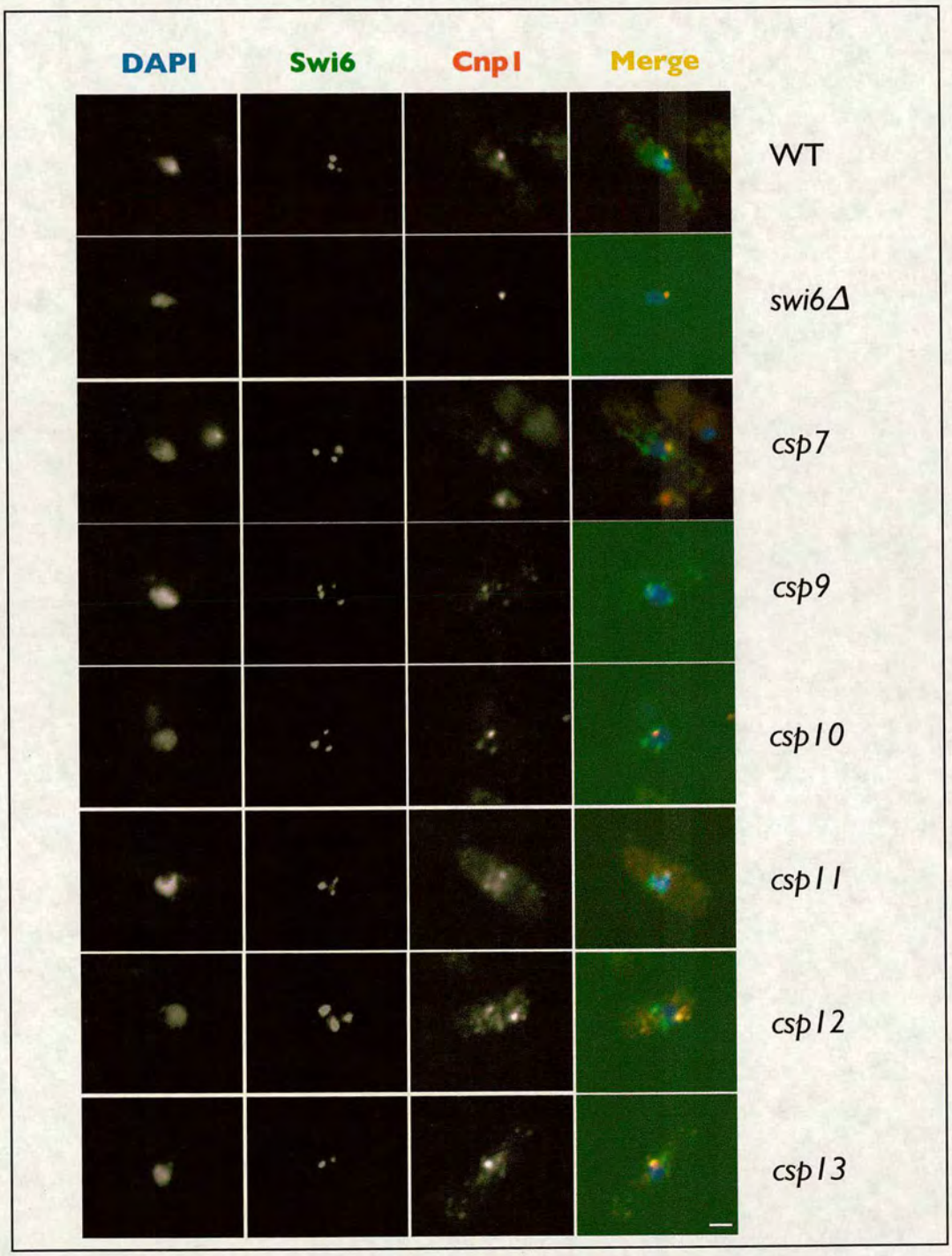


Figure 3.3. *csp* mutants retain Swi6 at centromeres.
Co-immunfluorescence staining using Swi6 antibody to stain heterochromatin and Cnp1 antibody to stain kinetochores shows that all of the *csp* mutants retain Swi6 localisation at centromeres, despite having defective centromere silencing. Scale = 5μM

analysis was carried out to analyse the levels of H3K9me2 on endogenous centromere sequence. The immunoprecipitated DNA was amplified using primers complementary to the centromeric outer repeats (*otr*) and a euchromatic negative control (*fbp*). Figure 3.4 shows that all of the *csp* mutants show a dramatic reduction in H3K9me2 levels on the centromeric outer repeats, consistent with previous data. However, the reduction is not quite as severe as that seen in a *clr4*Δ mutant and there appears to be some residual, albeit a small amount, of H3K9me2 on the outer repeats. In order to compare ChIP data with immunostaining, H3K9me2 staining was carried out. Wild type and *clr4*Δ mutants were stained with the same H3K9me2 monoclonal antibody used for ChIP under a variety of conditions (H3K9me2 antibody was a gift from Takeshi Urano). However, either the antibody or the fixation conditions were not suitable for immunostaining as a speckled pattern was observed in the nucleus and the cytoplasm of both wild type and *clr4*Δ mutant cells.

To further investigate the role of the *csp* mutant gene products in centromere function, it would be interesting to perform a Cnp1/heterochromatin establishment/maintenance assay in order to see how the *csp* mutant gene products behave. Cnp1 is the histone H3 variant which is found only at centromeres and is a key determinant of kinetochore assembly (Takahashi et al., 2000). Factors required for RNAi-mediated heterochromatin assembly have been shown to be essential for the establishment of Cnp1 chromatin and thus the kinetochore (Folco et al., 2008). To perform this type of analysis, the *csp* mutants would be transformed with plasmid containing unmodified central core and heterochromatin DNA to determine whether Cnp1 chromatin can be established in a particular *csp* mutant. To test whether the *csp* gene products are involved in maintaining Cnp1 chromatin at centromeres, the *csp* mutant strains would be crossed to a wild type strain containing a plasmid in which heterochromatin and Cnp1 chromatin had already been established (Folco et al., 2008). This could provide useful

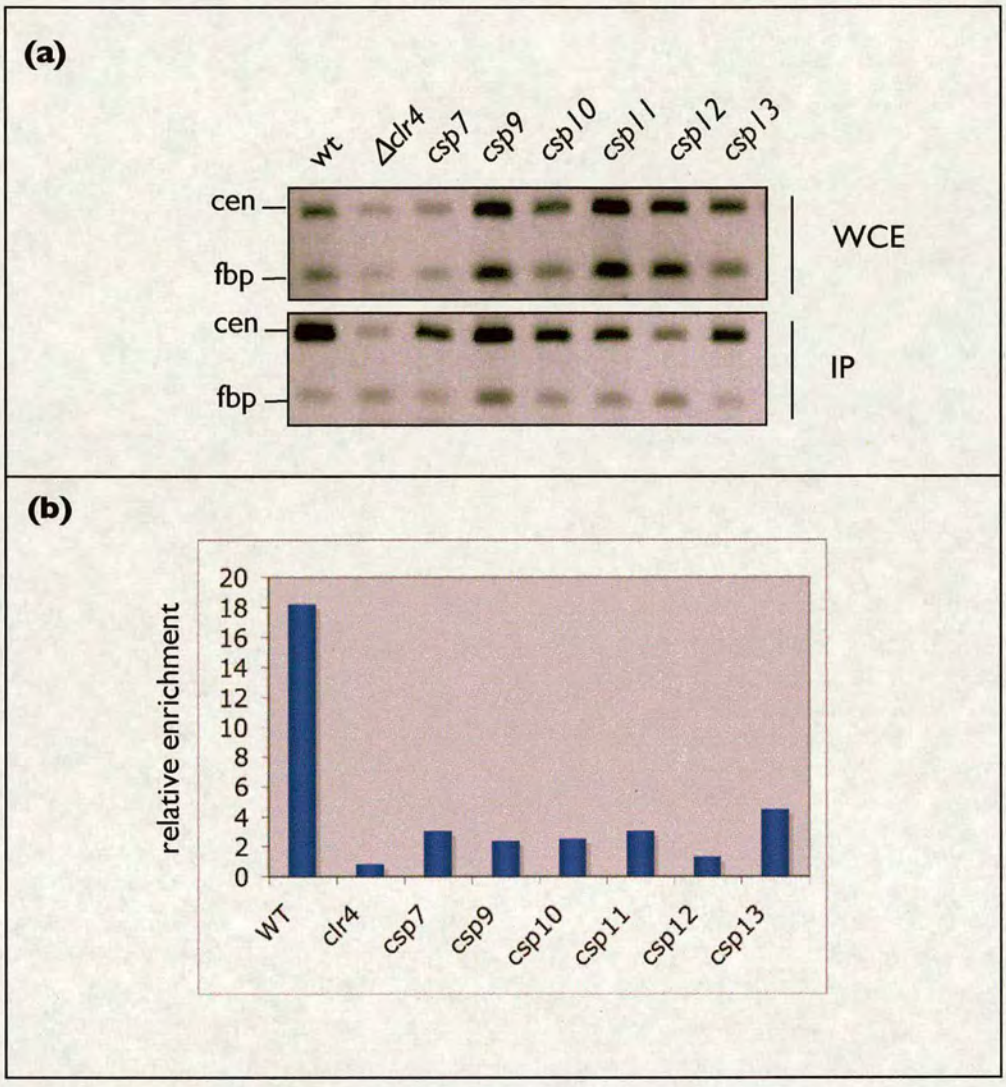


Figure 3.4. H3K9me2 is reduced at centromeres in the *csp* mutants.

(a) ChIP analysis shows that all of the *csp* mutants display reduced levels of H3K9me2 at centromeres. Multiplex PCR was performed and relative intensity of bands was calculated by dividing IP by WCE. Cen = Centro mere, Fbp = euchromatic control, WCE = whole cell extract, IP = immunoprecipitation. **(b)** Quantification of ChIP analysis showing that levels of H3K9me2 are greatly reduced but not to levels of a *clr4Δ* mutant.

information regarding the H3K9me2 state of centromeres in the *csp* mutants and how this contributes to centromere function.

3.2.4 *csp7* to *13* mutants accumulate non-coding centromeric RNA transcripts

RNAi mutants have been shown to accumulate non-coding RNA transcripts originating from the centromeric outer repeats that are normally processed into siRNAs (Volpe et al., 2002). These transcripts are derived from both the upper and lower strand of the *dg-dh* centromeric repeats. In wild type cells, only the lower strand is transcribed but this is barely detectable by RT-PCR as it is thought to be continually processed by the RNAi machinery (Volpe et al., 2002). Transcription of the upper strand is thought to be repressed by the formation of Swi6 heterochromatin as in a *swi6* mutant, only the upper strand accumulates (Volpe et al., 2002). In contrast, both strands are transcribed and accumulate to a high level in RNAi mutants (Volpe et al., 2002).

The centromeric transcripts are thought to act as a precursor to produce dsRNA which can then be processed by Dcr1 to produce siRNAs. siRNAs of between 20 and 24 nts, which are homologous to centromeric *dg-dh* repeats, have been detected in wild type cells (Cam et al., 2005; Reinhart and Bartel, 2002). These siRNAs are then incorporated into the RITS complex via Ago1 which somehow mediates targeting of chromatin-modifying activities to the homologous DNA/chromatin template to bring about heterochromatin assembly. However, deletion of any of the factors involved in RNAi-directed heterochromatin formation causes loss of siRNA production, consequent loss of H3K9me2 and thus heterochromatin formation. Due to the inherent interdependencies within the pathway, the order of events that trigger RNAi-mediated heterochromatin assembly are difficult to determine. However, the accumulated data has led to the proposal of a model whereby siRNA production and heterochromatin formation are intimately linked as

part of a self-enforcing RNAi feedback loop (Figure 3.5) (Noma et al., 2004; Sugiyama et al., 2005). It is therefore difficult to place mutants at a specific point along the pathway by examining their general phenotypes as they tend to be more or less identical. Nonetheless, analysing the overall phenotypes of mutants can provide useful information as to their general role in RNAi-directed heterochromatin assembly.

In order to investigate whether the *csp* mutants could be involved in RNAi-directed heterochromatin formation, levels of centromeric transcripts and siRNAs were examined. The position of strand-specific primers used to amplify centromeric transcripts is shown in Figure 3.6a. First strand cDNA synthesis reactions were primed using primers specific to either the forward or reverse strand of centromeric transcripts (cen for, which amplifies the lower strand or cen rev, which amplifies the upper strand) and with a primer complementary to the reverse strand of actin as a control. This RT-PCR analysis demonstrates that *csp7* to *13* accumulate centromeric non-coding RNA transcripts from both strands at levels similar to that seen in RNAi mutants (Figure 3.6b) (Motamedi et al., 2004; Volpe et al., 2003). In this instance cells lacking *dcr1* were used as a control.

The accumulation of centromeric transcripts was confirmed by northern analysis using a probe against the centromeric *dh* repeats (Figure 3.7a). The resulting phosphorimage reveals that all of the *csp* mutants accumulate centromeric transcripts to levels equivalent to those seen in an *rdp1*Δ mutant (Figure 3.7b). It should be noted that neither the RT-PCR or northern analysis is specific for a particular centromere as the *dg/dh* repeats share a high degree of homology and therefore the primers and probes indicated in Figures 3.6 and 3.7 will detect transcripts and siRNAs deriving from all three centromeres. In addition, regions of the mating-type locus and telomeres which share high homology with *dg/dh* repeats will be represented in these analyses.

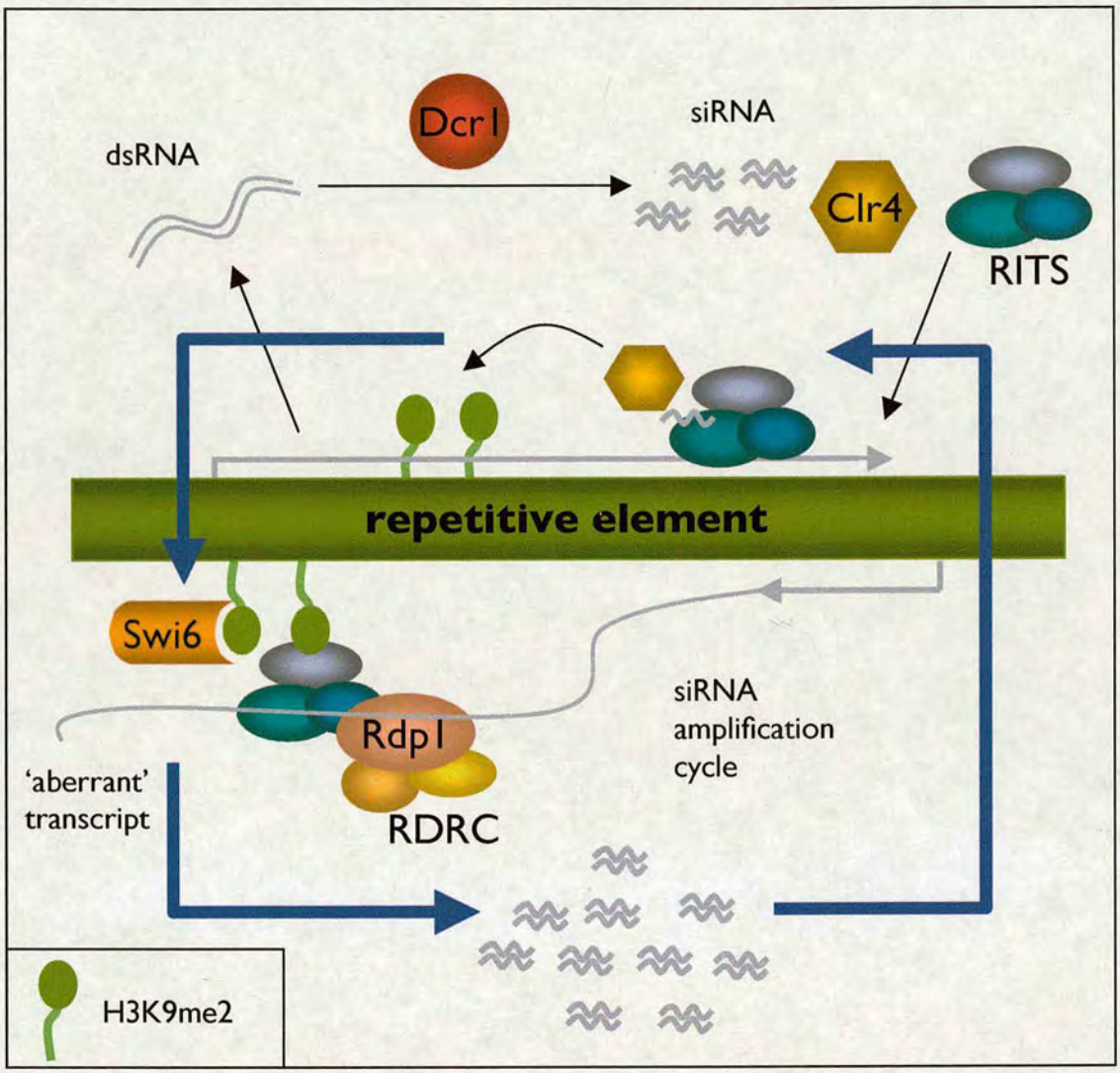


Figure 3.5. A self-enforcing loop couples heterochromatin assembly to siRNA production.
Bi-directional transcription of the centromeric outer repeats provides an initial source of dsRNA precursor which is processed by Dcr1 into primary siRNAs. These siRNAs are targeted to heterochromatin, presumably via the RITS complex which allows/acts in concert with chromatin modification factors such as Clr4. Clr4 methylates H3K9 and allows RITS to assemble on heterochromatin. Once this is achieved, the RDRC can assemble on RITS at heterochromatic loci. siRNAs may target Rdp1 to nascent transcripts to synthesise dsRNA. Dcr1 may be recruited to process these dsRNAs into secondary siRNAs which may act as amplification signals to recruit more heterochromatin assembly factors. *(Adapted from Sugiyama, 2005)*

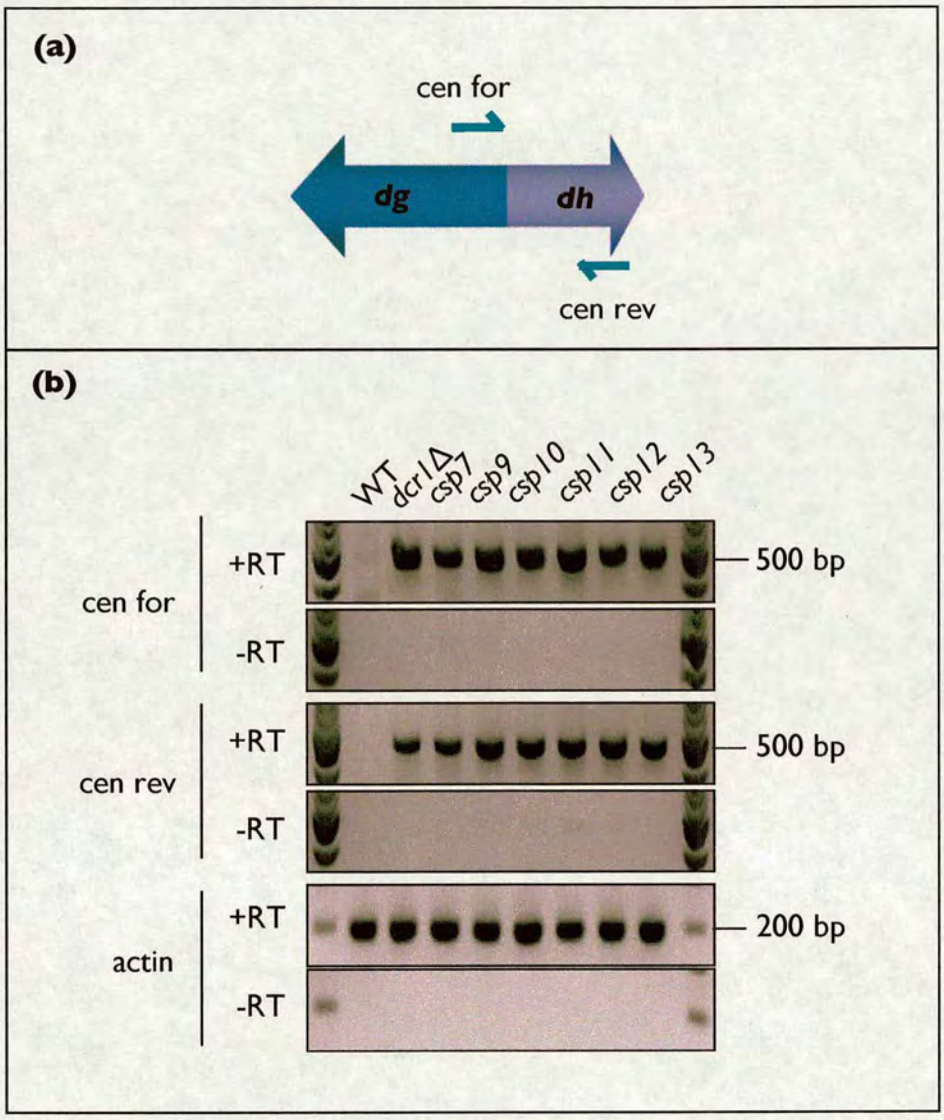


Figure 3.6. RT-PCR analysis of non-coding centromere transcripts.

(a) A diagram showing the position of primers used to initiate cDNA synthesis for RT-PCR. The cen for primer is used to detect anti-sense transcripts from the lower strand and the cen rev is used to detect sense transcripts from the upper strand. **(b)** Strand specific RT-PCR analysis in the presence (+RT) or absence (-RT) of reverse transcriptase demonstrates that all of the *csp* mutants accumulate centromeric transcripts to the same extent as an RNAi mutant, in this case *dcr1Δ* is used for comparison.

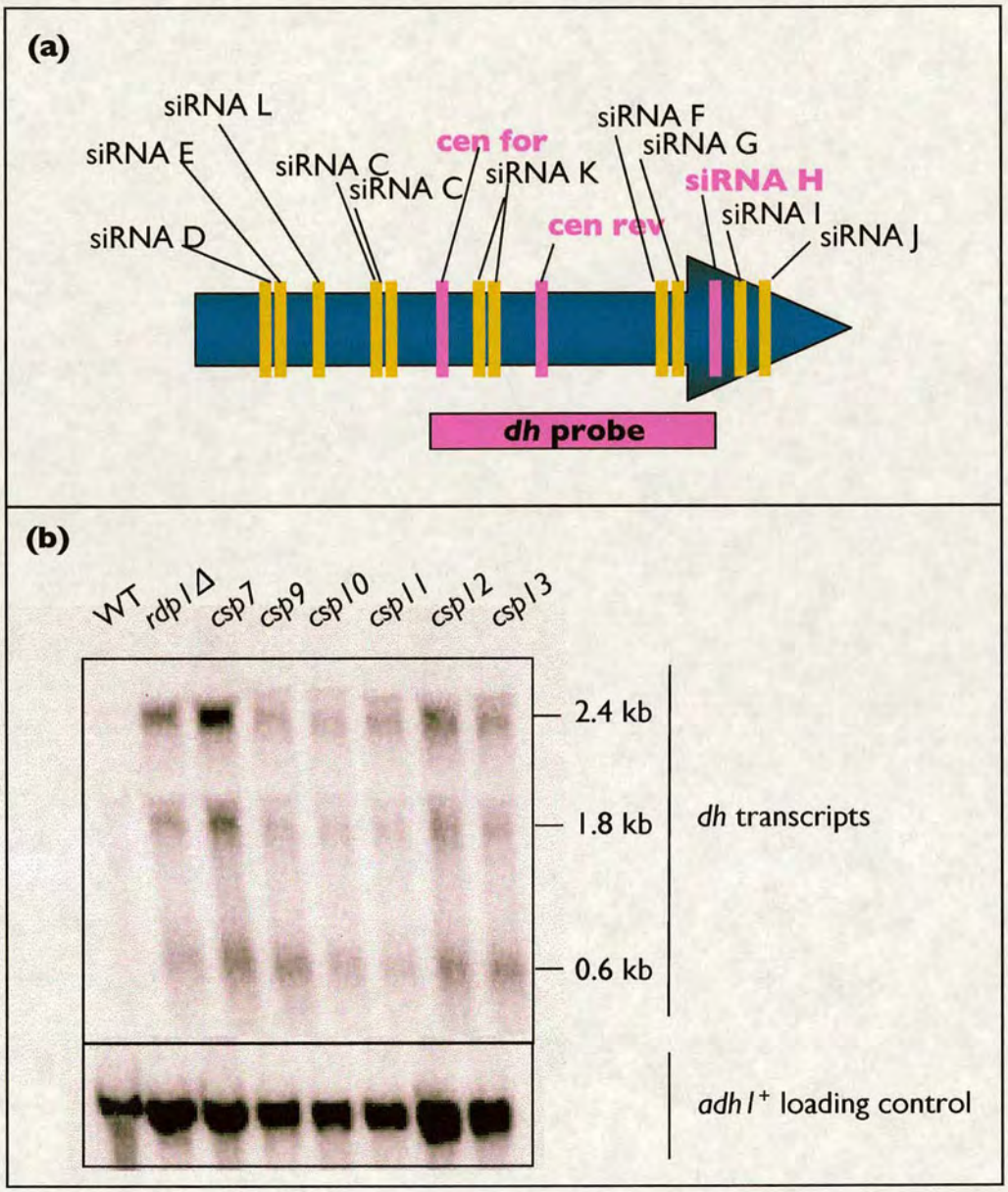


Figure 3.7. Northern analysis of centromeric non-coding transcripts.

(a) A schematic diagram showing the relative positions of sequenced siRNAs and primers used to generate a probe for northern analysis *(Reinhart and Bartel, 2002)* . Cen for and siRNA H oligos were used to generate a PCR fragment to probe the northern blot. Cen for and cen rev oligos are the same as those used for the RT-PCR analysis shown in Figure 3.6. **(b)** Northern analysis using the probe indicated in (a) confirms that all of the *csp* mutants accumulate centromeric transcripts.

3.2.5 *csp7* to *13* mutants are defective in siRNA production

Deletion of any of the components of the RNAi machinery results in a loss of centromeric siRNA production (Motamedi et al., 2004; Verdel et al., 2004). Centromeric siRNA production was examined by northern blotting using the same probe as for the non-coding RNA transcripts. As expected, none of the *csp* mutants are able to process the centromeric transcripts into siRNAs (Figure 3.8). In wild type cells, siRNAs are observed as a 'smear' upwards from around 24 nts to just above 30 nts. This is slightly larger than suggested by published sequence data. It may be that the siRNAs are modified in some way, perhaps phosphorylated as in *C. elegans*, although this has yet to be investigated (Pak and Fire, 2007; Sijen et al., 2007). In RNAi mutants this 'smear' of siRNAs disappears completely; this is also observed in all of the *csp* mutants (Figure 3.8).

Taken together with the transcript analysis, these data indicate that all of the *csp* mutants examined are defective in processing the centromere transcripts into siRNAs. As a result, all of the *csp* mutants are defective in RNAi-directed heterochromatin formation as they must be unable to target the RITS complex to centromeric outer repeats. Since most of the factors known to be involved in RNAi-directed heterochromatin formation display accumulation of centromere transcripts and loss of siRNA production it is difficult to ascertain where the *csp* mutants may act in this pathway.

3.2.6 siRNAs can be produced from an exogenous source in *csp* mutants

The *csp* mutants are defective in heterochromatin formation, that is, they fail to properly process centromere transcripts into siRNAs but still appear to be able to target low levels of H3K9me2 and Swi6 to the outer repeats. It is possible that the *csp* mutants are able to process dsRNA into siRNAs at an undetectable level and in this way cause some H3K9me2 and Swi6 targeting. However, the phenotypes observed for the *csp* mutants are similar to those

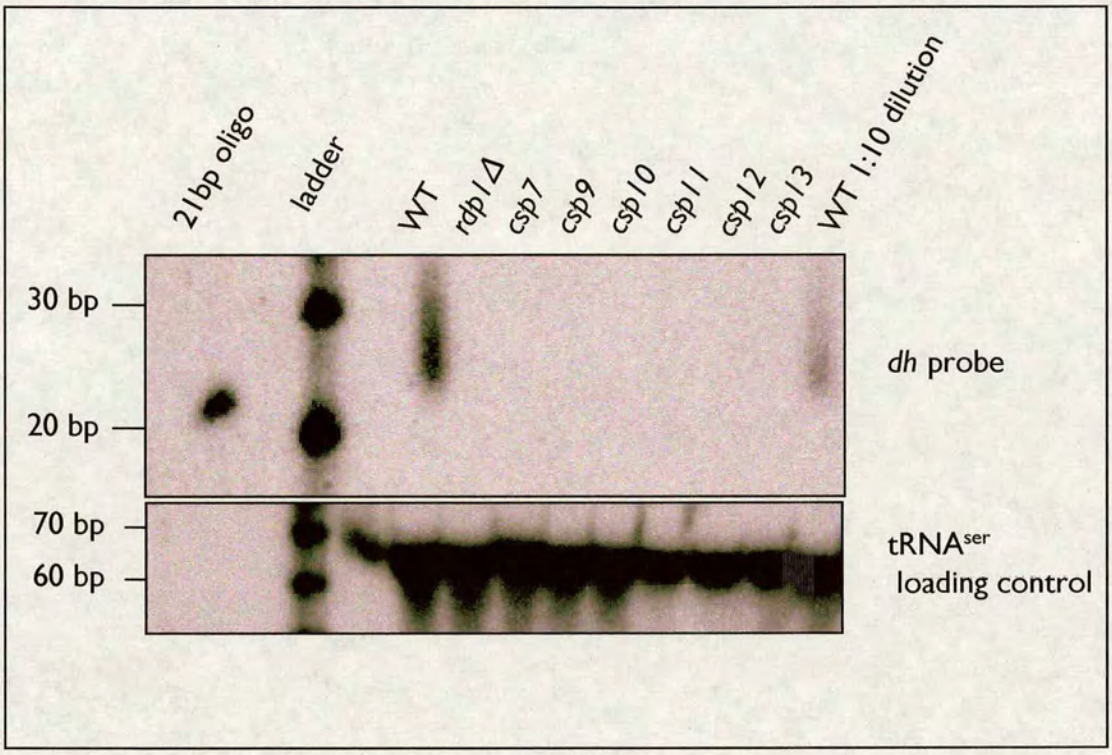


Figure 3.8. *csp* mutants fail to produce centromeric siRNAs. siRNAs originating from *dh* centromeric repeats accumulate in wild-type cells but not in RNAi mutants. *rdp1Δ* is included here for comparison. Centromeric siRNAs are undetectable in any of the *csp* mutants.

seen in a *dcr1Δ* mutant which is unable to produce any siRNAs so this hypothesis seems unlikely. Rather, it could be that the residual H3K9me2 and Swi6 binding are due to an RNAi-independent event.

In order to overcome some of the difficulties in determining where *csp* mutants may act to contribute to silent chromatin formation at centromeres, the following strategy was devised. Briefly, a double-stranded (ds) hairpin RNA was transformed into *csp* mutants to investigate; a) if they are capable of processing a dsRNA precursor into siRNAs and b) are these siRNAs able to target and silence a transgene, presumably via the RITS complex? To do this, a previously engineered GFP hairpin plasmid was utilised (Sigova et al., 2004). This construct contains the 760 bp ORF of GFP which was cloned as an inverted repeat separated by a 67 bp spacer region containing the first intron of the *rad9* gene which when spliced is thought to leave a loop of 14 nt between the GFP arms (Sigova et al., 2004). This GFP construct is transcribed from the *nmt1* promoter, one of the strongest in *S. pombe*. As this construct contained a uracil marker gene, the plasmid was modified to replace this uracil gene with leucine (gift from Femke Simmer) (Figure 3.9). In addition, a strain was constructed in which the GFP ORF was fused in frame to the *ura4*$^+$ gene at the arg3 locus, *arg3::ura4*$^+$*-GFP* (gift from Halim Boukaba) (Figure 3.9). This was done to allow investigation as to whether the GFP hairpin construct could, in the first instance target and silence the GFP transgene and secondly, whether this silencing could cause spreading of chromatin modifications upstream and trigger silencing of the *ura4*$^+$ marker gene (Figure 3.9).

Initially, the *csp* mutants were screened simply for their ability to process the ds GFP hairpin into siRNAs. *csp7, 9, 10* and *12* mutants are able to process the hairpin GFP RNA into siRNAs shown in Figure 3.10. However, *csp11* and *13* appear to be unable to produce siRNAs even when the source dsRNA is expressed at high levels. This demonstrates that Dcr1 is still capable of processing the hairpin RNA in some of the *csp* mutants. In *csp11* and *csp13*, no GFP siRNAs are evident which could imply that these genes are involved

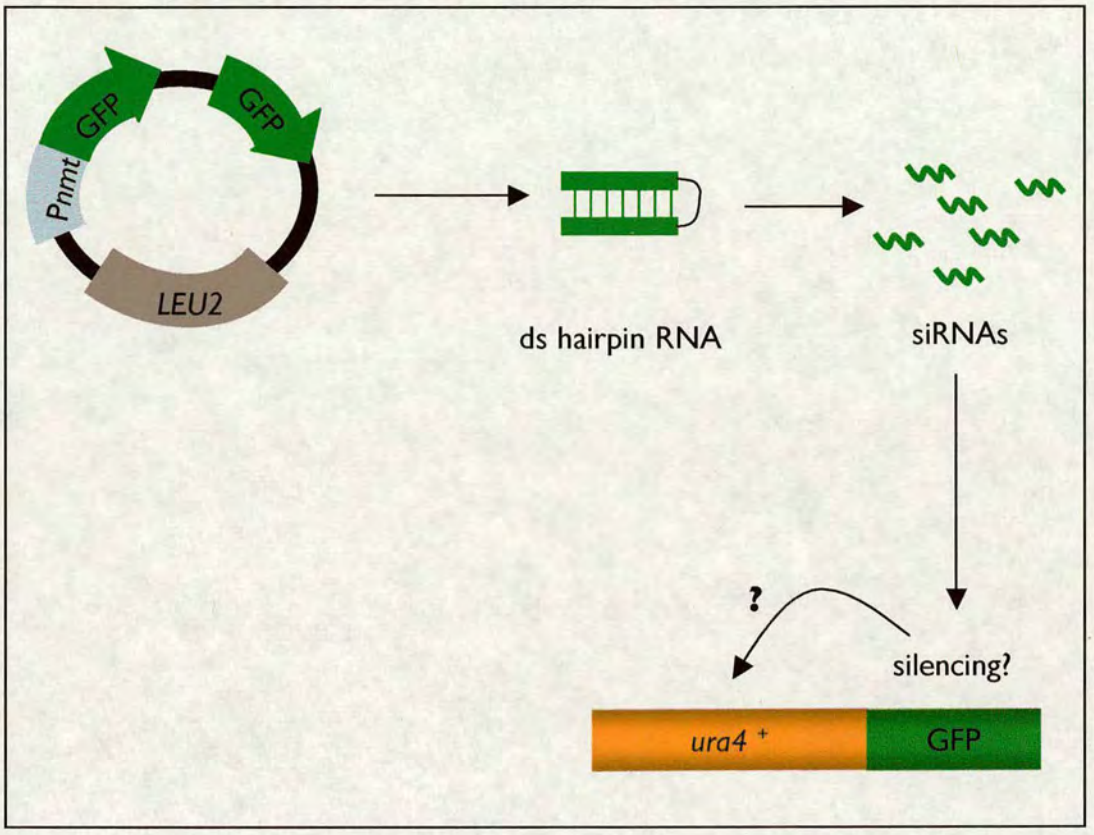


Figure 3.9. Can the *csp* mutants produce siRNAs from an exogenous source?
A double-stranded GFP silencing trigger is transcribed from the *nmt1* promoter as a hairpin RNA containing a 14 nt loop of *rad9* intron. This may be processed into siRNAs which effect silencing of a target marker gene. If silencing of the GFP transgene does occur, it seems feasible to assume that these siRNAs become incorporated into the RITS complex which could mediate heterochromatin formation across the transgene. Thus, it may be possible to mediate silencing of an upstream sequence, in this case the *ura4*$^{+}$ gene, through spreading of heterochromatin.

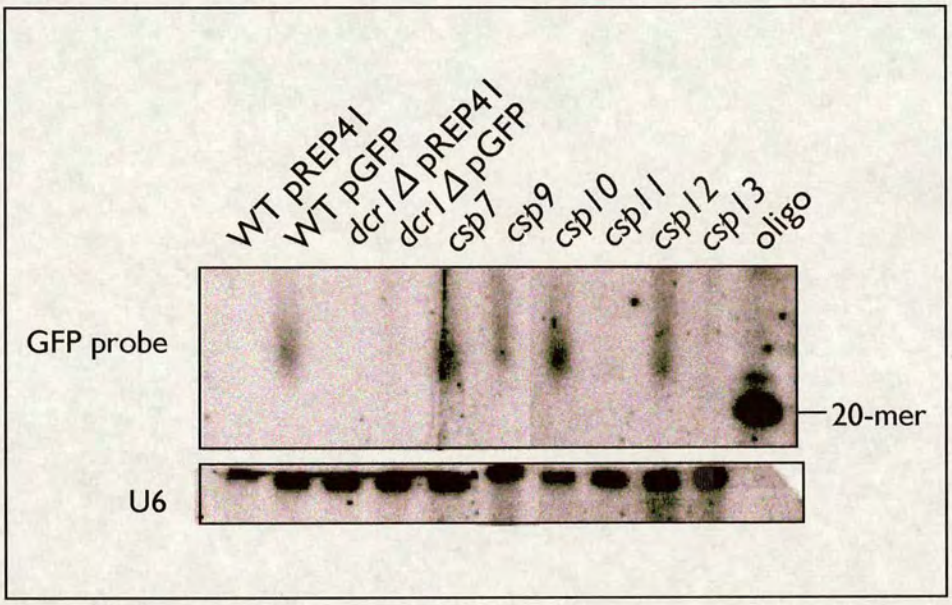


Figure 3.10. Production of siRNAs from a GFP hairpin in *csp* mutants.

A GFP hairpin was transformed into the *csp* mutants. WT and *dcr1Δ* were transformed with pREP41 empty plasmid as a control. In WT cells containing the GFP hairpin, GFP siRNAs are made. In a *dcr1Δ* control no siRNAs are seen, as expected. In the presence of the *ura4*:GFP target transcript *csp7, 9, 10* and *12* are able to process the GFP hairpin into siRNAs. *csp11* and *13* do not appear to produce any GFP siRNAs.

upstream of Dcr1 or may collaborate with Dcr1 to direct cleavage, and perhaps play a role in the transcription of the precursor RNA.

In order to assess whether these siRNAs can be targeted to induce silencing of the inserted *ura4*-GFP, the GFP hairpin plasmid was transformed into *csp* strains containing *arg3::ura4*$^{+}$*-GFP*. Levels of *ura4*-GFP transcription were analysed by northern blotting using several probes against both GFP and *ura4*$^{+}$ ORFs (not shown). These northerns were unsuccessful due to high background hybridisation levels. Previous serial dilution assays have demonstrated that plating assays are not sensitive enough to see any knockdown of the *ura4*$^{+}$-GFP transcript (Femke Simmer, pers. comm.). That is, plating wild type cells containing the *ura4*$^{+}$-GFP transgene and the GFP hairpin plasmid were still able to grow on plates lacking uracil and unable to grow on FOA. *dcr1*Δ cells which are unable to produce any siRNAs were used as a control and displayed the same phenotype as the wild type (Femke Simmer, pers. comm.). However, this lack of silencing in the wild type cells may be due to the fact that the GFP siRNAs are unable to spread effectively enough to induce strong silencing of the transgene.

The published studies using the GFP hairpin utilised FACS analysis to demonstrate that an *adh1*:GFP transgene is silenced via the GFP hairpin (Sigova et al., 2004). As northern analysis was unsuccessful, FACS analysis was attempted to examine whether the GFP siRNAs produced in the *csp* mutants were capable of inducing silencing of the *ura4*$^{+}$-GFP transgene. The data obtained was inconclusive as expression of the GFP hairpin RNA only resulted in a 20% knockdown of the GFP signal in wild type cells compared to *dcr1*Δ control cells. Another way to assess whether these siRNAs are actually functional would be to examine if the GFP siRNAs produced in the *csp* mutants become incorporated into the RITS complex and target chromatin modifications to the DNA. This remains to be tested.

Although these analyses did not determine whether the siRNAs produced are functional, it has provided some indication that *csp11* and *csp13* behave differently to the other *csp* mutants in that they appear to be unable to process the dsRNA hairpin into siRNA. This could mean that they are involved in a more upstream process such as transcription or processing of RNA prior to cleavage by Dcr1 or that they are co-factors which cooperate with Dcr1 to induce cleavage of dsRNA.

3.2.7 *csp* mutants are not sensitive to 6-azauracil

Since both *csp11* and *csp13* appear to be unable to process a dsRNA into siRNAs, it could be hypothesised that they may play an upstream role, perhaps during transcription or processing of RNA. A crude plating assay using 6-azauracil (6AU) was performed to test this. 6AU treatment causes the inhibition of transcriptional elongation by depleting intracellular pools of GTP and UTP (Ishiguro et al., 2000). This is not in itself lethal but when combined with mutations affecting transcription can block growth. The *csp* mutants were therefore tested for growth on 6AU to examine whether they had any defect in transcriptional elongation. *tfs1*Δ, a transcriptional elongation factor, was used as a control. As demonstrated in Figure 3.11, none of the *csp* mutants appeared to show any sensitivity to 6AU which indicates that they are unlikely to be involved in transcriptional elongation.

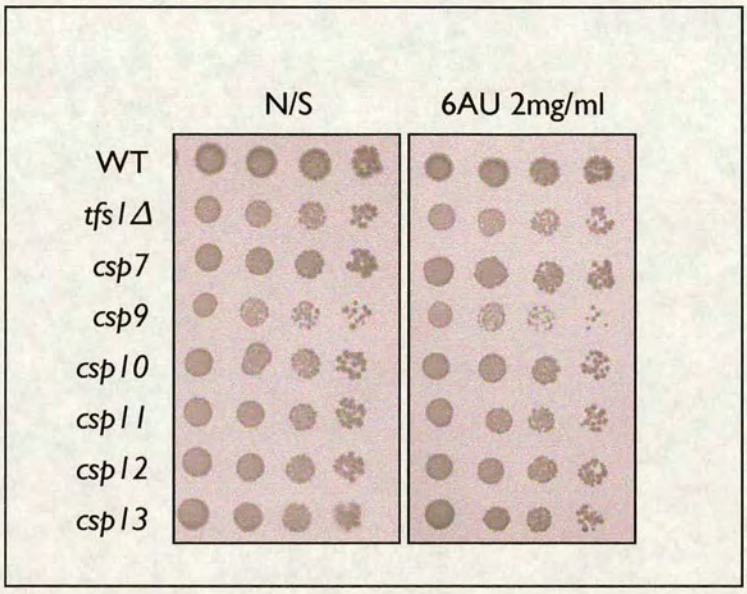


Figure 3.11. *csp* mutants are not involved in transcription.
None of the *csp* mutants show increased sensitivity to 6-azauracil. Although this is a crude assay, it appears unlikely that any of the *csps* are involved in transcriptional elongation.

3.3 Discussion

Taken together, the data described here clearly indicate a role for the *csp* proteins in RNAi-directed heterochromatin formation, at least at centromeres. A summary of the *csp* mutant phenotypes discussed here is shown in Table 3.1. However, due to the fact that removal of any component involved in RNAi-directed heterochromatin formation causes collapse of the entire pathway, interpretation of these results with regards as to where the mutant gene products may act is limited.

Like other RNAi mutants, all of the *csps* display an accumulation of centromeric non-coding RNA transcripts and a concomitant loss of siRNA production. One would expect that this loss of siRNA production would be accompanied by a loss of Swi6 and H3K9me2 across the centromeric outer repeats. However, like the observations of the RNAi mutants which retain small amounts of Swi6 and H3K9me2, this is not the case (Sadaie et al., 2004). In some instances, the *csp* mutants appear to retain at least some H3K9me2. Presumably this is enough to allow some Swi6 to bind but not enough to attract a high enough concentration of cohesin resulting in the aberrant chromosome segregation phenotypes observed. All of the *csp* mutants display high rates of chromosome loss and this demonstrates the instability of centromeres within these mutants (Ekwall et al., 1999). The *csps* also display chromosome segregation defects; chromosome missegregation and lagging chromosomes on late anaphase spindles (Ekwall et al., 1999). This points toward centromeric defects but could also point to defects in spindle formation and/or the spindle checkpoint (Cleveland et al., 2003). Indeed, these chromosome segregation phenotypes may occur due to the loss of centromere-microtubule interactions.

	Alleviation of silencing at *otr*	Swi6 localisation	H3K9me2 *otr*	Centromere transcripts	Centromeric siRNAs	GFP siRNAs
wt	×	√	√	×	√	√
clr4Δ	√	×	×	√	×	-
dcr1Δ	√	√	√	√	×	×
csp7	√	√	√	√	×	√
csp9	√	√	√	√	×	√
csp10	√	√	√	√	×	√
csp11	√	√	√	√	×	×
csp12	√	√	√	√	×	√
csp13	√	√	√	√	×	×

Table 3.1. A summary of the *csp* mutant phenotypes.
All of the *csp* mutants alleviate silencing of *otr1R(Sph1)ade6*$^{+}$ at the outer repeats. All of the mutants retain Swi6 localisation and some H3K9me2 at centromeres. The mutants all accumulate centromeric non-coding RNA transcripts and are unable to synthesise centromeric siRNAs. Only *csp11* and *csp13* are unable to process a dsRNA hairpin into siRNAs.

It may be that a small number of centromeric transcripts are processed or are aberrantly processed to allow the production of an undetectable amount of siRNAs. This could possibly be enough to target chromatin-modifying enzymes such as histone deacetylases and Clr4 to the centromere, allowing small patches of heterochromatin to form. This is consistent, at least in the case of *csp7, 9, 10* and *12,* with the data obtained using the GFP hairpin whereby these mutants clearly have the ability to process a dsRNA into siRNAs. Whether these siRNAs are able to direct chromatin-modifying activities to the target region remains to be investigated. On the other hand, residual H3K9me2 and Swi6 binding may be due to RNAi-independent recruitment of chromatin-modifying activities much like that observed at the mating-type locus and telomeres (Jia et al., 2004; Kanoh et al., 2005; Kim et al., 2004). At the mating-type locus, the ATF/CREB proteins Pcr1 and Atf1 are required for the maintenance of heterochromatin in the absence of RNAi components. Deletion of either of the genes encoding Atf1 or Pcr1, in combination with the deletion of RNAi components, causes heterochromatin formation to be completely abolished (Jia et al., 2004; Kim et al., 2004). This suggests that the two pathways act in parallel and that Atf1/Pcr1 act to retain specific factors such as Clr4 and hence Swi6 once they have been delivered to the locus by the RNAi machinery. This supports the idea that these DNA binding proteins act to maintain the silent state at the mating type locus in the absence of RNAi.

One could hypothesise that a similar parallel pathway may exist at centromeres. Indeed, three CENP-B homologs, which in humans bind α-satellite DNA, have been implicated in the formation of centromeric heterochromatin. These CENP-B homologs have been proposed to act to bind Swi6 or to attract chromatin-modifying activities to centromeres (Nakagawa et al., 2002). Moreover, Cid14, a poly(A) polymerase, has been shown to be involved centromeric heterochromatin gene silencing but it also acts at the mating-type locus (Buhler et al., 2007). The discovery of these

factors affecting centromeric heterochromatin offer tantalising insights as to alternative pathways involved in centromere structure/function.

The characterisation of the *csp* mutants has given clues as to their role in centromeric heterochromatin function. The *csps* were discovered using an *ade6*$^{+}$ marker gene insertion at the centromeric outer repeats which allowed their isolation on the basis of colour selection. However, due to the variegation of expression of this *ade6*$^{+}$ gene and the fact the *csp* mutant phenotype is not completely penetrant, it has not been possible to uncover the genes responsible for the phenotypes observed until recently. Had the identities of the *csps* cloned so far been uncovered sooner it would have provided a great insight into the connections between the RNAi pathway, general RNA metabolism, and centromeric heterochromatin in fission yeast. However, the *csp* mutants still provide some interesting possibilities for investigation as there are many questions remaining unanswered. Even so, the uncloned non-ts *csp* mutants provide us with some further insights into RNAi-directed heterochromatin assembly at fission yeast centromeres. The cloning and identification of the non-ts *csp* mutant genes is discussed in Chapter 4.

CHAPTER 4

Identifying defective genes in the *csp* mutants

4.1 Introduction

4.1.1 Mutations in the *csp* genes are unknown

The *csp* (*c*entromere: *s*uppressor of *p*osition effect) mutants were isolated as mutations which specifically alleviate the silencing of marker genes inserted into the heterochromatic repeats of centromeres (Ekwall et al., 1999). In Chapter 3, I described the examination of the *csp* mutant phenotypes with a view to providing some clues as to what the *csp*$^+$ genes may be doing and whereabouts in the pathway they contribute to heterochromatin formation at centromeres. However, the affected genes in the *csp* mutants are unknown. Since they have been generated by EMS mutagenesis they are predicted to be point mutants. It was essential to identify in which genes these mutations occur so as to fully elucidate their role in heterochromatic gene silencing and centromere function. Previous attempts to use existing yeast genomic libraries or genetic crosses to clone the *csps* were unsuccessful. In this chapter I will discuss the cloning and identification of the *csp* mutants using a newly available yeast genomic library.

4.2 Results

4.2.1 Cloning of the *csp* genes

The strategy adopted was to use a new high-copy yeast genomic library to clone the mutants by complementation (Figure 4.1). The library used contains *Sau3AI* fragments of fission yeast genomic DNA cloned into the multi-copy plasmid pAL-KS and is known colloquially as the 'Shimoda' library (Tanaka, 2000; Nakamura et al., 2001). It contains approximately 60,000 independent clones with an average insert size of 8kb.

The *csp* strains used for library screening contained the *otr1R(SphI)ade6*$^+$ insertion within *cen1*. Initial screening was performed by transformation of library DNA via electroporation but this was found to produce a high level of background red and pink colonies even using only an empty plasmid

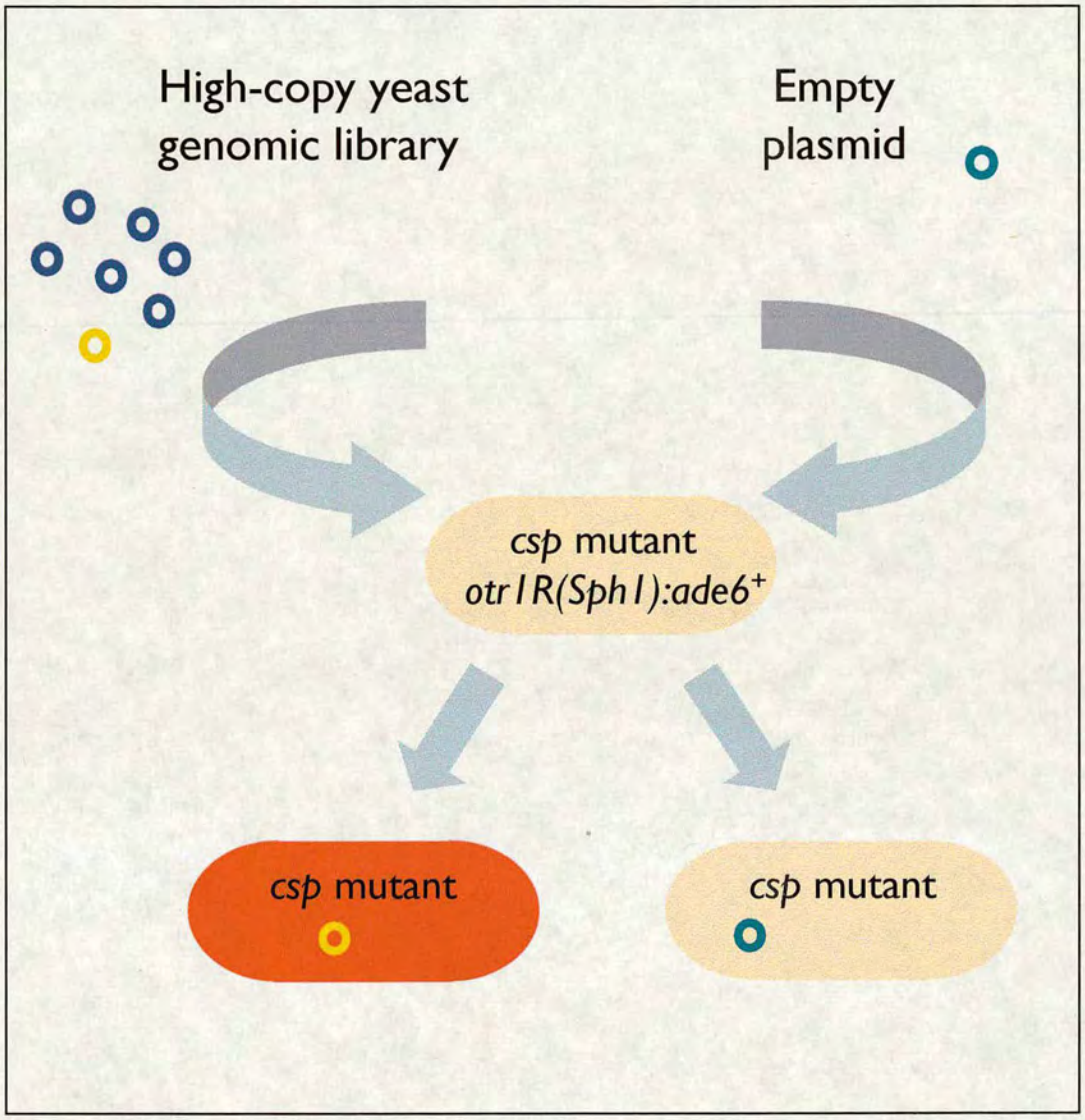


Figure 4.1. Strategy for cloning the *csp*$^+$ genes.
An overview of the screening strategy used to clone the *csp*$^+$ genes. *csp* mutants containing *otr1R(Sph1):ade6*$^+$ at centromere 1 were transformed with a yeast high-copy genomic library. In parallel, mutants were transformed with empty plasmid in order to determine the level of background false positives. Red colonies which display restoration of centromere silencing were selected and subjected to further analysis.

(pAL-KS or pLEU2) as a control. This indicated that the process of electroporation itself somehow affects the *csp* mutants and causes them to variegate.

Electroporation and lithium acetate are the most commonly used methods to transform *S. pombe*. Electroporation has the advantage of being very fast with high transformation efficiency but the results are not always reproducible. Lithium acetate is comparatively slower but has a higher transformation efficiency than electroporation (Nurse). Therefore, the *csp* mutants were transformed using a standard lithium acetate protocol and screened for red colonies in which centromeric silencing was restored. This method resulted in an acceptable background level of red and pink false positive colonies upon transformation of the empty pAL-KS plasmid of approximately 1-5 in 10,000 colonies depending on the mutant. A schematic representation of the pAL-KS plasmid is shown in Figure 4.2. This library was chosen over other available resources for several reasons. The plasmid contains an autonomous replication sequence (*ars1*) which is more mitotically stable than those based on the *S. cerevisiae* 2μM origin. The pAL-KS plasmid is also less prone to rearrangements and has a higher copy number than those based on 2μM (Nurse). In addition, the 'Shimoda' library has a large insert size which gives better coverage of the genome than other available libraries.

The complementing plasmids were rescued from *S. pombe* and digested with *SalI* and *NotI* restriction enzymes to check for inserts. Repression of centromere silencing was verified by re-transforming the plasmid back into the *csp* mutant to ensure that complementation was reproducible. Inserts were identified by sequencing using vector-specific primers directly adjacent to the insert. Once restoration of silencing was confirmed and the inserts were identified, the mutation itself was identified by sequencing candidate ORFs in the *csp* mutant background, usually in more than one strain (Figure 4.3).

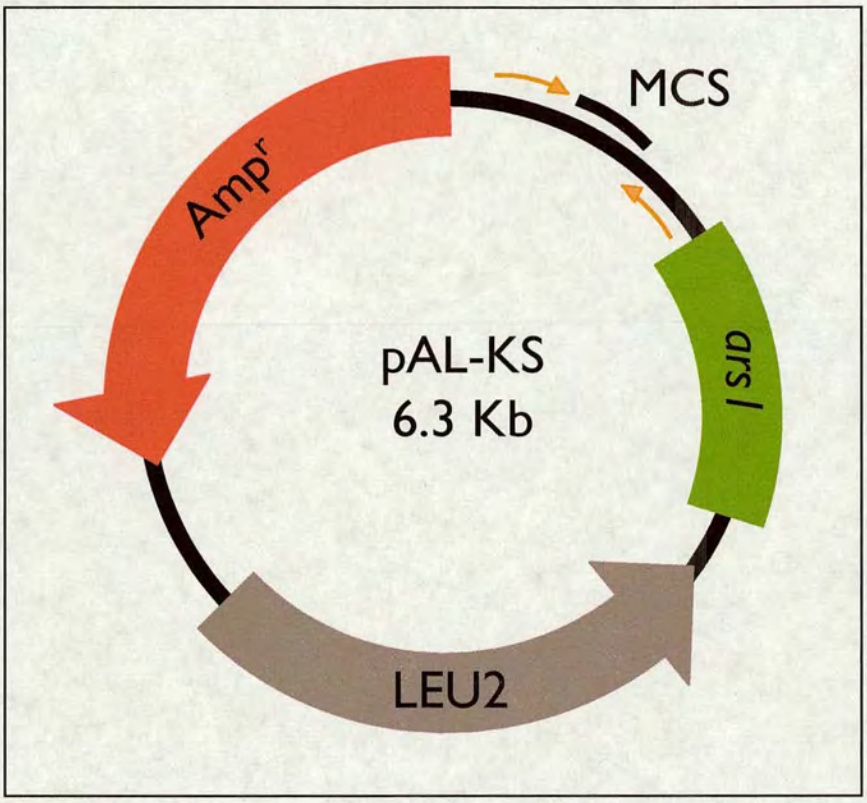


Figure 4.2. Plasmid map of pAL-KS vector.
The pAL-KS plasmid is 6.3 Kb in size and contains an ampicillin resistance gene for selection in *E.coli* and a LEU2 marker gene for selecting yeast transformants. It has an autonomous replication sequence, *ars1*$^+$, which allows a high transformation efficiency and low copy number. The multiple cloning site, MCS, is as follows; *ApaI, XhoI,* ***SalI****, HindIII, PstI, SmaI, BamHI, SpeI,* ***NotI****, SacIII and SacI.* The *SalI* and *NotI* sites are shown in bold as these are the sites immediately flanking the inserts. Orange arrows denote the position of the vector-specific oligos used to PCR and sequence the inserts.

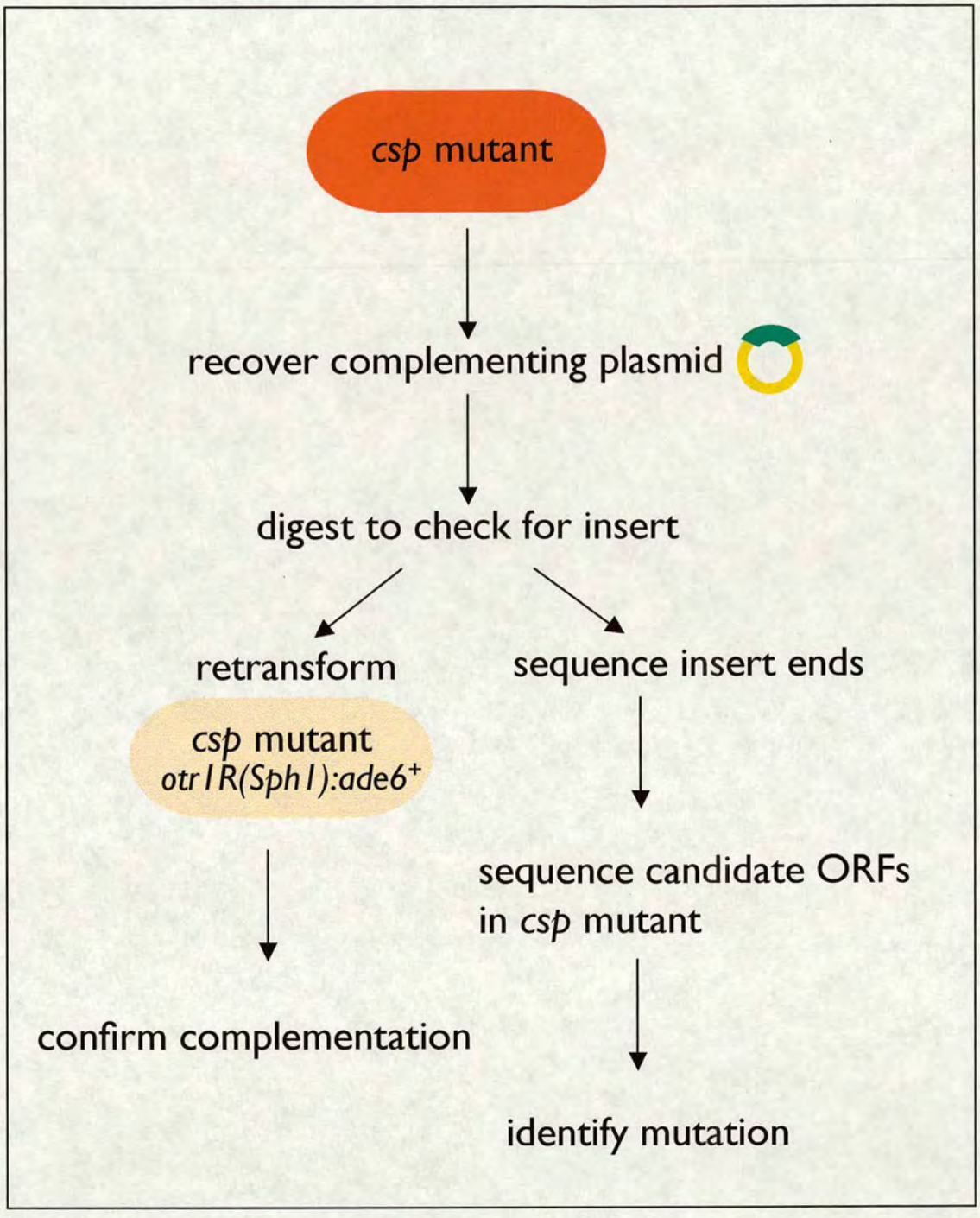


Figure 4.3. Strategy to identify the *csp*$^+$ genes and mutations.
Plasmids were recovered from complemented *csp* colonies by growing for two days in selective media, plasmid DNA recovered and transformed into *E. coli* to amplify. Plasmids were then recovered from *E. coli* and digested to check for the presence of an insert. The plasmid was re-transformed into *S. pombe* to confirm complementation and the ends of the insert were identified by sequencing with vector-specific primers. Candidate ORFs were sequenced in the *csp* mutant to identify the mutation.

4.2.2 *csp7* is an allele of *rdp1*,$^+$ an essential component of the RNAi pathway

Identification of the affected genes in the *csp7* to *13* mutants was undertaken using the 'Shimoda' library as described above. The original transformations were carried out by electroporation but this was unsatisfactory as a high number of false positives were obtained. The initial strategy to overcome this was to cross the *csp* mutants containing *otr1(SphI):ade6* with a strain containing another marker gene in *cen1*, *imr1L(NcoI):ura4*, in order to make a secondary evaluation of the red colonies initially selected. The *ura4*$^+$ insertion is at the opposite side of *cen1*, 15 kb away from *ade6*$^+$ as seen in Figure 4.4. In wild type strains, colonies repressing this *ura4*$^+$ gene can be identified by counter-selection on 5-FOA. This strategy worked well for *csp7* but not for the other *csp* mutants. By transforming a *csp7* strain containing two marker genes with the genomic library, complemented red colonies which are 5-FOA resistant were selected. Thus, plasmids which restore silencing of both centromeric marker genes were isolated.

Two plasmids were recovered using this approach. When these were digested with restriction enzymes *SalI* and *NotI* they were found to have identically sized genomic inserts of 8.7 kb (Figure 4.5a). However, as the insert contains a *SalI* site at 2110 bp and the empty plasmid is 6.3 kb, after digestion two bands of around 6 kb and 2 kb are observed on a gel (Figure 4.5a). Sequencing and subsequent BLAST searches revealed that both of the plasmids contain part of *S. pombe* chromosome 1 cosmid SPAC6F12. This encompasses 336 bp of the gene encoding Tom20, a mitochondrial outer membrane translocase protein, the whole ORF of a hypothetical coiled-coil containing protein, the whole ORF of Rdp1, the RNA-dependant RNA polymerase and approximately 1 kb of the gene encoding Ade3, predicted to be involved in purine biosynthesis. Transformation of the individual plasmids back into the *csp7* mutant produced a much higher frequency (around 70-80%) of red colonies than the original screen (0.03%) or empty plasmid (Figure 4.5b). This confirmed that the insert contained in these

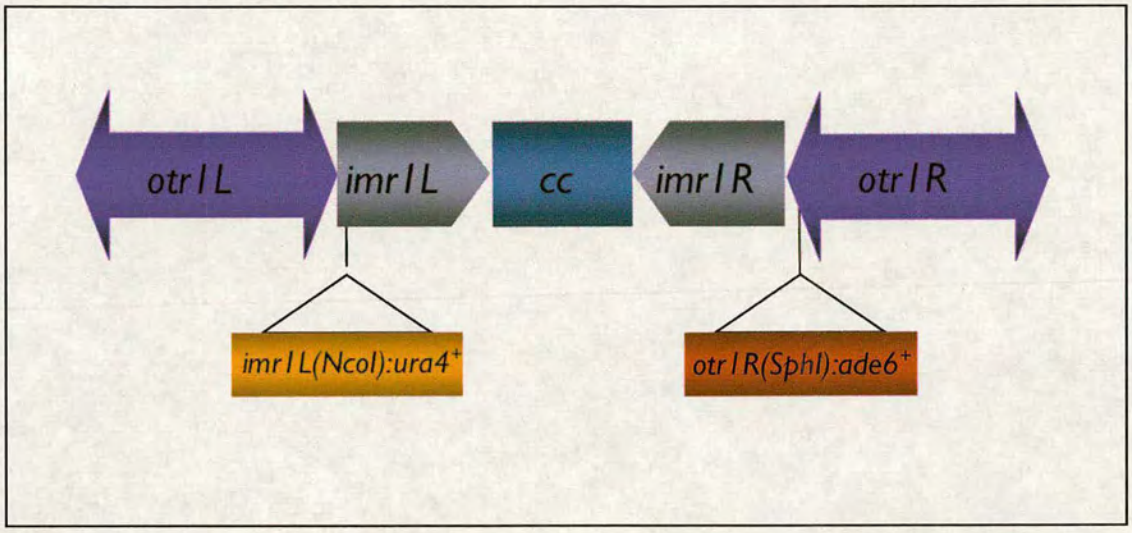


Figure 4.4. *csp* mutants containing two centromeric marker gene insertions.

Each of the *csp* mutants containing the *otr1R(SphI):ade6*$^+$ marker gene insertion was crossed to a strain containing the *imr1L(NcoI):ura4*$^+$ marker gene 15 kb away to have double selection for complementation of the loss of silencing by plasmids from the genomic library.

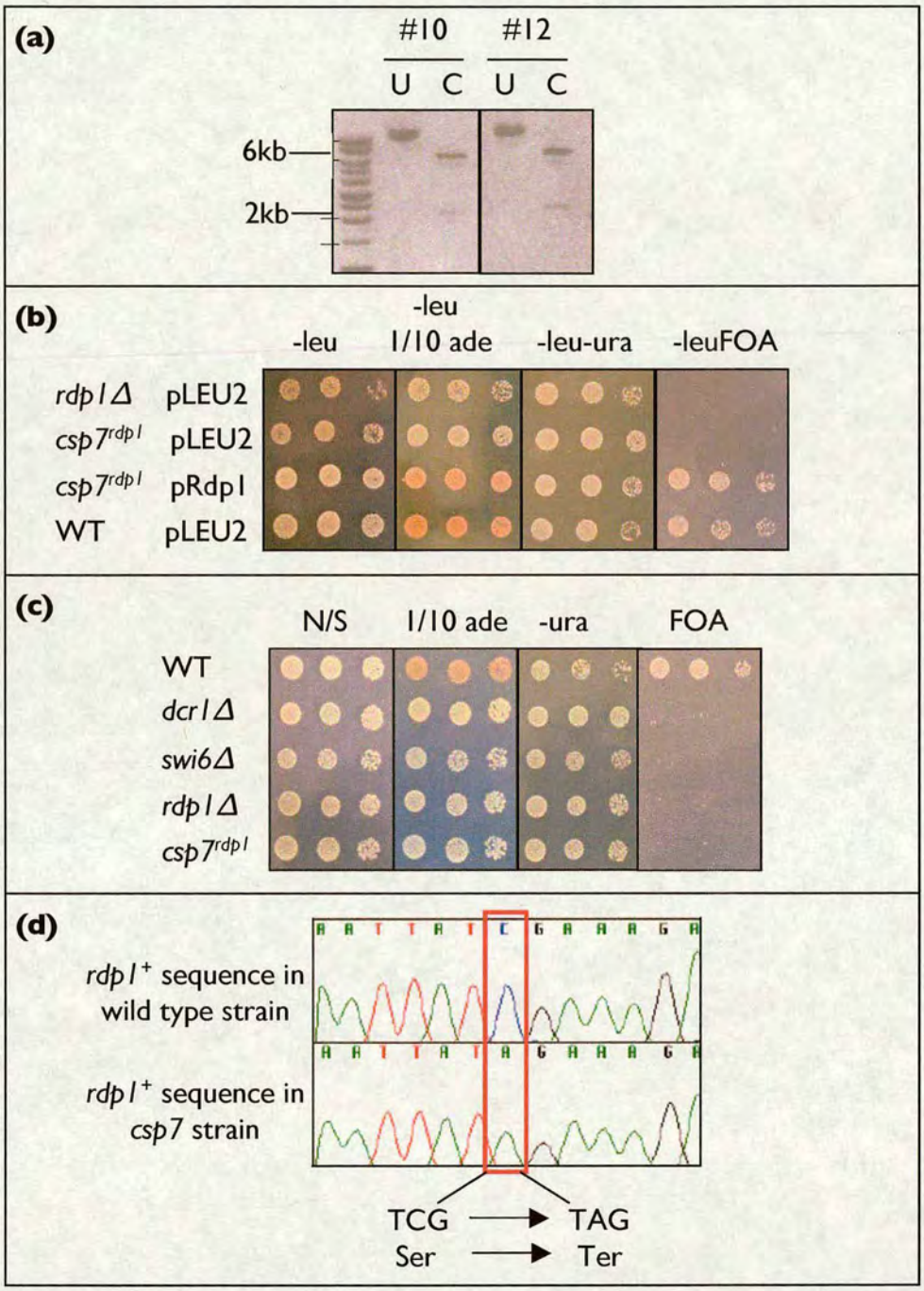


Figure 4.5. Cloning and initial characterisation of *csp7*$^{+}$.
(a) Two independently isolated plasmids (numbered 10 and 12) complement the *csp7* silencing defect. U; uncut plasmid, C; digested with *SalI* and *NotI* restriction endonucleases to release the plasmid insert. **(b)** Sequencing the ends of the inserts using vector-specific primers revealed that the plasmids contained the full ORF of *rdp1*$^{+}$. pRdp1 (10 or 12) complemented the *csp7* defect in centromere silencing. **(c)** *csp7* and *rdp1Δ* display the same centromeric silencing defect. **(d)** Sequence analysis confirms that *csp7* is an allele of Rdp1.

plasmids complements the *csp7* defect in silencing. The plasmids were named pRdp1. The complementation of *csp7* by pRdp1 is variable as *csp7* colonies containing the pRdp1 plasmid do not return to a wild type red colour but are slightly paler in colour and in fact show a range from pale pink through to almost red. This could be explained by the fact that the ORFs contained in the plasmid are overexpressed and that expression levels vary in each colony.

Surprisingly, the pRdp1 plasmids are not able to complement the defective silencing of an *rdp1*Δ mutant (Figure 4.6). It was hypothesised that overexpression of *rdp1*$^+$ had some detrimental effect to the cells and interfered in some way with centromeric silencing so the following approach was used. Linearised pRdp1 plasmid was transformed into the *rdp1*Δ strain in the hope that homologous recombination of a single copy of the plasmid would complement the defective silencing. However, complementation was not reproducibly observed (not shown). It is conceivable that the *rdp1*Δ strain contains a mutation in another gene and this somehow affects centromere silencing or the *ade6+* pathway and thus complementation cannot be observed. pRdp1 was also transformed into *ago1*Δ and *dcr1*Δ strains but again no complementation was observed (Figure 4.6).

It is likely that suppression of the *csp7* phenotype is mediated by Rdp1 as Rdp1 has previously been shown to be required for transcriptional silencing at the centromere (Volpe et al, 2002; Volpe et al., 2003). Consistent with this, the centromeric silencing defects displayed by *rdp1*Δ mutants and *csp7* are similar therefore the *rdp1*$^+$ ORF was sequenced from DNA amplified from *csp7* cells (Figure 4.5c). This demonstrated that *csp7* is an allele of *rdp1*$^+$ since it contains a point mutation at nucleotide 1232 of the ORF which creates a premature STOP codon at residue 411 (serine 411 STOP), just upstream of the polymerase domain (Figure 4.5d and Figure 4.7a).

Normally, allelism is tested by genetic crossing whereby the unknown

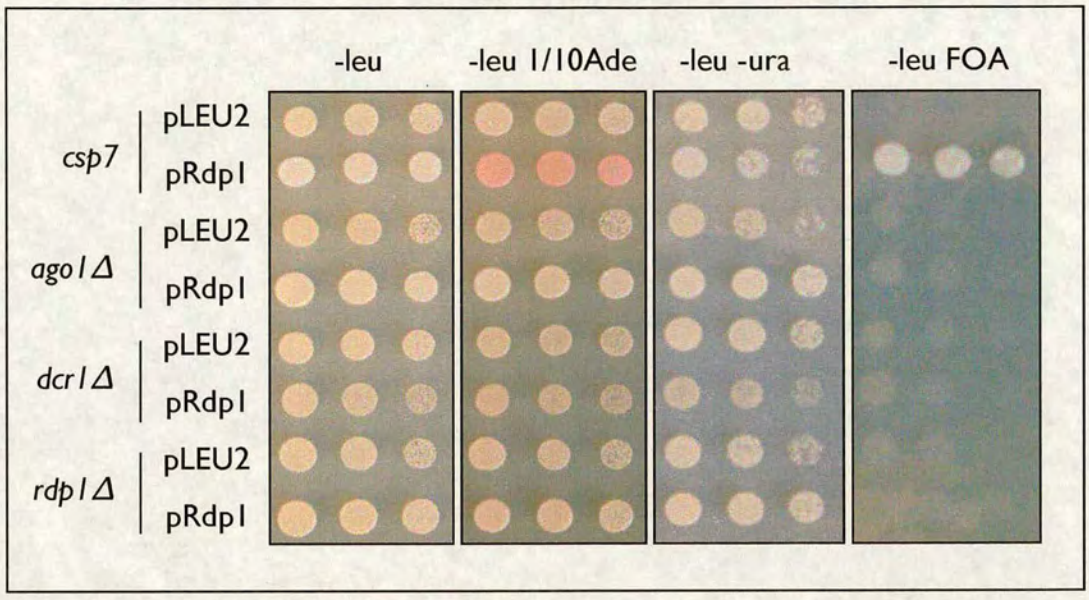


Figure 4.6. pRdp1 does not complement RNAi mutants.
Overexpression of pRdp1 in an *rdp1Δ* mutant does not rescue the silencing defect of this mutant. The silencing defect of *ago1Δ* or *dcr1Δ* are not affected by pRdp1 expression.

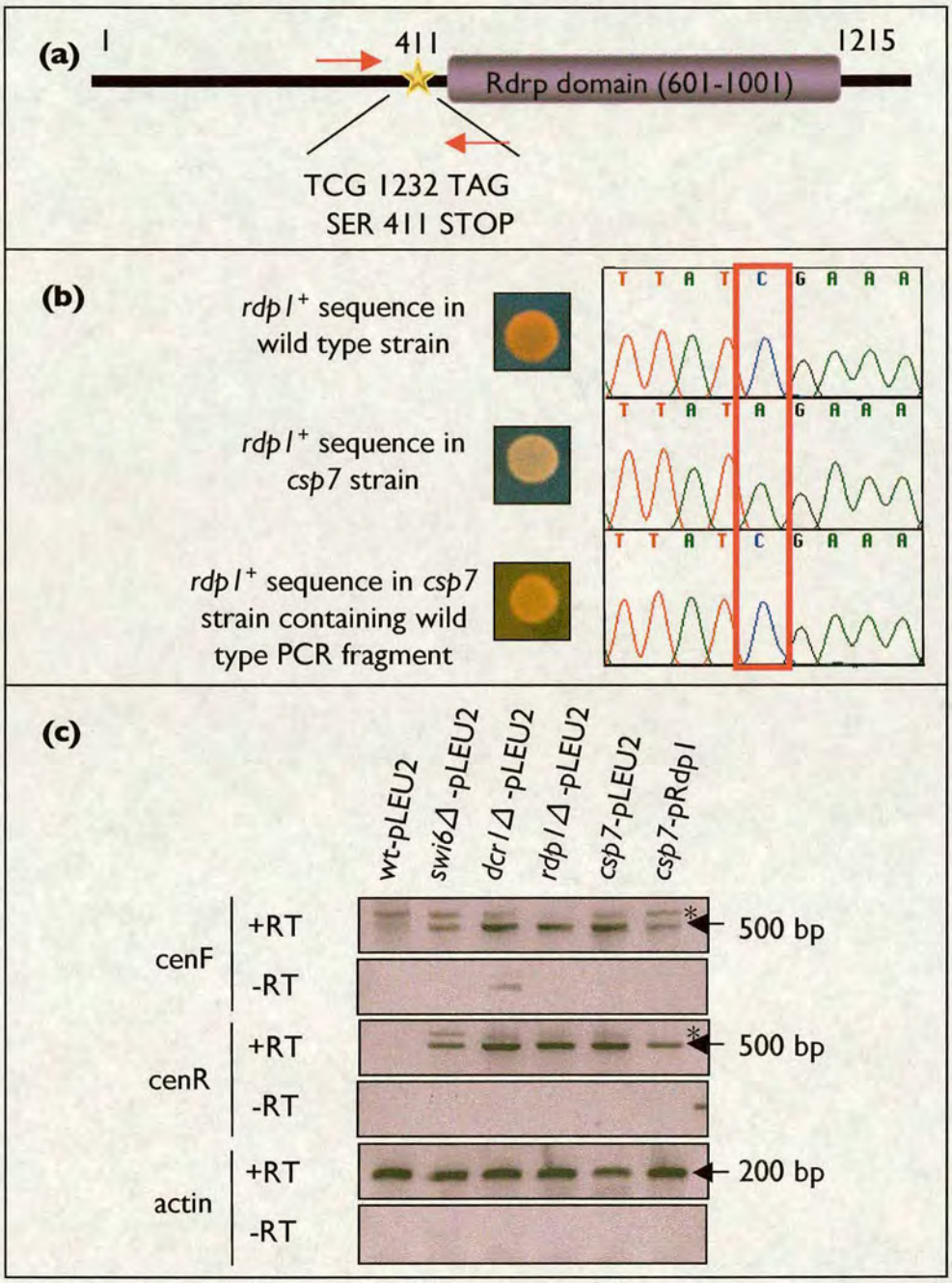


Figure 4.7. *csp7*$^+$ is an allele of Rdp1.

(a) *csp7* contains a premature STOP codon indicated by a star just upstream of the polymerase domain. The red arrows indicate the position of primers used to make the PCR fragment referred to in (b). **(b)** A 300bp wild type PCR fragment of Rdp1 complements the *csp7* phenotype. *csp7* colonies containing the PCR fragment re-establish silencing of the *ade6*$^+$ gene. **(c)** Strand-specific RT-PCR analysis shows that *csp7* is only partially complemented by pRdp1. RNAi mutants which alleviate centromere silencing accumulate non-coding RNA transcripts originating from both the sense and anti-sense strands of the centromere, as does *csp7*. Expression of Rdp1 in *csp7* does lessen this accumulation but not to wild type levels. Arrows indicate specific products, asterisks denote non-specific products.

mutant is mated with a known knockout and should therefore produce only mutant progeny if the two mutations are indeed in the same ORF. However, as the *csp7* strain containing *otr1(SphI):ade6*$^{+}$ mated to itself produces variable colony colour this approach was unreliable. To confirm that *rdp1*$^{+}$ and *csp7* were indeed allelic, a PCR fragment of wild type *rdp1*$^{+}$ was transformed into *csp7* containing *otr1(SphI):ade6* to replace the mutation by homologous recombination. Colonies that had taken up the PCR fragment were shown to have restored silencing of the marker gene and were red in colour (Figure 4.7b).

Non-coding RNA transcripts originating from the centromeric outer repeats accumulate in fission yeast RNAi mutants such as *rdp1*Δ, *ago1*Δ and *dcr1*Δ but are constantly turned over in wild type cells (Volpe et al, 2002; Volpe et al., 2003). Only upper transcripts accumulate in a *swi6*Δ mutant. Strand-specific RT-PCR analysis of centromeric transcripts demonstrates that both strands accumulate in an *rdp1*Δ mutant but are barely detectable in a wild type strain (Figure 4.7c). *csp7* cells containing the pRdp1 plasmid display only partial reduction in the accumulation of centromeric transcripts compared to an empty plasmid control, further demonstrating that the complementation of *csp7* by pRdp1 is not fully penetrant (Figure 4.7c). Again, this could be due to a variation in Rdp1 expression levels in different colonies and may indicate that overexpression of a single component of the pathway may alter or interfere with silencing by altering the balance of factors involved.

At the time of discovering that *csp7* and *rdp1*$^{+}$ were allelic, little information was available about Rdp1. The proposed strategy was to use a tandem-affinity purification (TAP) tagging procedure with mass spectrometry to identify factors interacting with both full-length Rdp1 and the truncated *csp7*^{rdp1} protein. Full-length and truncated Rdp1 were TAP-tagged using a modified Bahler cassette containing a noursethrecin (cloNAT) marker gene (a gift from Stuart McNeill) (Sato et al., 2005). Tagged strains were checked for functionality using centromere silencing and TBZ sensitivity assays. The

full-length Rdp1 behaved as wild type with respect to centromere silencing and TBZ sensitivity and the truncated *csp7*^{rdp1} protein behaved like the untagged *csp7* mutant indicating that the tagged proteins were functional (Figure 4.8). Tagged strains were also verified by Southern blotting (not shown). At this point I planned to further characterise Rdp1 and associated proteins to dissect their function. Unfortunately, shortly after this time Moazed *et al* published the biochemical purification and characterisation of the RNA-Directed RNA polymerase complex (RDRC) (Motamedi et al., 2004). Rdp1 is an RNA-directed RNA polymerase thought to be responsible for the amplification of double stranded RNA (dsRNA), and thus siRNAs, in the RNAi pathway (Motamedi et al., 2004; Sugiyama et al., 2005; Volpe et al., 2003). It has been shown to associate with the putative poly(A) polymerase Cid12, and the putative helicase Hrr1 in the RDRC (Motamedi et al., 2004). The RDRC interacts with the RITS complex in a Dcr1-dependant manner.

It has been demonstrated that Rdp1 can act as an RNA-dependent RNA polymerase (Motamedi et al., 2004; Sugiyama et al., 2005). A point mutation within the polymerase domain which destroys this activity renders Rdp1 defective in centromere silencing and unable to associate with its binding partners in a complex. Since the mutation in *csp7* truncates the entire polymerase domain it would seem reasonable to assume that even if any protein were produced it would result in a similar phenotype which is indeed the case.

4.2.3 *csp9*$^{+}$ is an allele of *ago1*$^{+}$, an essential RNAi component

Genetic analysis had previously revealed that *csp9* was closely linked to the gene encoding Argonaute1 (Ago1) (pers. comm. R.Allshire and W. Richardson). Ago1 is a component of the RITS complex and is required for heterochromatin formation at centromeres (Noma et al., 2004; Verdel et al., 2004). It contains a PAZ domain thought to be required for RNA binding and a PIWI domain which is required to cleave or 'slice' RNA (Figure 4.9a) (Irvine et al., 2006).

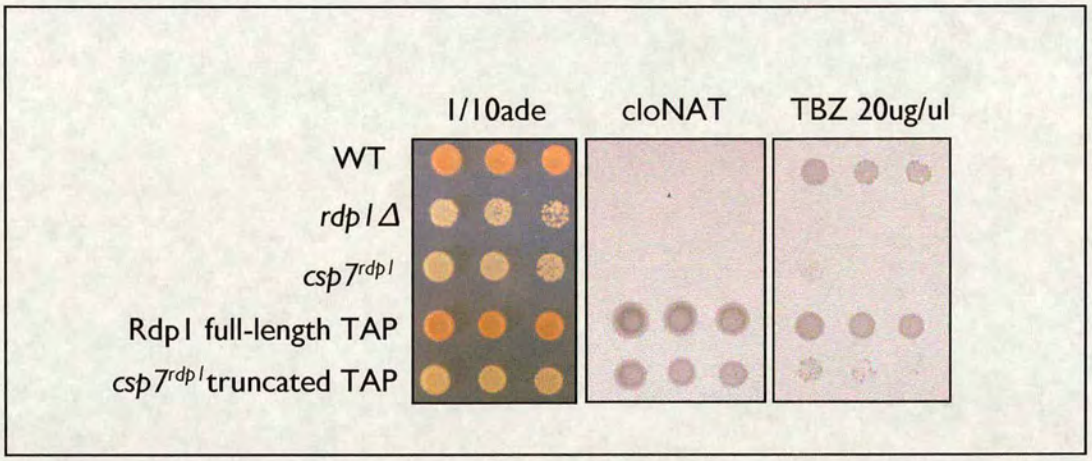


Figure 4.8. TAP tagging of Rdp1.
Strains which exhibit intact centromere silencing are red and show resistance to TBZ. Full-length TAP tagged Rdp1 does not alleviate centromere silencing and is resistant to TBZ, indicating that the tagged protein is functional. Tagged truncated protein behaves as a *csp7* mutant displaying alleviation of centromere silencing and sensitivity to TBZ.

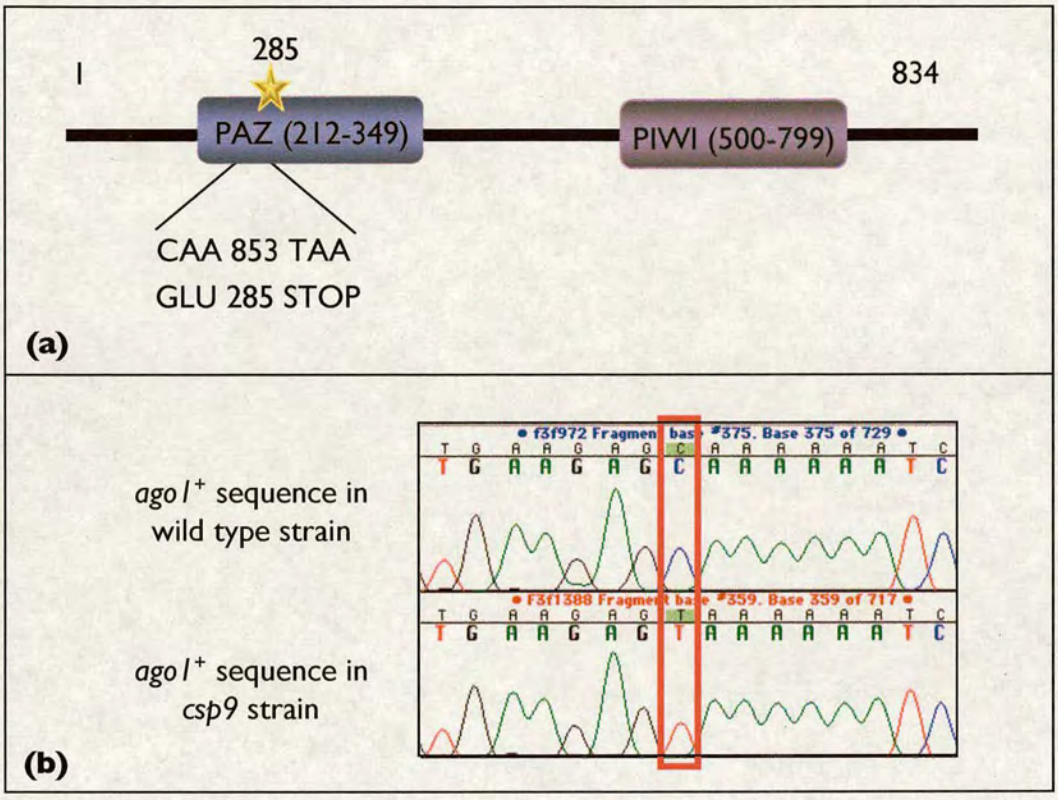


Figure 4.9. Identification of *csp9*$^{+}$.
(a) Previous genetic analysis revealed that *csp9* was closely linked to *ago1*$^{+}$, which contains a PAZ and a PIWI domain (pers. comm R. Allshire and W. Richardson). **(b)** Sequence analysis confirms that *csp9* contains a premature STOP codon indicated in (a) by a star almost halfway through the PAZ domain.

It seemed likely that *csp9* could be an allele of Ago1. The *ago1*$^+$ ORF was amplified from DNA extracted from *csp9* cells and sequence analysis of *ago1*$^+$ was carried out. Simultaneously, the same library screen used to identify *csp7* was carried out to identify plasmids complementing *csp9*. The library screen was unsuccessful in recovering any complementing plasmids. However, sequence analysis confirmed that *csp9* is an allele of *ago1*$^+$. *csp9* contains a C to T mutation at nucleotide 853 which causes a glutamine to STOP mutation of codon 285, truncating the protein approximately halfway through the PAZ domain (Figure 4.9b).

4.2.4 *csp10*$^+$ is an allele of *cid12*$^+$, a component of the RDRC, which associates with *rdp1*$^+$

The *csp10*$^+$ gene was isolated in a similar manner to *csp7*$^+$. The *csp10* strain containing *otr1R(Sph1):ade6* was transformed with the Shimoda library and 3 red colonies were recovered from around 10,000 transformants. One of the plasmids had no insert when recovered from *S. pombe* however, the remaining two were found to have inserts of 6 kb and 3 kb (Figure 4.10a). When these individual plasmids were re-transformed into the original *csp10* strain containing *otr1R(Sph1):ade6*$^+$ they consistently complemented the defect in centromere silencing (Figure 3.10b). Both plasmids contained sequence from the same *S. pombe* chromosome 3 cosmid SPCC663. The 3 kb insert was found to contain part of an ORF of a sequence orphan SPCC663.11, the ORF of the gene encoding Cid12 and the ORF of a gene encoding an N-acetyltransferase. The 6 kb plasmid also contains the sequence orphan SPCC663.11, Cid12, the N-acetyltransferase, a hypothetical protein SPCC663.13, and another sequence orphan, SPCC663.14. As Cid12 associates with Rdp1 in the RDRC it seemed like the most obvious ORF to complement the *csp10* phenotypes. To test this, the *cid12*$^+$ ORF was amplified from DNA extracted from *csp10* and sequenced. Sequence analysis confirmed that *csp10* and *cid12*$^+$ are indeed allelic. *csp10* contains a C to T mutation at nucleotide 256 which results in an arginine to STOP mutation in codon 86 (Figure 4.10c and d) This truncates the protein approximately a third of the way into the putative nuceotidyltransferase domain. This

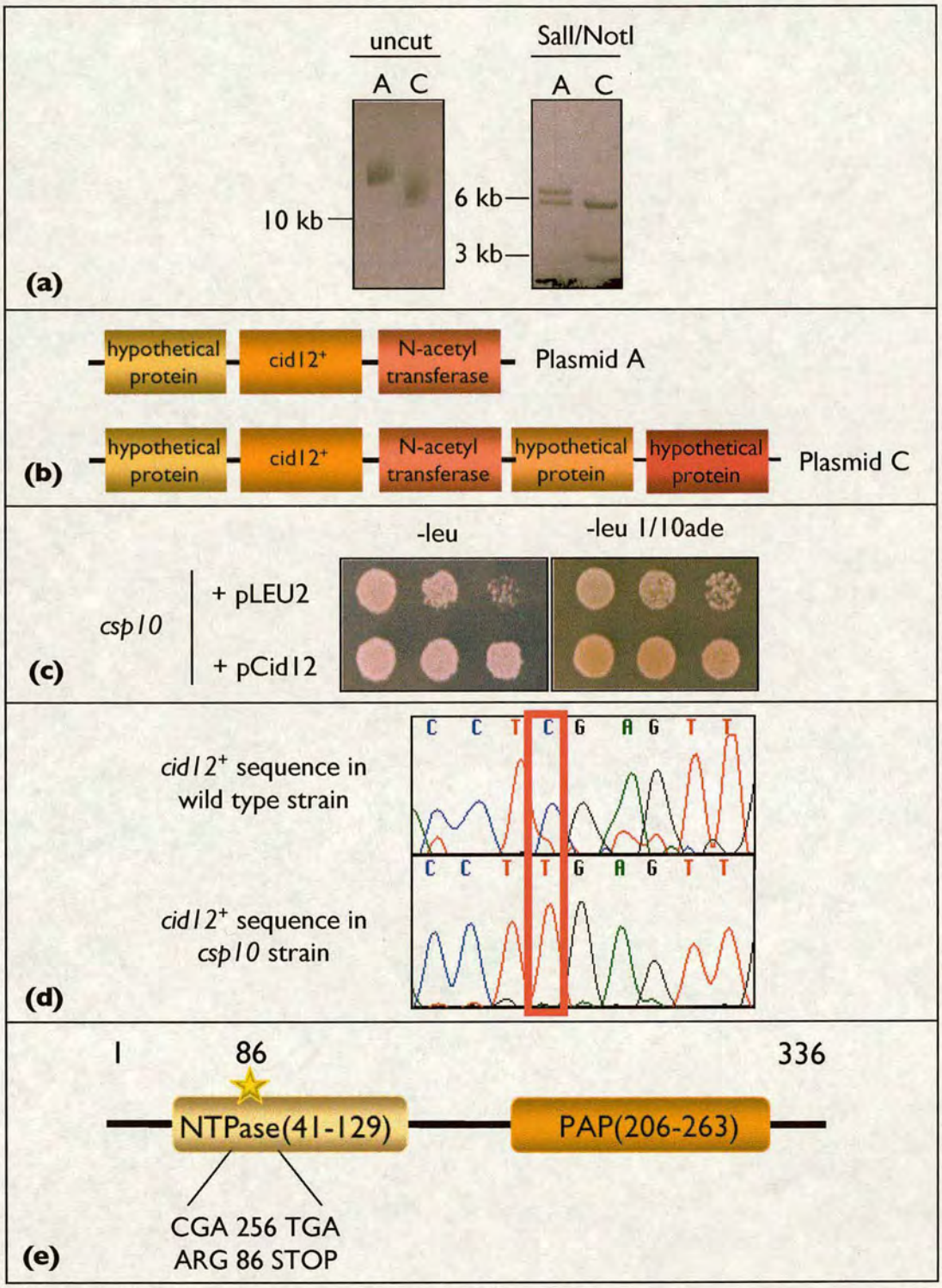


Figure 4.10. Identification of *csp10*$^+$.

(a) Two independently isolated plasmids (A and C) were digested and found to have distinct inserts. **(b)** Schematic representation of plasmids A and C showing ORFs. **(c)** Both of the plasmids complement the *csp10* silencing defect, only C is shown here. **(d)** Sequence analysis demonstrates that *csp10*$^+$ and *cid12*$^+$ are allelic. *csp10* contains an arginine to STOP mutation in codon 86. **(e)** The mutation in *csp10* causes a premature STOP codon approximately a third of the way into the nucleotidyltranferase domain, indicated by a star.

domain is characterised by the sequence *h*G[G/S]X9–13D*h*[D/E]*h* (where X = any amino acid and *h* = hydrophobic residue) which is proposed to use the three aspartic acid residues to coordinate a divalent metal ion in the catalytic site (Iyer et al., 2003). The STOP codon causes the truncation of the last of these aspartic acid residues. The phenotypes of *csp10* and analysis of the Cid12 protein will be discussed in greater detail in Chapters 5 and 6.

4.2.5 *csp11* is unknown

csp11 was transformed with the Shimoda library and positive colonies were selected. Three separate transformations yielded a total of 10 positive colonies, of which two plasmids could not be rescued into *E. coli*. The remaining 8 plasmids were rescued and digested to ensure they contained inserts. However, upon re-transformation into a *csp11* strain, none of these plasmids restored silencing. This may be due to variegation of the *csp11* strain as an effect of transformation. As yet, *csp11* remains unidentified.

4.2.6 *csp12* is an allele of *arb1*$^+$ which associates with *ago1*$^+$

csp12$^+$ was transformed with the Shimoda library and positive colonies were selected. Two plasmids were isolated but on re-transformation only one containing a 6 kb insert was found to complement *csp12* (Figure 4.11a). This plasmid was found to contain most of the ORF of the gene encoding Gar2, a protein involved in nucleolar structure and function, the complete ORFs of genes encoding two hypothetical proteins, SPAC140.03 and SPAC140.04 and part of a serine/threonine kinase.

Interestingly, the same plasmid was isolated in another screen to identify factors required for outer repeat and central core silencing. Four alleles of *cos2* were found in a screen to identify factors involved in central core silencing and outer repeat silencing. The pGar2 plasmid was found only to complement the *cos2* defect in outer repeat silencing but had no effect on central core silencing. pGar2 also complements the TBZ sensitivity of the

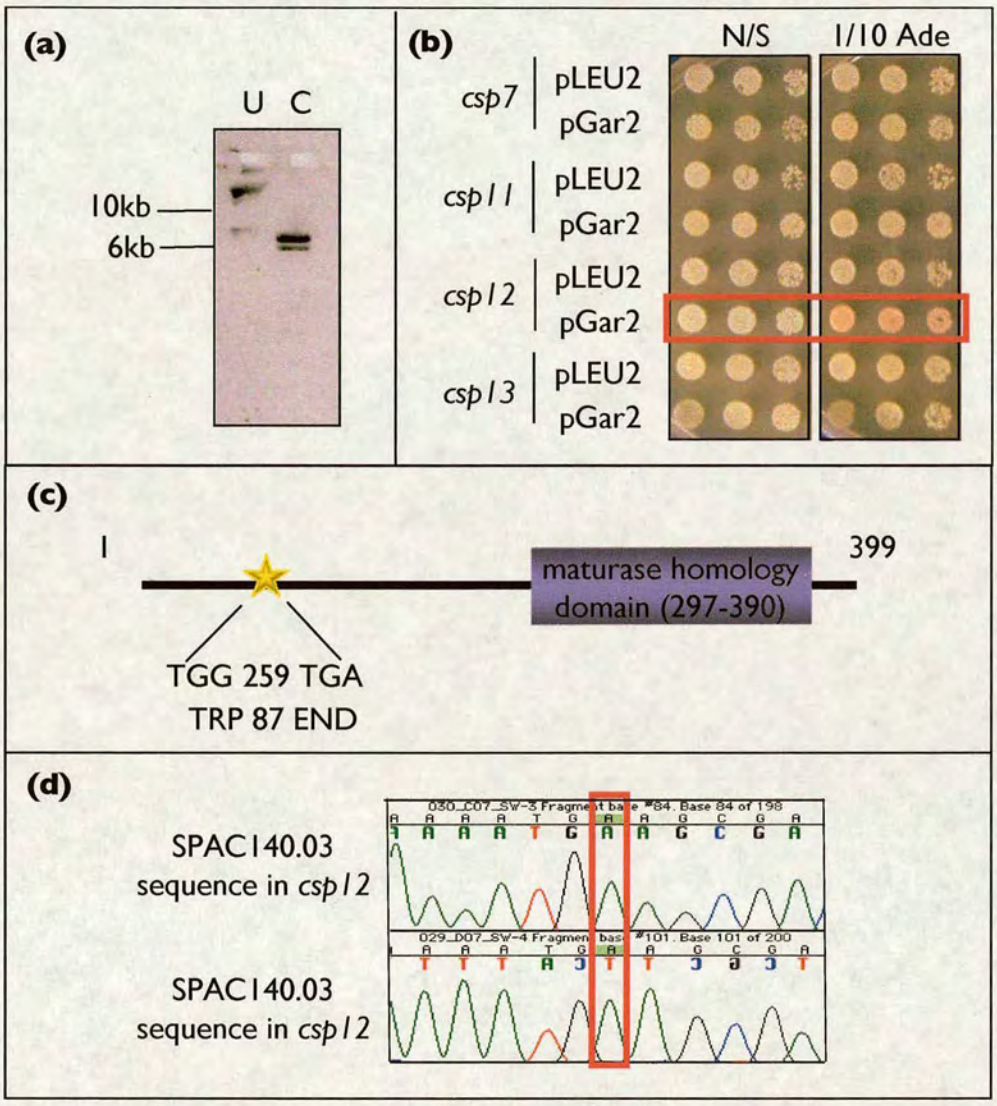


Figure 4.11. Identification of *csp12*$^{+}$.
(a) A plasmid containing a 6 kb insert was found to complement *csp12*. **(b)** The complementation is specific to *csp12* and does not suppress other *csps*. **(c)** *csp12* contains an tryptophan to STOP mutation in causing truncation of around two-thirds of the protein. **(d)** Sequence analysis confirms that *csp12* and Arb1 are alleles.

cos2 strains (Dunleavy, 2007). As the same plasmid was found to complement two distinct mutations affecting outer repeat silencing, it was a concern that the complementation of *csp12* by pGar2 was non-specific. Therefore several other *cos* mutants and *csp* mutants were transformed with pGar2. However, the complementation appeared to be specific for only *cos2* and *csp12* (Figure 4.11b). As nothing was known about the two hypothetical ORFs encoded by this pGar2 plasmid it was decided to first sequence the Gar2 ORF. Gar2 has a GR-rich domain and is a conserved protein. It is related to nucleolin in vertebrates and is thought to play a role in the assembly of ribosomal components. It is required for processing of 35s pre-rRNA and its disruption affects normal cell growth (Gulli et al., 1995; Leger-Silvestre et al., 1997). However, sequence analysis of the ORF of Gar2 from DNA extracted from both *csp12* and *cos2* mutants did not reveal the presence of any mutations (Dunleavy, 2007).

Sequence analysis of the other two ORFs encoding the hypothetical proteins was carried out. The *csp12* cells were found to contain a G to A mutation of nucleotide 259 in the N-terminal part of SPAC140.03. BLAST searches indicated that this protein had homology to maturases which are involved in the self-splicing of introns (Figure 4.11c). The mutation results in a tryptophan to STOP mutation in codon 87 (Figure 4.11c and d). This ORF was also sequenced in four *cos2* mutant alleles but it appears that this mutation is not the cause of the *cos2* phenotype.

Again, a TAP-tagging approach was intended to be carried out so as to allow further characterisation of this protein. However, before this work could begin, SPAC140.03 was identified as Arb1 which interacts with Ago1 and Arb2 in a complex named ARC for Argonaute siRNA chaperone (Buker et al., 2007). Indeed, Arb1 is described as a conserved protein containing a C-terminal domain similar to organellar maturases. The ARC complex, in contrast to RITS, contains mostly double-stranded siRNA (Buker et al., 2007). Arb1 and Arb2 are thought to inhibit the release of an siRNA passenger

strand from Ago1 and hence the 'slicer' activity of Ago1 although the cellular purpose of this as yet remains unclear. Deletion of either Arb1 or Arb2 results in the loss of H3K9me2 and Swi6 from the centromere, in addition to defective siRNA production (Buker et al., 2007). *csp12* displays similar phenotypes to *arb1Δ; the csp12* mutation causes alleviation of silencing at centromere 1 as an *arb1Δ* mutant and is also sensitive to the microtubule destabilizing drug thiabendazole (Figure 4.12a and b).

4.2.7 *csp13* is linked to *dcr1*$^+$

Previous genetic analysis revealed that *csp13* was closely linked to *dcr1*$^+$ (pers. comm. R.Allshire and W. Richardson). Dcr1 is the ribonuclease thought to be responsible for the initiation of the RNAi pathway in fission yeast. Dcr1 cleaves dsRNA into siRNAs which can then be incorporated into the RITS complex to target homologous RNAs and induce heterochromatin assembly (Motamedi et al., 2004; Noma et al., 2004; Sugiyama et al., 2005).

Complementation of the *csp13* mutant using the Shimoda library has so far proved to be unsuccessful. This is perhaps in part due to the high background level, i.e. the high proportion of pink/red colonies observed when *csp13* strains are transformed with empty plasmid alone. Therefore, sequence analysis of *dcr1*$^+$ in a *csp13* mutant was carried out. Sequencing of the *dcr1*$^+$ ORF did not reveal any mutations in a *csp13* mutant. Previously, it was thought that histone H2B contained a mutation in the *csp13* background (pers comm. R. Allshire and W. Richardson). However, upon repeating the sequence analysis, no mutations were discovered in the H2B ORF. In addition, as *dcr1*$^+$ is close to *prp45*$^+$, a splicing factor involved in pre-mRNA processing, this ORF was also amplified from DNA extracted from *csp13* and sequenced. However, no mutation was evident and to date the *csp13* mutation remains unknown.

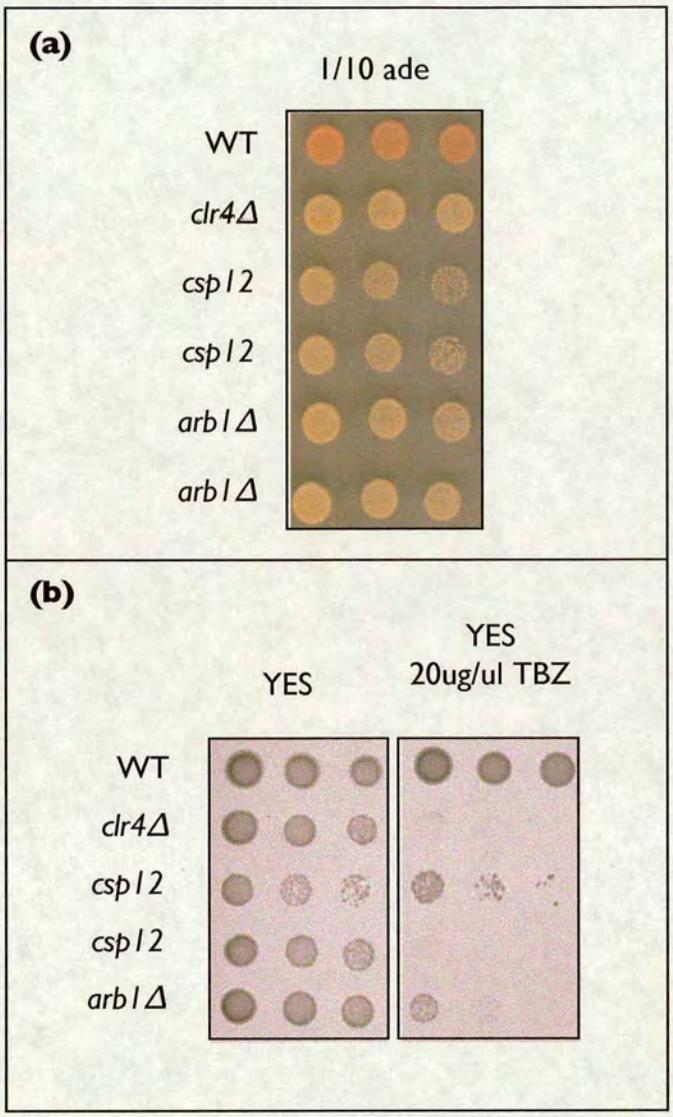


Figure 4.12. *csp1 2* and *arb1 Δ* display the same phenotypes.
(a) *csp1 2* and *arb1 Δ* display the same defect in centromere silencing. **(b)** *csp1 2* and *arb1 Δ* are both sensitive to TBZ.

4.2.8 Swi6 localises to centromeres in the *csp* mutants and their null alleles

As demonstrated in Chapter3, all of the *csp* mutants retain Swi6 localisation at the centromere. It has previously been shown that RNAi mutants lose at least some Swi6 association with centromeres by ChIP (Volpe, 2003; Buker, 2007). In order to examine whether any differences could be seen between the *csp* mutants and their equivalent null alleles, immunofluoresence staining using the Swi6 and Cnp1, the fission yeast CENP-A homolog, antibodies was carried out. This analysis showed that both the *csp* point mutants and the null alleles behaved in a similar manner and that Swi6 was retained at centromeres despite defects in centromere silencing (Figure 4.13).

4.2.9 *csp* mutants and their null alleles can produce siRNAs from an exogenous source.

As shown in Chapter 3, *csp7, 9, 10* and *12* are all able to process a dsRNA hairpin into siRNAs. Whether these siRNAs are taken up by RITS and thus capable of targeting chromatin-modifying activities to homologous DNA sequence remains to be investigated. As well as examining the *csp* mutants, their equivalent null alleles were also analysed for their ability to produce siRNAs from an exogenous source as it has previously been shown that they are unable to produce centromeric siRNAs (Motamedi, 2004; Verdel, 2004). GFP siRNAs are evident in all of the null alleles examined and also in a catalytic mutant of Rdp1 (Figure 4.14). siRNAs are produced to varying degrees but this may be due to different levels of expression of the GFP hairpin in different mutants.

4.2.10 Summary of identification of the *csp* genes

A summary of the *csp* mutants identified so far is presented in Table 4.1. In-depth analysis and cloning of *csps1* to *6* was undertaken by a former PhD student, Manuela Portoso (Portoso, 2005). The *csp3*$^{+}$ and *csp4*$^{+}$ genes were identified by Karl Ekwall (Djupedal et al., 2005). The affected genes in the *csp* mutants identified so far are all involved in fundamental pathways of

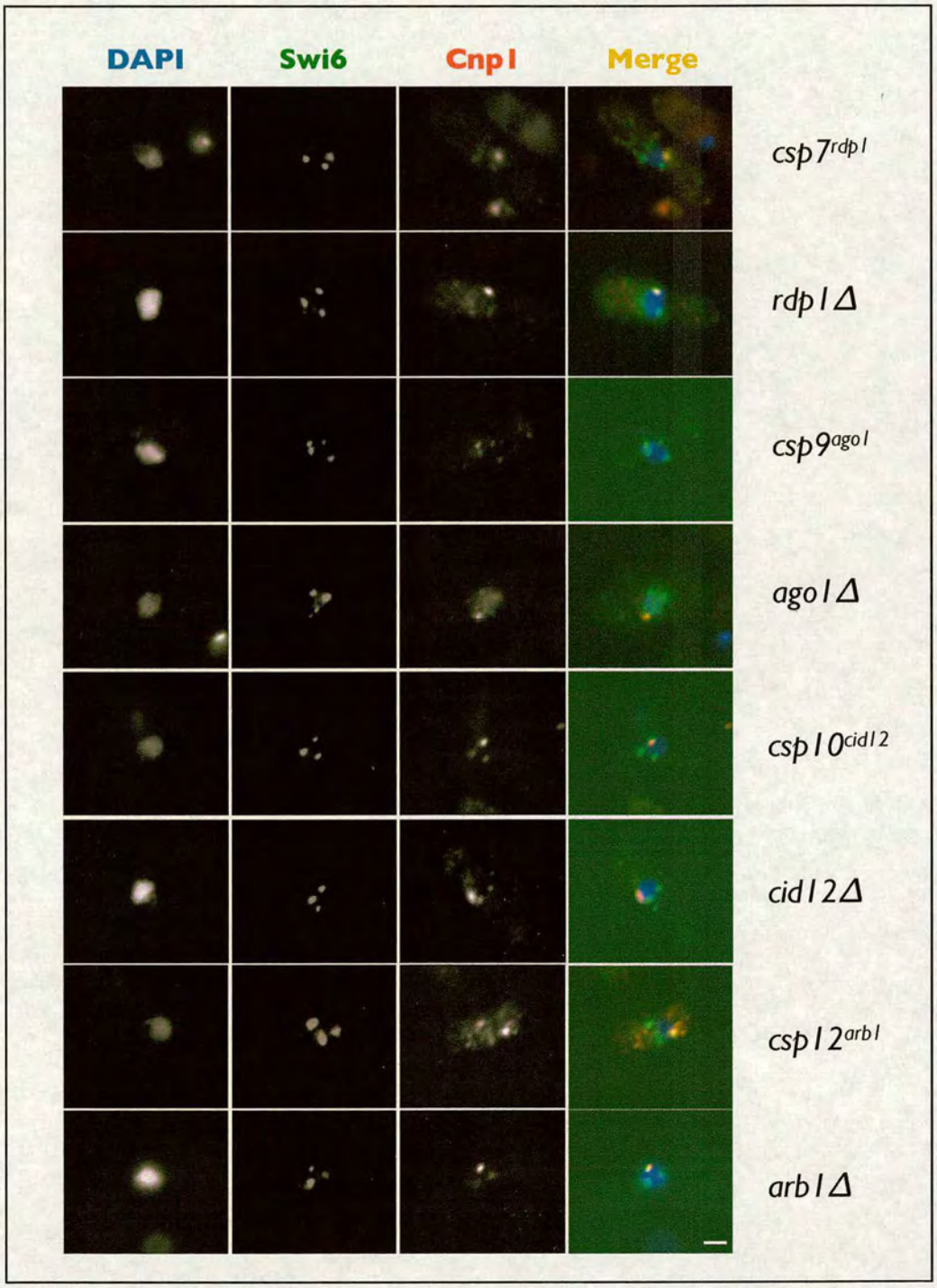


Figure 4.13. *csp* mutants and their alleles retain Swi6 localisation.

Co-immunfluorescence staining using Swi6 antibody to stain heterochromatin and Cnp1 antibody to stain kinetochores shows that all of the *csp* mutants and their equivalent null mutants retain Swi6 localisation at centromeres, despite having defective centromere silencing. Scale = 5μM

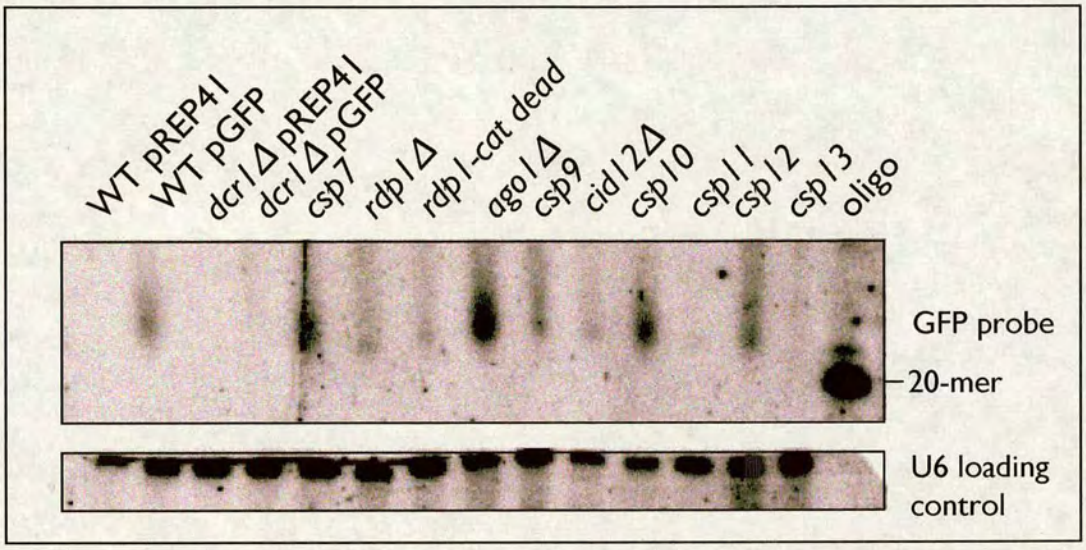


Figure 4.14. *csp* mutants and their alleles can produce siRNAs from an exogenous source.

As shown in Chapter 3, *csp 7, 9, 10* and *12* mutants are able to produce siRNAs from an exogenous source. Their equivalent null mutants also appear to be able to process the dsGFP hairpin, although to varying degrees. The *rdp1-cat dead* mutant contains a point mutation which renders its polymerase domain inactive, however it can also still produce siRNAs.

csp1$^+$	unknown
csp2$^+$	unknown
csp3$^+$	RNA polymerase II subunit, Rbp7
csp4$^+$	splicing factor, Cwf10
csp5$^+$	splicing factor, Prp39
csp6$^+$	unknown, suppressed by Hsp70 proteins
csp7$^+$	RNA-dependant RNA polymerase, Rdp1
csp9$^+$	RITS component, Ago1
csp10$^+$ = *csp8*$^+$	RDRC component, Cid12
csp11$^+$	unknown
csp12$^+$	ARC component, Arb1
csp13$^+$	unknown

Table 4.1 Cloning of the *csp*$^+$ genes.
The progress of identification of the genes responsible for the *csp* mutant phenotypes so far. The ts *csps* are shaded in red and the non-ts are shaded in blue.

RNA metabolism and processing. It is therefore unsurprising that these genes are essential. *csp3* is an allele of Rbp7 which is a conserved subunit of RNA polymerase II required to promote pre-siRNA transcription and RNAi-mediated chromatin silencing (Djupedal et al., 2005). *csp4* is an allele of Cwf10 which is orthologous to the *S. cerevisiae* splicing factor Snu114.

Snu114 is a GTP-binding component of the U5 snRNP which is involved in U4/U6 unwinding during spliceosome activation. *csp5* is an allele of Prp39 which is a U1-associated protein involved in pre-mRNA splicing. The affected genes in the *csp1, 2* and *csp6* mutants remain to be identified although the phenotype of *csp6* was found to be suppressed by the overexpression of several Hsp70 heat-shock proteins (Portoso, 2005). *csp7* is an allele of the RNA-dependant RNA polymerase Rdp1, *csp9* is an allele of the RITS component, Ago1, *csp10* is an allele of the putative poly(A) polymerase Cid12, and *csp12* is an allele of Arb1. So far, all of the identified non-ts *csps* have been found to be key factors of the RNAi-mediated heterochromatin assembly pathway (Figure 4.15).

4.3 Discussion

The use of genetic screens to identify new factors involved in heterochromatin formation and centromere function in *S. pombe* has proven to be highly successful. The *csp* screen was carried out in order to identify novel factors involved in heterochromatin formation, specifically at centromeric outer repeats, to avoid the isolation of factors which play a general role in heterochromatin assembly. In the case of the *csp* screen however, it has taken a huge effort to clone cognate genes. This may be because the *csps* are point mutants and show some variegation under normal growth conditions. The recent commercial availability of whole genome *S. pombe* knock-out sets should allow such screens to become less difficult as this potentially allows mutants to be screened against more than one marker gene fairly quickly.

Despite difficulties in identifying the mutants, the *csp* screen has provided a great resource of factors involved in heterochromatin formation and centromere function. The non-ts *csp* mutants display phenotypes reminiscent of those seen in RNAi mutants and indeed all of those cloned so far appear to play a central role in RNAi-dependant heterochromatin assembly at centromeres (Figure 4.15). This supports the specificity of the screen as previously characterised RNAi components are known to have a minimal effect on silencing at the mating-type locus and at telomeres due to parallel pathways existing at these loci. It seems plausible the two remaining uncloned non-ts mutants will be involved in the same processes.

The ts *csp* mutants have provided a previously unknown link between RNA processing and centromere function although whether this effect is specific remains to be seen. Elizabeth Bayne is currently following up the link between splicing factors and heterochromatin silencing and the effect does seem to be specific as mutations in several other splicing factors tested show no defect in heterochromatin silencing. Defects in silencing in these mutants occur both at restrictive and permissive temperatures whereas the splicing defect occurs only at restrictive temperatures. In addition, microarray analysis does not show any upregulation of unspliced ORFs which may be involved in heterochromatin assembly (pers. comm. Elizabeth Bayne and Karl Ekwall). Another fact which links splicing to heterochromatin assembly is that Cid12 is known to associate with splicing factors in the absence of *rdp1*$^{+}$ (Motamedi et al., 2004). This may indicate distinct roles for Cid12 within the cell or may point to a more general role in RNA processing.

In summary, genetic screening for novel mutants involved in centromere function has been greatly successful. The *csp* screen has provided insights into heterochromatin assembly at centromeres in fission yeast, despite the difficulty in their identification. The cloning of the remaining *csp* mutants may uncover further links between RNA processing and heterochromatin formation.

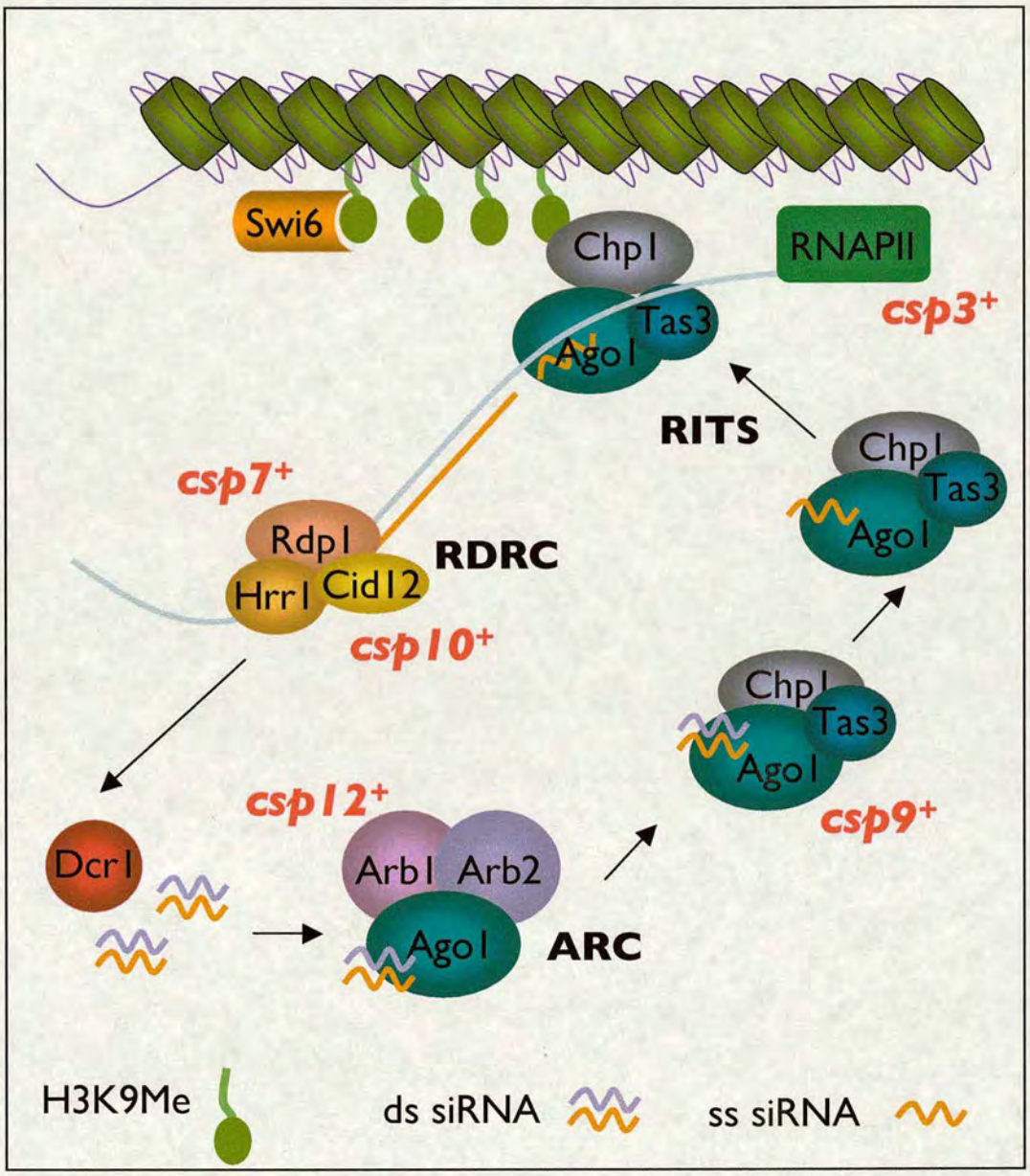


Figure 4.15. Non-ts *csp* mutants are involved in RNAi-directed heterochromatin formation.

Outer repeats are transcribed by RNAPII, of which *csp3*$^+$ encodes a conserved subunit. The RITS complex containing Ago1, encoded by *csp9*$^+$, targets single-stranded siRNAs in order to slice transcripts. The RDRC containing Rdp1 and Cid12, encoded by *csp7*$^+$ and *csp10*$^+$ respectively, is involved in processing the centromere transcripts, perhaps by generating dsRNA substrates for Dcr1 or by amplification of the siRNAs themselves. The ARC complex containing Arb1, encoded by *csp12*$^+$, may be required for the transfer of double-stranded siRNA from Dcr1 to Ago1 *(adapted from Moazed, 2007)*.

CHAPTER 5
Investigating the role of the putative poly(A) polymerase Cid12 in RNAi-mediated heterochromatin formation

5.1 Introduction

5.1.1 Cid12 is part of the RDRC complex and is required for centromeric silencing, chromosome segregation and checkpoint control

Cid12 is a conserved, putative poly(A) polymerase which has been identified as a component of the RNA-dependant RNA polymerase complex (RDRC). It was found to associate with Rdp1, the RNA-dependant RNA polymerase, and Hrr1, a putative helicase, by mass spectrometry analysis (Motamedi et al., 2004). As expected, Cid12 localises predominantly to the nucleus but can be observed by immunofluorescence to a lesser extent in the cytoplasm (Motamedi et al., 2004). All of the components of the RDRC are required for siRNA generation and complete methylation of histone H3 on lysine 9 and Swi6 association with heterochromatic loci (Motamedi et al., 2004; Sugiyama et al., 2005). Purifications of Cid12 in an *rdp1*Δ mutant background revealed that Cid12 also associates with 26 peptides which match known splicing factors (Motamedi et al., 2004). However, a detailed description of these splicing factors has not been published. Unpublished analysis suggests a role for several splicing factors in heterochromatic gene silencing and confirms that specific splicing factors associate with Cid12. Defects in several splicing factors, including Cwf10^{csp4} and Prp39^{csp5} are known to affect silencing at centromeres but have variable effects on heterochromatin structure (Bayne et al, Allshire lab submitted).

What is the role of the Cid12 protein? As a putative poly(A) polymerase one could reasonably expect Cid12 to play a role in mRNA processing and/or regulation. Polyadenylation is known to target transcripts for degradation. Moreover, some mRNA transcripts are polyadenylated in the cytoplasm which stabilises them by preventing their degradation. It is possible that Cid12 is involved in regulating the level of non-coding centromere repeat transcripts as it is known that all the components of the RDRC, including Cid12, associate with these RNA transcripts (Motamedi et al., 2004). One

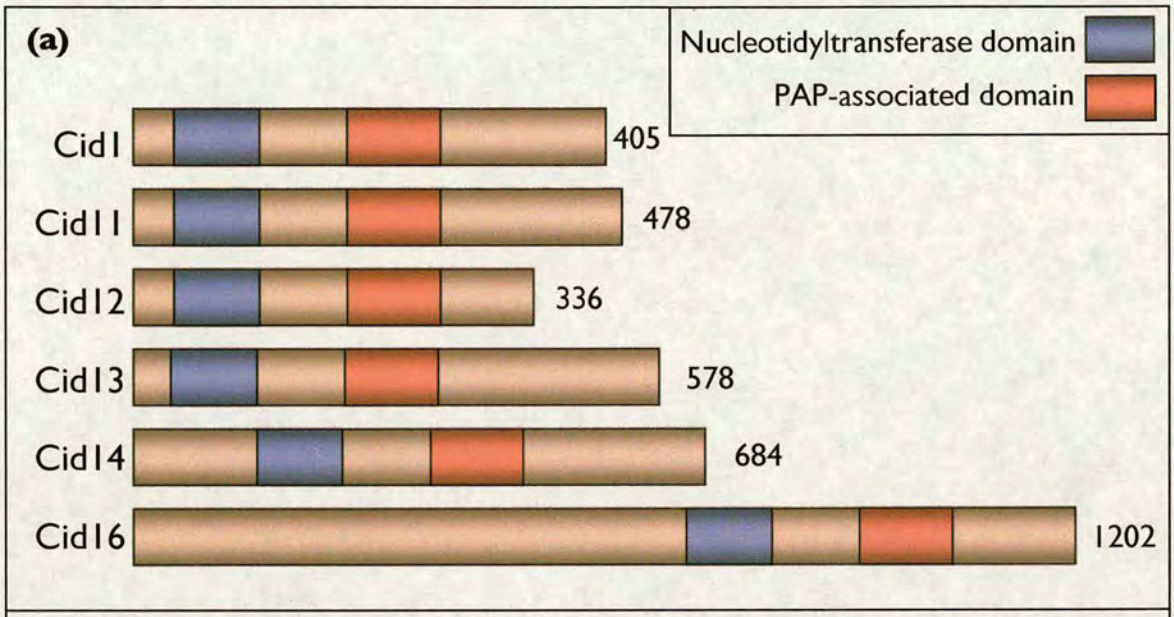
idea is that Cid12 binds the 3′ end of transcripts, producing a poly(A) tract which is able to prime RNA synthesis by Rdp1. A related poly(A) polymerase in *C. elegans*, RDE-3, has also been shown to be required for siRNA production and efficient RNAi (Chen et al., 2005). Polyadenylation by Cid12 might also play a role in RNA degradation since short poly(A) tails are known to attract the exosome and mediate 3′-5′ degradation (Anderson, 2005).

During the course of this work, Win et al (2006) demonstrated that in addition to its role in the RNAi-induced silencing pathway, Cid12 is required for correct chromosome segregation and plays a role in DNA replication checkpoint control. Furthermore, cells lacking Cid12 were shown to accumulate polyadenylated transcripts originating from centromeric heterochromatin (Win, et al., 2006). In this chapter, I will describe the phenotypes of *cid12* mutants and my attempts to elucidate the enzymatic activity of Cid12 in order to understand its role in RNAi-directed heterochromatin formation.

5.1.2 Cid12 belongs to a family of non-canonical poly(A) polymerases

The Cid family of proteins was identified initially through Cid1, a cytoplasmic poly(A) polymerase (Read et al., 2002). The S-M checkpoint is required to delay mitosis until DNA replication is complete. When cells defective in this checkpoint are also inhibited in DNA replication they lose viability. When overexpressed, Cid1 (caffeine induced death suppressor) overcomes the detrimental effects of the combination of hydroxyurea which blocks replication and caffeine which overcomes the S-M checkpoint (Read et al., 2002). The other proteins in the Cid1 family were discovered via BLAST searches of the *S. pombe* database (Read et al. 2002). In total there are six members of the Cid1-like family in fission yeast, *cid1, cid11, cid12, cid13, cid14* and *cid16* (Figure 5.1a) (Read et al., 2002; Stevenson and Norbury, 2006).

The Cid1-like proteins belong to the polymerase β superfamily of enzymes and contain a nucleotidyltransferase (NTPase) domain and a poly(A)



(b)

Protein	Localisation	Function
Cid1	Cytoplasmic	checkpoint response to replication block
Cid11	Nuclear and cytoplasmic	unknown
Cid12	Nuclear and cytoplasmic	RNAi-dependant transcriptional silencing
Cid13	Nuclear and cytoplasmic	maintains dNTP pools via ribonucleotide reductase
Cid14	Nuclear/nucleolar	mRNA targeting for exosome-mediated degradation/RNAi-independent silencing
Cid16	Mitochondrial	mitochondrial RNA processing

Figure 5.1. The Cid1-like family of non-canonical poly(A) polymerases.

(a) The *S. pombe* Cid1-like family of non-canonical poly(A) polymerases contains a total of 6 members. The Cid1-like proteins all belong to the polymerase β family and contain the catalytic nucleotidyltransferase domain, shown as a shaded blue box, and a PAP-associated domain, shown as a shaded pink box. The predicted size in amino acids is also shown. **(b)** A summary of Cid1-like protein functions highlighting their varied roles within the cell (*adapted from Stevenson and Norbury, 2006).*

polymerase (PAP) domain. Polyadenylation of mRNAs is an essential process in eukaryotic cells and fulfills several functions. Poly(A) tails are required for mRNA stability, efficient transport of mRNA from the nucleus to the cytoplasm and for proper translation (Stevenson and Norbury, 2006). Shortening of the poly(A) tail can lead to translational repression and rapid degradation of the transcript (Stevenson and Norbury, 2006). The Cid1-like family of proteins have diverse functions within the cell and act both in the nucleus and the cytoplasm to target specific RNAs. The Cid1-like proteins are known to play vital roles in the checkpoint response to replication block, RNAi-mediated heterochromatin formation and in RNA surveillance pathways such as exosome-mediated degradation (Stevenson and Norbury, 2006). A summary of their roles is shown in Figure 5.1b.

As polyadenylation is an essential RNA processing event in eukaryotes, it is unsurprising that the Cid1 family is highly conserved from yeast through to humans. An alignment of the Cid1-like protein family from *S. pombe* is shown in Figure 5.2a. The polymerase β superfamily to which these proteins belong are characterised by the sequence hG[G/S]X9-13D*h*[D/E]*h* (where X = any amino acid and *h* = hydrophobic residue) which forms the active site of the enzyme (Iyer et al., 2003). A conserved glycine serine motif is contained in a helical turn and the three aspartic acid residues form a catalytic triad. These conserved residues coordinate divalent metal cations which direct the formation of a phosphodiester bond between a 5′ nucleoside triphosphate and a 3′ hydroxyl of the preceding nucleotide (Iyer et al., 2003). All of the Cid1 family proteins contain this motif.

3-D modelling has suggested that the secondary structure of Cid1 is most similar to the central catalytic 'palm' domain of rat Polβ. However, many nucleotidyltransferases share this same 'palm' motif despite a lack of amino acid conservation, except for the motif described above (Date et al., 1991; Martin and Keller, 1996). The 3D structure of a classical C-shaped poly(A) polymerase with the three aspartic acid residues essential for catalysis in poly(A) polymerases and Polβ shaded in yellow is shown in Figure 5.2b

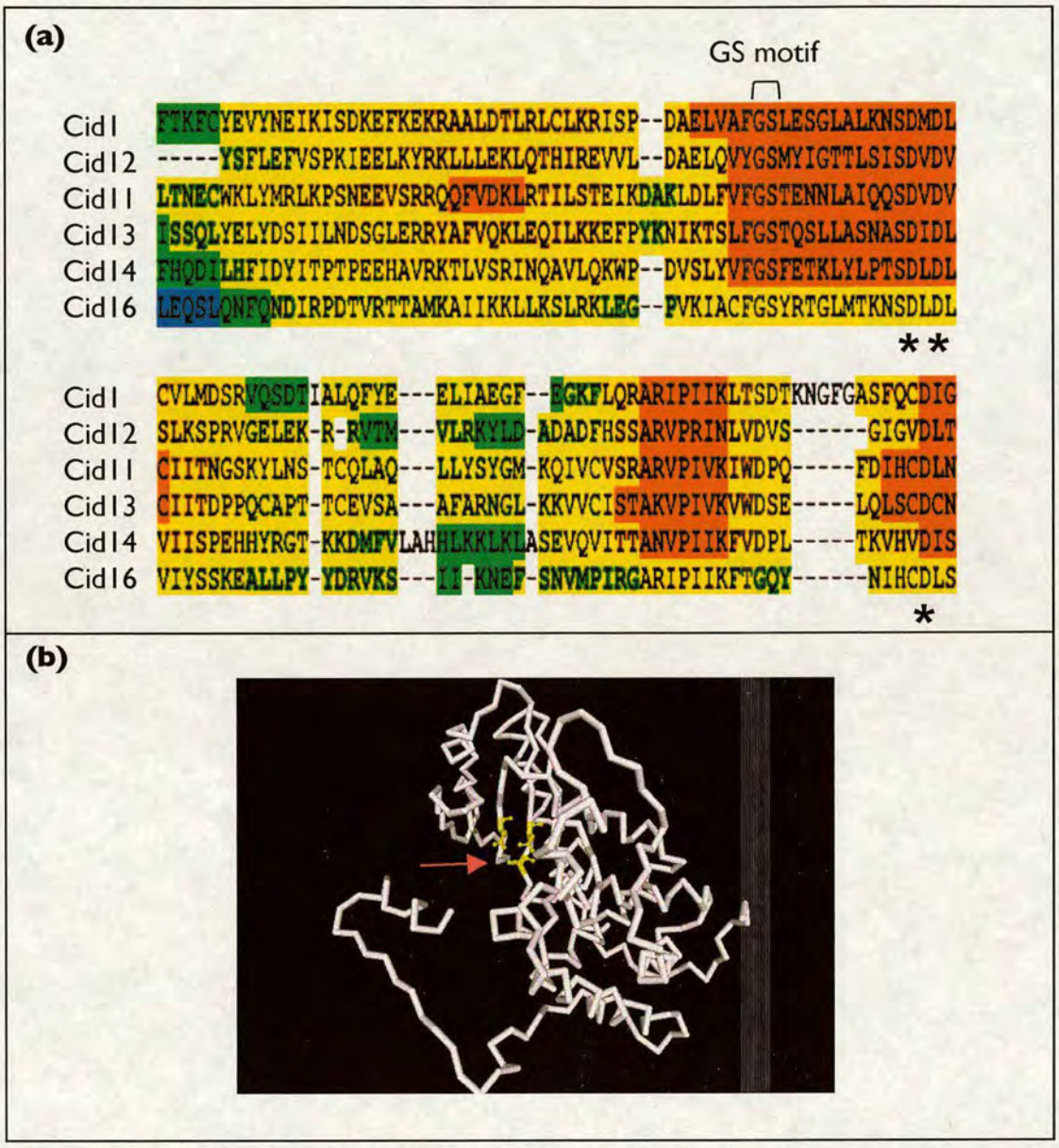


Figure 5.2. Cid1-like proteins share high homology.
(a) The six Cid1-like proteins share homology over the nucleotidyltransferase domain. Three aspartic acid residues which are involved in coordinating the divalent cation in the active site are highlighted with an asterisk. It has been shown, at least for some of the Cid1-like proteins, that mutation of these residues causes a loss of poly(A) polymerase activity. Cid proteins were aligned using ClustalW. **(b)** A 3-D representation of a poly(A) polymerase showing the residues required for NTP binding and catalysis in yellow and highlighted by a red arrow. This structure was predicted using MODBASE (Pieper et al 2006).

(Date et al., 1991; Martin and Keller, 1996). Cid1 has been shown to possess both poly(A) and poly (U) polymerase activity. Mutation of the first two of the catalytic aspartic acid residues has been shown to abolish the polymerase activity of Cid1 (Read et al., 2002; Rissland et al., 2007).

Other Cid1 family members have been shown to have poly(A) polymerase activity. Cid13 is a cytoplasmic poly(A) polymerase involved in regulating ribonucleotide reductase mRNA and has been shown to interact with the poly(A) binding protein Pab1 (Saitoh et al., 2002). Cid14 is the functional homolog of the *S. cerevisiae* poly(A) polymerase Trf4. Cid14 is found in the nucleolus and is required for polyadenylation of rRNAs prior to their degradation via the exosome (Win et al., 2006). Additionally, Cid14 has been found to mediate the silencing of genes inserted into heterochromatin and is defective in siRNA production (Buhler et al., 2007). However, it is not required to maintain the structural integrity of heterochromatin (Buhler et al., 2007). Its role in gene silencing is dependant on its enzymatic activity, as mutation of two aspartic acid residues known to be required for catalytic activity, D298 and D230, abolishes heterochromatic gene silencing (Buhler et al., 2007). So far, no enzymatic activity has been demonstrated for Cid11, Cid12 or Cid16.

5.2 Results

5.2.1 *csp10* is an allele of *cid12*

The *csp* mutants were placed in separate complementation groups by crossing to each other in all pairwise combinations (Ekwall et al., 1999). *csp8* and *csp10* were subsequently determined to allelic. However, *csp10* has been identified as an allele of *cid12* as detailed in Chapter 4. Sequence analysis of *csp10* demonstrated that *cid12* contains a C to G mutation which causes a premature STOP codon approximately halfway into the NTPase domain. Surprisingly, this same mutation was found in an independently isolated mutant, *csp8* (Figure 5.3). Originally, *csp8* and *csp10* were put into separate complementation groups. However, subsequent analysis has demonstrated these mutants are allelic. This error could be due to the fact that the *csp*

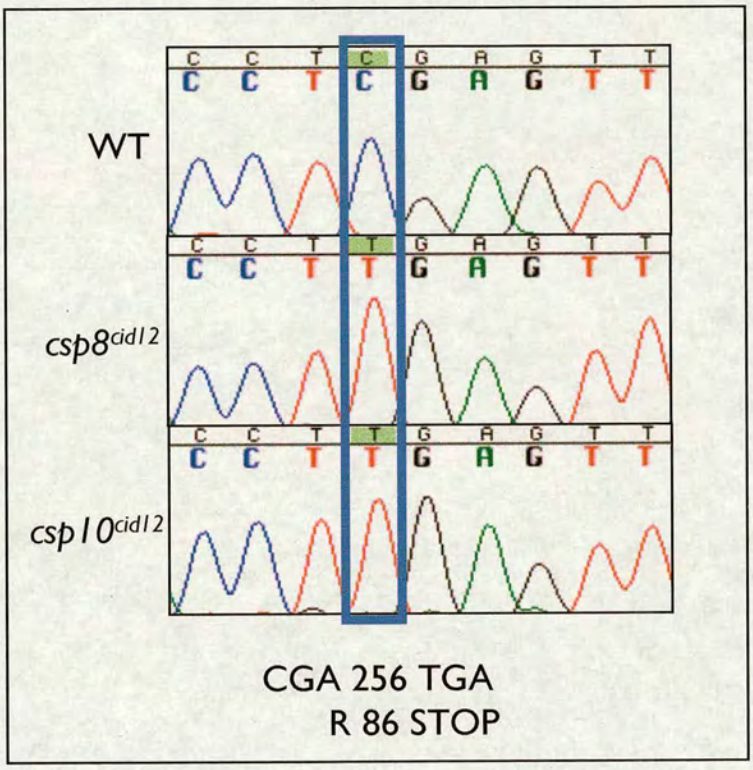


Figure 5.3. *csp8* and *csp10* have identical mutations in *cid12*.
Sequence analysis of *csp8* and *csp10* shows that the two mutants share the same mutation, despite having originally been identified as independent alleles.

mutants display variegated expression of marker genes inserted at centromeric outer repeat sequences during mating. This variegation could be due to the occurrence of spontaneous mutations which affect the adenine biosynthesis pathway or may be due to a mutation which causes the variegating phenotype.

5.2.2 *cid12Δ* alleviates silencing of *ade6*$^+$ in centromeric outer repeats

csp10^{cid12} was found in a screen to identify mutants which specifically alleviate silencing at the centromeric outer repeats. During the course of this study, *cid12*Δ was also shown to alleviate outer repeat silencing (Motamedi et al., 2004). As *csp10*^{cid12} and *cid12* are allelic, *cid12*Δ and several other members of the Cid1 family were crossed to introduce the *otr1R(SphI):ade6*$^+$ marker gene into the mutants to test their effect on centromere silencing. *cid1*Δ and *cid11*Δ were provided by Chris Norbury (University of Oxford). The *cid12*Δ and *cid14*Δ strains were provided by Shao-Win Wang (University of Oxford). *cid13*Δ was kindly donated by Paul Russell (Scripps Research Institute). Only *cid12*Δ displays strong alleviation of silencing as illustrated by Figure 5.4a. However, a recent publication has demonstrated that *cid14*Δ alleviates silencing of a *ura4*$^+$ marker gene inserted within the outer repeats (Buhler et al., 2007). Mutants which have defective centromere function are often sensitive to the microtubule destabilising drug TBZ. All of the Cid1-family members which were tested for centromere silencing were also tested for TBZ sensitivity. Again, only *cid12*Δ showed sensitivity to TBZ comparable to that seen in a *clr4*Δ mutant (Figure 5.4b). These data suggest that *cid12* is involved in centromeric heterochromatin formation and suggest that the other Cid1 related proteins do not have a role here.

5.2.3 *cid12Δ* centromeric silencing defects are partially complemented by an overexpressing plasmid

In order to investigate the role of Cid12 in RNAi-mediated heterochromatin formation, plasmids overexpressing wild type Cid12 and a putative catalytically dead *Cid12*^{dada} from the *nmt1* promoter in pREP1 were obtained from Shao-Win Wang (University of Oxford). The *Cid12*^{dada} mutant has the

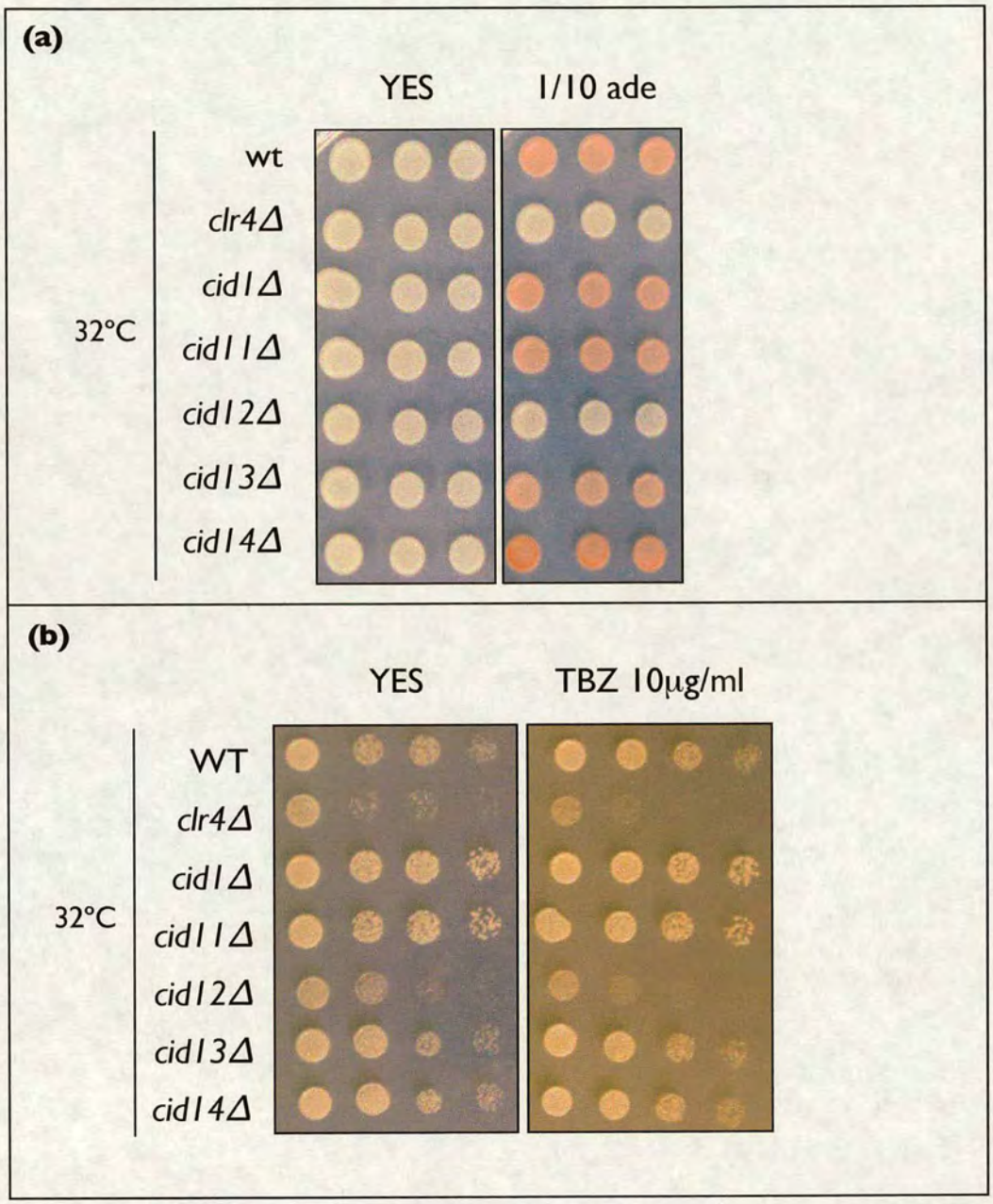


Figure 5.4. *cid12Δ* displays defects consistent with a role in centromere function.

(a) The Cid1-like family members containing the marker gene *otr1R(Sph1):ade6*$^{+}$ were tested for alleviation of silencing. Wild type colonies repress expression of this marker gene and therefore are red. Mutant colonies alleviating silencing are white. Only *cid12Δ* was found to have a strong effect. **(b)** Cid1-like family members were tested for sensitivity to the microtubule destabilising drug TBZ. Only *cid12Δ* cells appear to be TBZ sensitive.

first two aspartic acid residues of the NTPase domain at codons 77 and 79 mutated into alanine residues. This would be expected to abolish any predicted poly(A) polymerase activity of the Cid12 enzyme. These plasmids were used to investigate whether the putative enzymatic activity of Cid12 is required for its role in heterochromatic gene silencing.

Overexpression of Cid12 is lethal in *cid12*Δ mutants but promoter activity can be attenuated by growing cells transformed with the plasmid in 15 μM thiamine (pers. comm. Shao-Win Wang). pREP1-Cid12 is able to rescue the TBZ sensitivity of *cid12*Δ as demonstrated in Figure 5.5a. However, *cid12Δ* containing the mutated plasmid, pREP1-*Cid12*^{dada}, grows poorly on TBZ plates, indicating that these residues may be required for the function and activity of Cid12. *cid12*Δ also tends to grow less well than wild type strains in general (Figure 5.4b YES plate).

These plasmids were also tested for their ability to restore the alleviation of silencing observed at the centromeric outer repeats in a *cid12*Δ mutant containing *otr1R(Sph1):ura4*$^{+}$. pREP1-Cid12 caused partial restoration of silencing of the *ura4*$^{+}$ insertion as shown by slight growth on plates containing FOA (Figure 5.5b).

5.2.4 *cid12*Δ defects in the RNAi pathway are not complemented by an overexpressing plasmid

To further examine the effect of Cid12 mutation on centromere function, I carried out centromeric transcript and siRNA northern analysis. *cid12*Δ has previously been shown to accumulate non-coding centromeric transcripts from the outer repeats to levels similar to those seen in other RNAi mutants and to be defective in centromeric siRNA production (Motamedi et al., 2004)

*cid12*Δ accumulates centromere transcripts by RT-PCR, at levels similar to a *dcr1*Δ mutant (Figure 5.6a). Upon transformation with either pREP1-Cid12 or pREP1-*Cid12*^{dada}, centromeric transcript levels decrease. However, in *cid12*Δ strains expressing pREP1-Cid12, transcript levels do not return to that

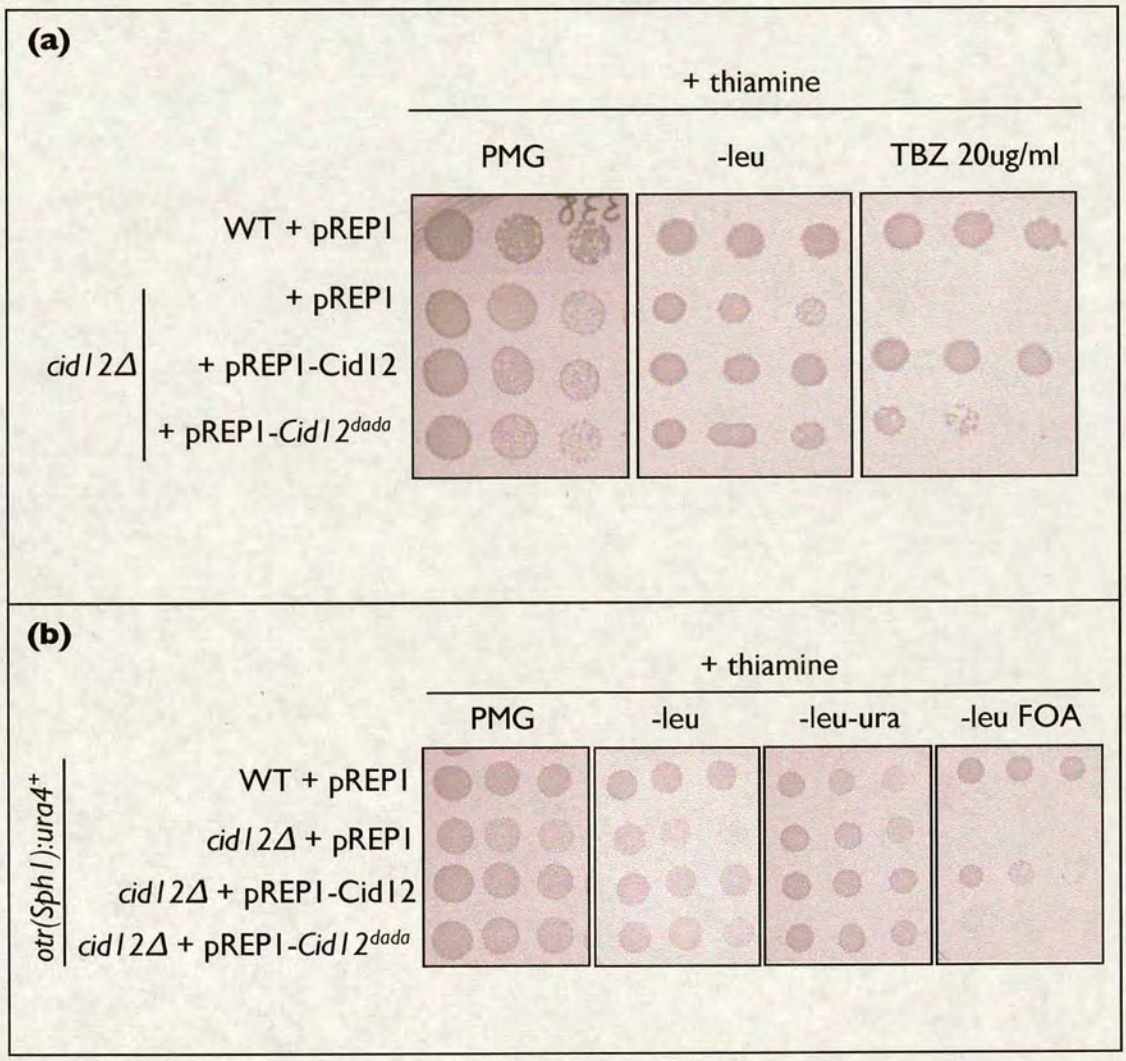


Figure 5.5. *cid12Δ* is partially complemented by a plasmid containing pREP1-Cid12.

(a) The TBZ sensitivity of *cid12Δ* is complemented by pREP1-Cid12 in the presence of 15μM thiamine. Overexpression of Cid12 is lethal (pers. comm. Shao-Win Wang). Mutation of the two first predicted catalytic aspartic acid residues, pREP1-*Cid12*^{dada}, results in less growth on TBZ. **(b)** *cid12Δ* alleviates silencing of a *ura4*$^{+}$ gene, *otr1R(Sph1):ura4*$^{+}$, inserted at centromere 1, as indicated by increased growth on -ura and less growth on the counter-selective FOA. This alleviation is partially repressed by pREP1-Cid12 but not by pREP1-Cid12^{dada}, as indicated by increased growth on FOA and slower growth on -ura. Both (a) and (b) were carried out at 32°C.

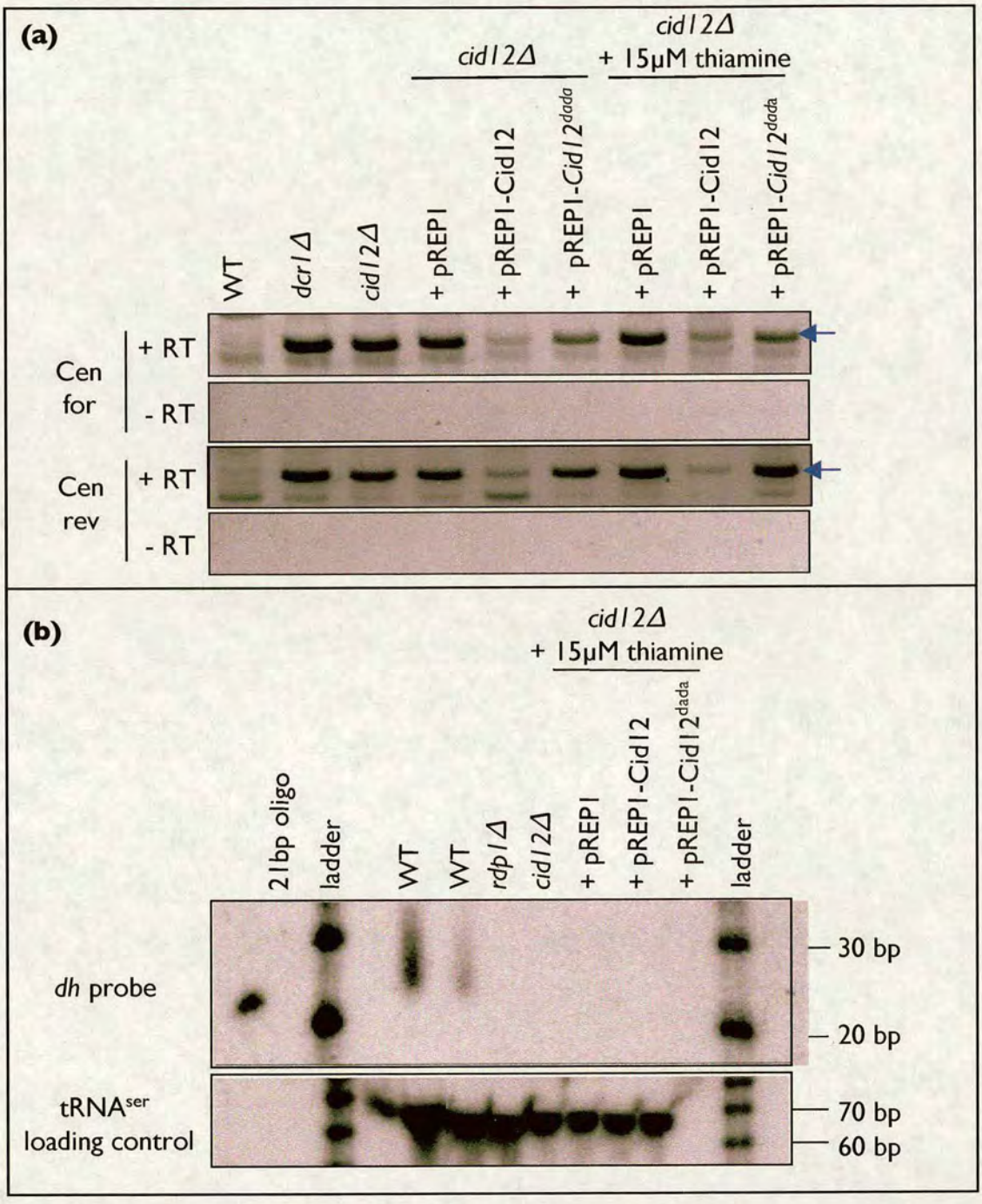


Figure 5.6. Complementation of *cid12Δ* by pREP1-Cid12 is partial.
(a) *cid12Δ* accumulates non-coding centromere transcripts from both strands, like other mutants defective in RNAi. The specific centromere transcript band is indicated by an arrow. In *cid12Δ* strains containing a plasmid expressing wild type Cid12, centromere transcript levels are reduced but not to levels seen in wild type cells. Transcript levels remain high in cells containing a plasmid expressing *Cid12^{dada}* mutant protein.
(b) Centromeric siRNAs are undetectable in *cid12Δ* comparable to other RNAi mutants. In *cid12Δ* cells containing a plasmid expressing wild type Cid12 the ability of *cid12Δ* to synthesise centromeric siRNAs is not restored, indicating that complementation by this plasmid is only partial.

of wild type indicating that complementation by this plasmid is only partial (Figure 5.6a).

Northern analysis confirms that, like an *rdp1*Δ mutant, the *cid12*Δ mutant is unable to produce detectable levels of centromeric siRNAs. On transformation of either pREP1-Cid12 or pREP1-*Cid12*^{dada} into *cid12*Δ, no siRNAs are observed (Figure 5.6b). It could be that the levels of siRNAs produced are so small as to be undetectable by this method or that misexpression of Cid12 causes an imbalance which causes collapse of the RNAi-induced heterochromatic silencing pathway. This data again demonstrates that the complementation of *cid12*Δ by these plasmids is not complete and is consistent with previous results that inappropriate expression of Cid12 fails to completely restore silencing of a marker gene inserted into centromeric heterochromatin.

5.2.5 A comparison of plasmids which complement *cid12*Δ

Previously, a plasmid containing *cid12*, pAL-Cid12, was found to complement *csp10*^{cid12}. This has been described as plasmid A in Chapter 4, Figure 4.9b. This plasmid has an *ars1* element and therefore copy number varies from cell to cell. pAL-Cid12 was used in a side-by-side comparison with pREP1-Cid12 to examine levels of complementation with respect to *otr1R(SphI):ade6*$^{+}$. pAL-Cid12 complements alleviation of silencing of the marker gene well in both *csp10*^{cid12} and *cid12*Δ (Figure5.7). However, expression of pREP1-Cid12 does not completely complement either *csp10*^{cid12} or *cid12*Δ, in accordance with previous results, which indicates that inappropriate expression of Cid12 may be detrimental to centromere silencing.

5.2.6 Generating a putative catalytically dead *cid12*^{dada} mutant

Since complementation with of *cid12Δ* with pREP1-Cid12 is partial, it is difficult to assess the real impact of the *Cid12*^{dada} mutation. A more rigorous test is to generate a putative catalytically dead *cid12* in the endogenous gene expressed from its own promoter. To do this, the mutated *cid12*^{dada} ORF was

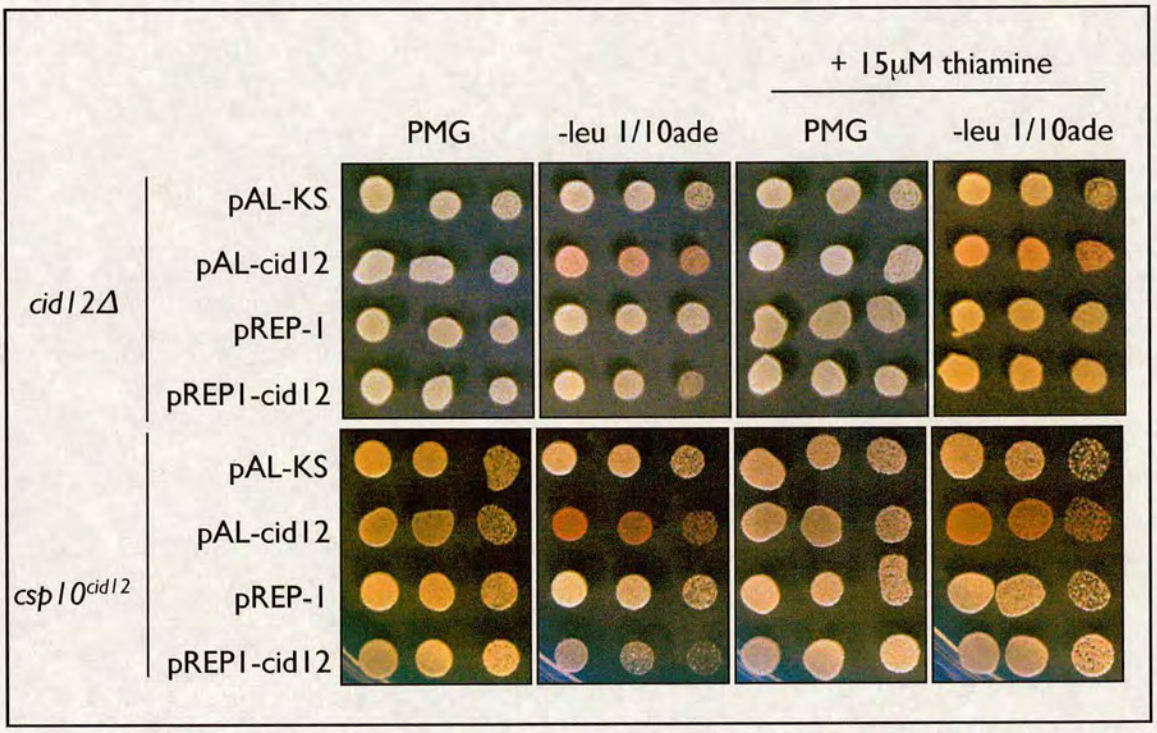


Figure 5.7. Complementation of *cid12Δ* by pREP1-Cid12 is partial. The centromere silencing defects of *csp10* are complemented by a plasmid, pAL-cid12, which was recovered from a screen using a yeast high-copy genomic library (shown in Figure 4.9b as plasmid A). pAL-cid12 complements the silencing defect at *otr1R(SphI)ade6*$^{+}$ in both *csp10* and *cid12Δ* strains. However, using this marker gene, only very slight restoration of silencing is observed when either *csp10* or *cid12Δ* is transformed with the pREP1-cid12 plasmid, showing further evidence that misexpression of Cid12 is detrimental to silencing at centromeres.

PCR amplified using proofreading Taq from the pREP1-*cid12*^{dada} plasmid with primers which have 80 bp homology to either side of the endogenous *cid12* ORF. This was co-transformed into a *cid12::ura4*$^{+}$ knockout strain along with a pLEU2 plasmid in order to recover transformants. Colonies which were leu+ and FOA resistant, and therefore should have the *ura4*$^{+}$ gene replaced by the mutated PCR fragment, were selected and sequenced for the presence of the mutations (Figure 5.8). The mutations changed codon 77 from GAT to GCT and codon 79 from GAC to GCT, both causing a D to A change (Figure 5.8). The mutant strain is therefore named *cid12*^{dada}.

5.2.7 Phenotypes of the *cid12*^{dada} mutant

Analysis of the *cid12*^{dada} mutant was carried out to examine its phenotypes. The *cid12*^{dada} mutant is sensitive to the microtubule poison TBZ to the same extent as a *cid12*Δ mutant (Figure 5.9a). In addition, neither the *cid12*Δ or the *cid12*^{dada} mutant are able to synthesise detectable centromeric siRNAs just like the *csp10*^{cid12} mutant and other mutants in components of the RDRC (Figure 5.9b).

Analysis of non-coding RNA transcripts originating from the centromere shows that all of the mutants in components of the RDRC and the *cid12*^{dada} mutant accumulate these transcripts. *hrr1*Δ consistently shows less centromere transcripts than the other components, perhaps suggesting that some of the transcripts may be processed or that in an *hrr1*Δ strain fewer transcripts are produced (Figure 5.10).

It is possible that the *cid12*^{dada} mutation affects silencing but not chromosome segregation. To determine if the *cid12*^{dada} mutant affects segregation to the same degree as *cid12*Δ chromosome segregation was quantified. The *csp10*^{cid12} mutant has previously been shown to have high rates of chromosome missegregation and was used here as a positive control (Ekwall et al., 1999; Pidoux et al., 2000). As expected, the *cid12*Δ mutant shows a similar percentage (around 20%) of lagging chromosomes on late anaphase spindles as the *csp10*^{cid12} mutant (Figure 5.11a and b). The *cid12*^{dada} mutant shows

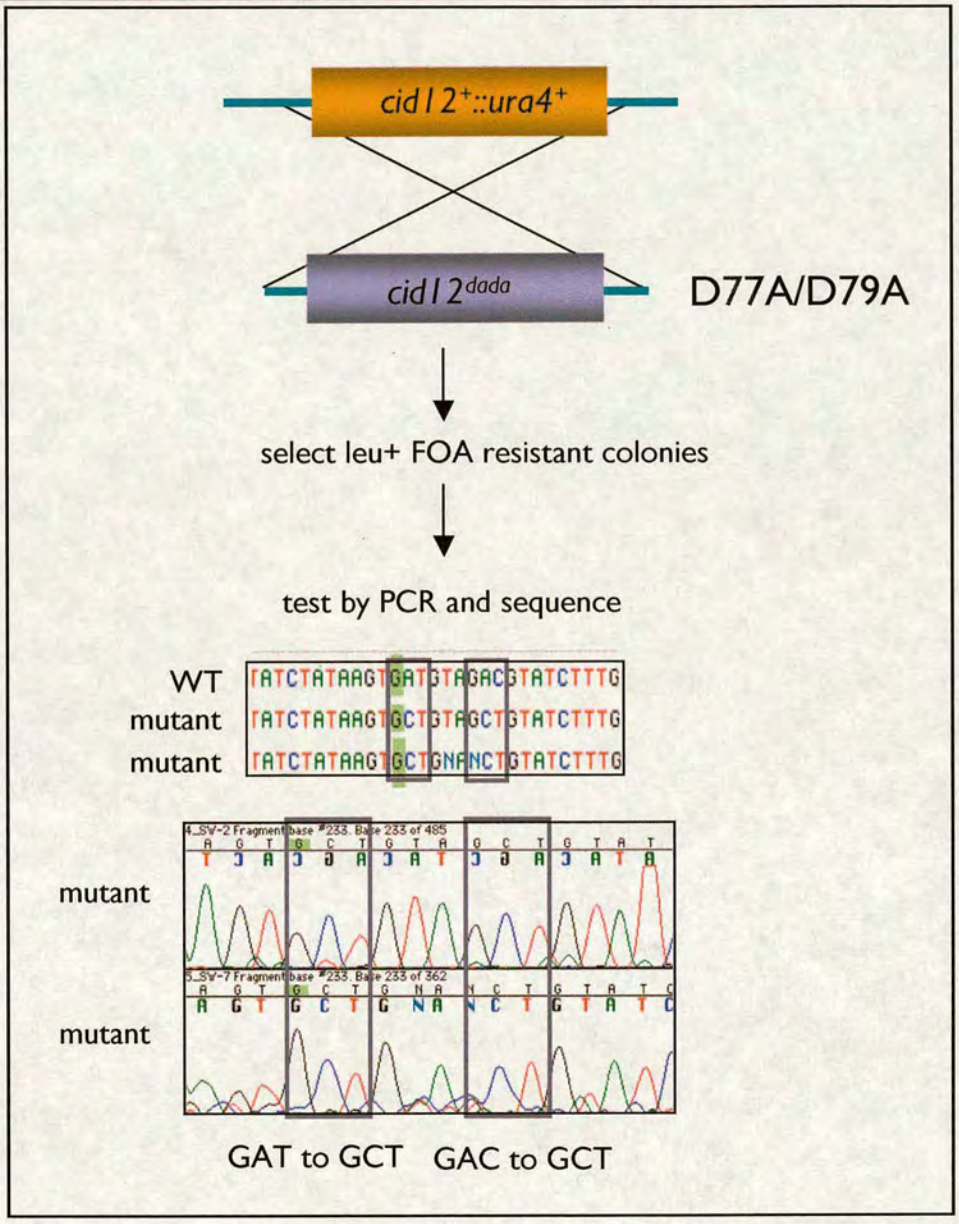


Figure 5.8. Generation of a putative catalytically dead genomic *cid12* mutant. A strain with *Cid12* knocked out with the *ura4*$^{+}$ was transformed with a mutated Cid12 PCR product. The PCR product was mutated at codon 77, GAT to GCT, and codon 79, GAC to GCT, to produce an aspartic acid to alanine change in both cases. The *cid12Δ* strain was co-transformed with a LEU2 plasmid and the mutated ORF, and transformants which were leu+ and FOA resistant were selected. These were then tested for the presence of the ORF by PCR and then sequenced to ensure the correct mutations were present. These mutants were called *cid12*^{dada}.

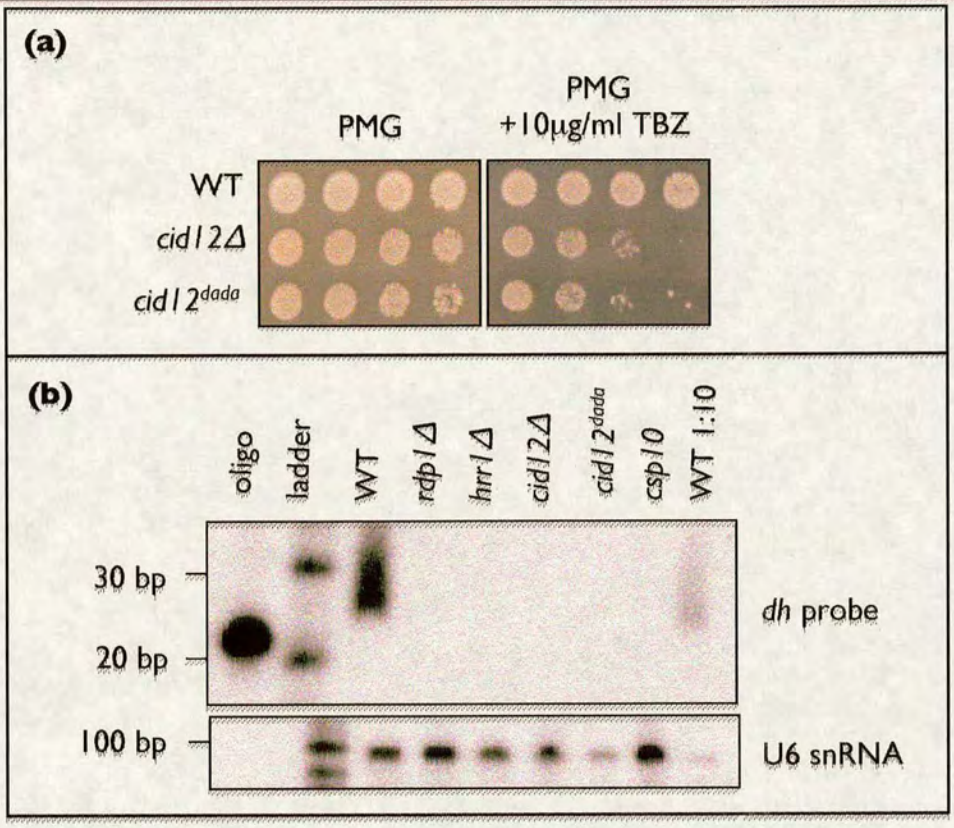


Figure 5.9. *Cid12*^{dada} mutants display similar phenotypes to the null mutant. (a) *cid12*^{dada} mutants are sensitive to TBZ. **(b)** *cid12*^{dada} mutants are unable to synthesise centromeric siRNAs.

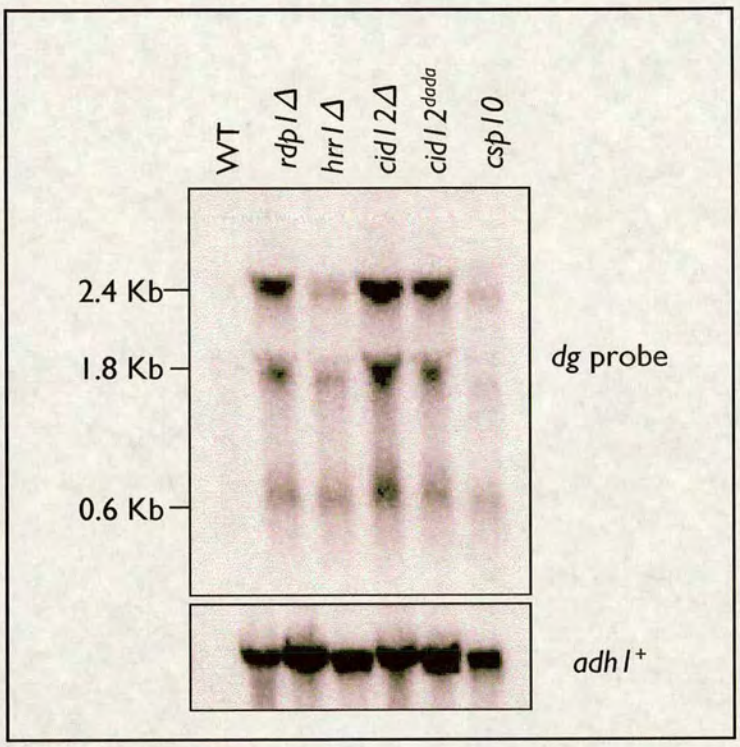


Figure 5.10. *Cid12*^{dada} mutants accumulate centromere transcripts. Mutants in each of the RDRC components accumulate centromere transcripts. *hrr1Δ* consistently shows lower levels of accumulation than the other components. *Cid12*^{dada} accumulates transcripts to the same levels as *cid12Δ*.

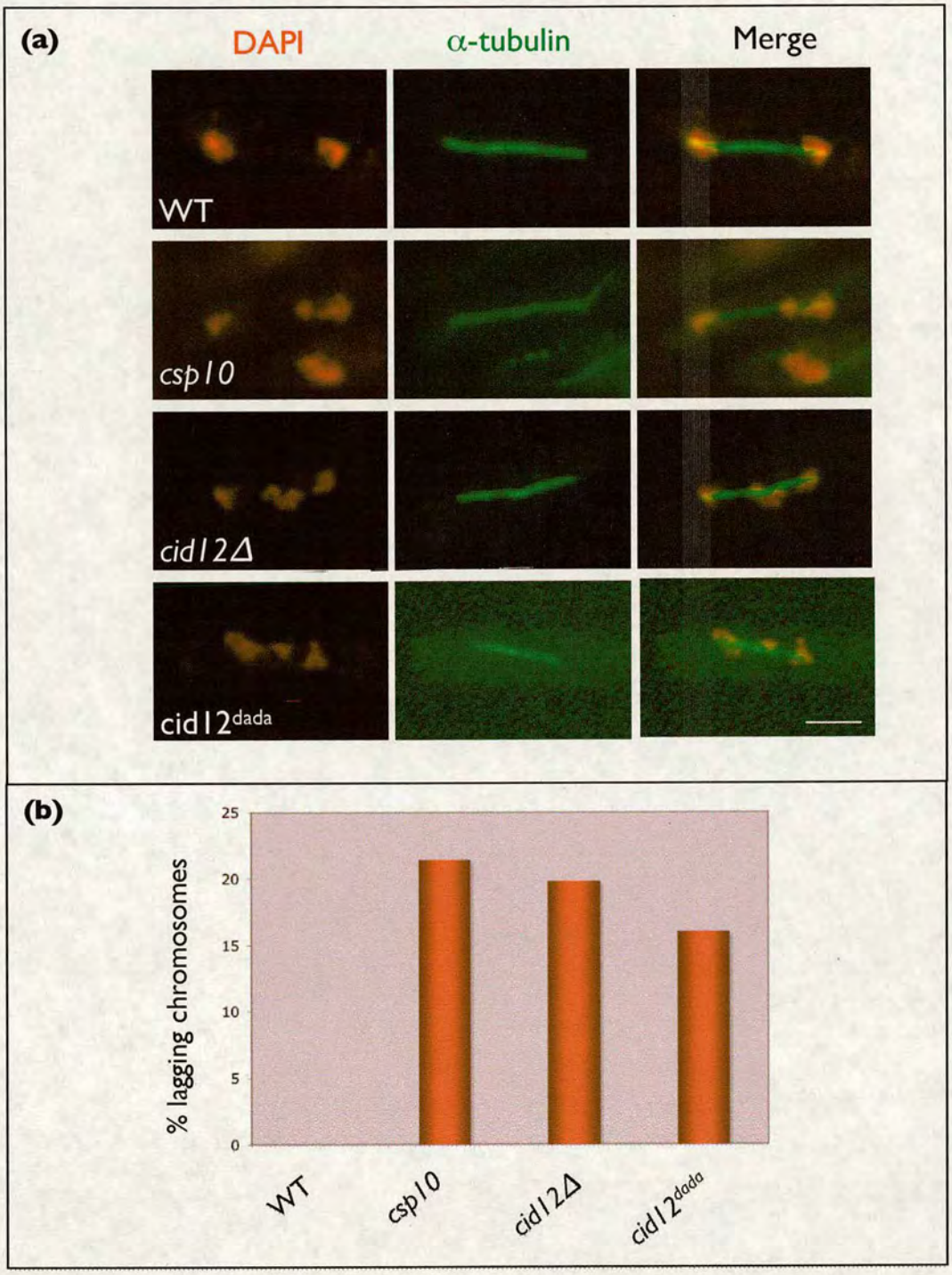


Figure 5.11. *Cid12*^{dada} mutants display chromosome segregation defects. **(a)** Cells were stained with α-tubulin to mark spindle microtubules and DAPI to stain DNA. Wild type cells show normal chromosome segregation whilst *csp1O*, *cid12Δ* and *cid12*^{dada} mutants all display lagging chromosomes. **(b)** Quantification of lagging chromosome analysis shows that wild type cells display no lagging chromosomes on late anaphase spindles but *csp1O* and *cid12Δ* have around 20%. *Cid12*^{dad} mutants show a slightly lesser defect with around 16% lagging chromosomes. 200 cell were analysed for each sample. Scale bar = 5μM.

similar but slightly fewer lagging chromosomes (16%) than the *cid12*Δ or *csp10*^{cid12} mutants.

5.2.8 Expressing epitope-tagged *cid12*^{dada}

As no antibodies to Cid12 were available, the mutated gene was tagged in order to carry out biochemical analysis and to check the stability of the mutated protein in the cell. Cid12 has previously been shown to associate with splicing factors therefore it was proposed to FLAG-tag the *Cid12*^{dada} protein in order to compare the effect of this mutation on its interactions with the wild type protein. The wild type *cid12* gene had previously been FLAG-tagged by Alexander Kagansky (Allshire Lab). The mutated *cid12*^{dada} gene was tagged using the same strategy. Briefly, the pFA6a-FLAG-NATMX6 vector was used as a template in a PCR reaction containing primers with 80 bp homology just upstream and downstream of the STOP codon of the mutated *cid12*^{dada} gene. The PCR product was transformed into the *cid12*^{dada} mutant and cloNAT resistant colonies were selected. These were screened by PCR for insertion of the FLAG tag and sequenced to ensure no further mutations had occurred at any stage in the Cid12 ORF or tag (Figure 5.12).

Colonies which were positive for the FLAG tag by PCR were analysed further. A *cid12*^{dada} -FLAG strain was tested for TBZ sensitivity as shown in Figure 5.13a. The *cid12*^{dada} -FLAG mutant may be expected to behave as a null mutant and indeed the mutant is sensitive to TBZ, like *cid12Δ* (Figure 5.13a).

Western blotting was carried out to determine the protein levels of wild type and mutant protein. Wild type Cid12-FLAG protein is readily detected on a western blot (Figure 5.13b). As the FLAG antibody produces a cross-reacting band at the size predicted for the Cid12-FLAG protein, the FLAG-tagged proteins were first enriched using M2-FLAG agarose and eluted with peptide. Unfortunately, no *cid12*^{dada} -FLAG protein could be observed (Figure 5.13b). This could mean that *cid12*^{dada} protein is itself unstable or that the tag causes the mutant protein to become unstable.

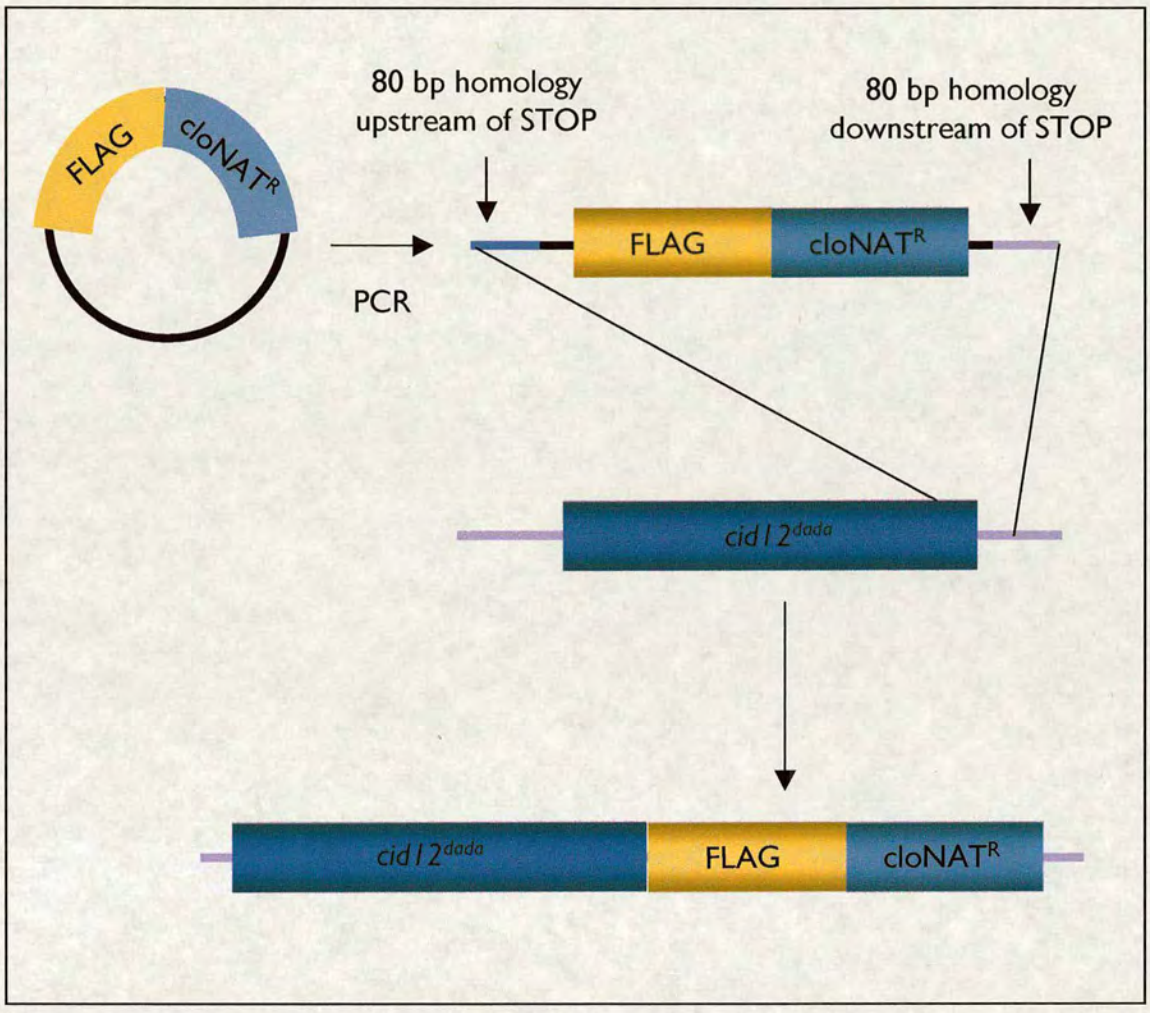


Figure 5.12. Tagging the *cid12*^{dada} mutant.
A PCR product using a vector based on the pFaA-FLAG-NAT cassette as a template was made with 80bp homology both up and downstream of the *cid12*^{dada} ORF. cloNAT resistant colonies were selected and checked by PCR for the presence of the FLAG tag.

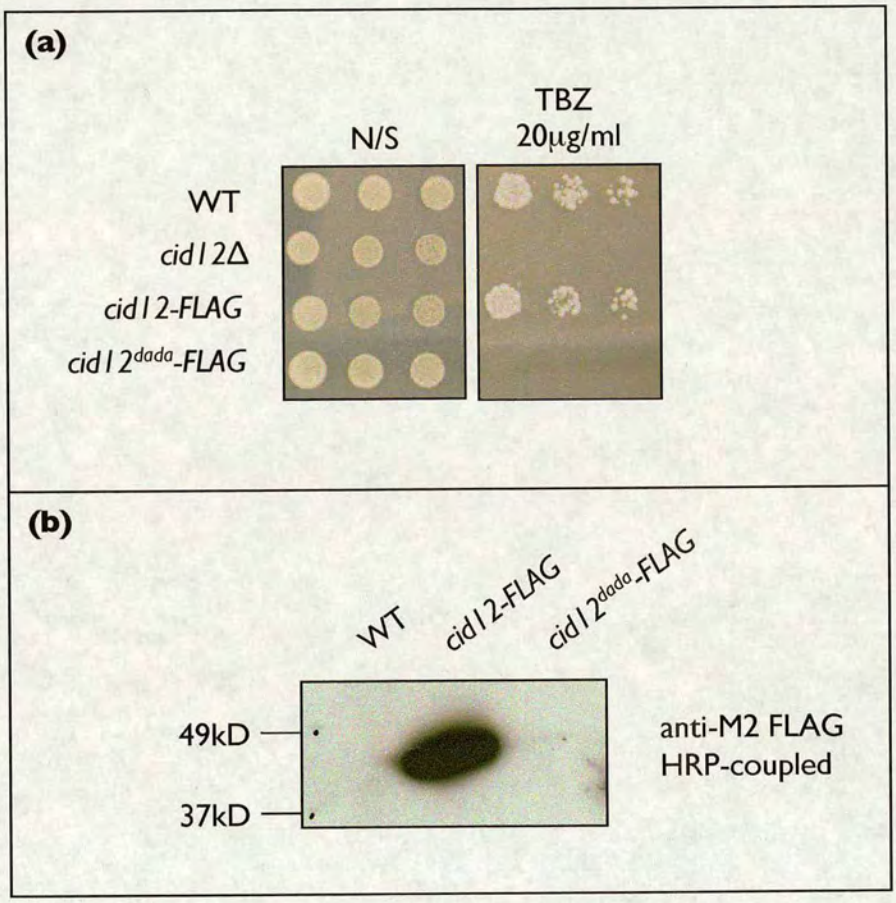


Figure 5.13. Absence of *Cid12*^{dada} protein suggests that mutant protein is unstable. **(a)** Wild type *Cid12* was FLAG-tagged by Alexander Kagansky. *cid12*^{dada} was similarly FLAG-tagged on the C-terminus. *cid12-FLAG* grows as wild type on TBZ media and *cid12*^{dada}*-FLAG* is TBZ sensitive. **(b)** Western blotting with anti-M2 FLAG antibody (Sigma) shows that wild type *Cid12*-FLAG is expressed but *cid12*^{dada}*-FLAG* is not.

To test whether the *cid12*^{dada} protein was unstable due to the FLAG tag, another tag was substituted (Figure 5.14a). A TAP tag was inserted at the C-terminus of the ORF and western analysis carried out to check for the production of protein. However, changing the tag on the mutant did not appear to make any difference and no *cid12*^{dada}-TAP protein was detectable although wild type protein was observed (Figure 5.14b). To combat these problems, full-length Cid12 has been sent for immunisation to produce antibody against the protein and this is ongoing at the time of writing.

5.2.9 *In vitro* characterisation of the nucleotidyltransferase activity of recombinant Cid12 proteins

Mutation of the putative catalytic residues of Cid12 appears to render the protein unstable in *S. pombe*. Recombinant Hexahistidine-tagged Cid12 wild type and *cid12*^{dada} mutant proteins were expressed in *E. coli* and purified by nickel chelate chromatography. Coomassie stained gels of purified His-tagged Cid12 and *cid12*^{dada} protein demonstrate that both the mutant and wild type protein are expressed and appear to be stable in *E. coli* (Figure 5.15). The identity of the proteins were confirmed by MALDI mass spectrometry (Andy Cronshaw, University of Edinburgh).

As Cid12 contains a putative nucleotidyltranferase domain it seemed likely that, like Cid1, Cid12 would possess ATPase activity. The assay used takes advantage of the fact that ATP hydrolysis is coupled to NADH oxidation which causes a decrease in absorbance at 340_{nm}. This pathway is summarised in Figure 5.16a. Hexahistidine-tagged Cid1 plasmid was a kind gift from Chris Norbury. Cid1 shows a modest ATPase activity, confirming published results, whereas both wild type and mutant Cid12 protein show levels equivalent to background (Figure 5.16b) (Read et al., 2002). This assay was carried out in the presence and absence of an RNA substrate but in both cases, Cid12 proteins failed to display any detectable ATPase activity (Figure 5.16b). It is possible that partner proteins are required or that *in vivo* posttranslational modification, such as phosphorylation, of the substrate is needed to activate Cid12.

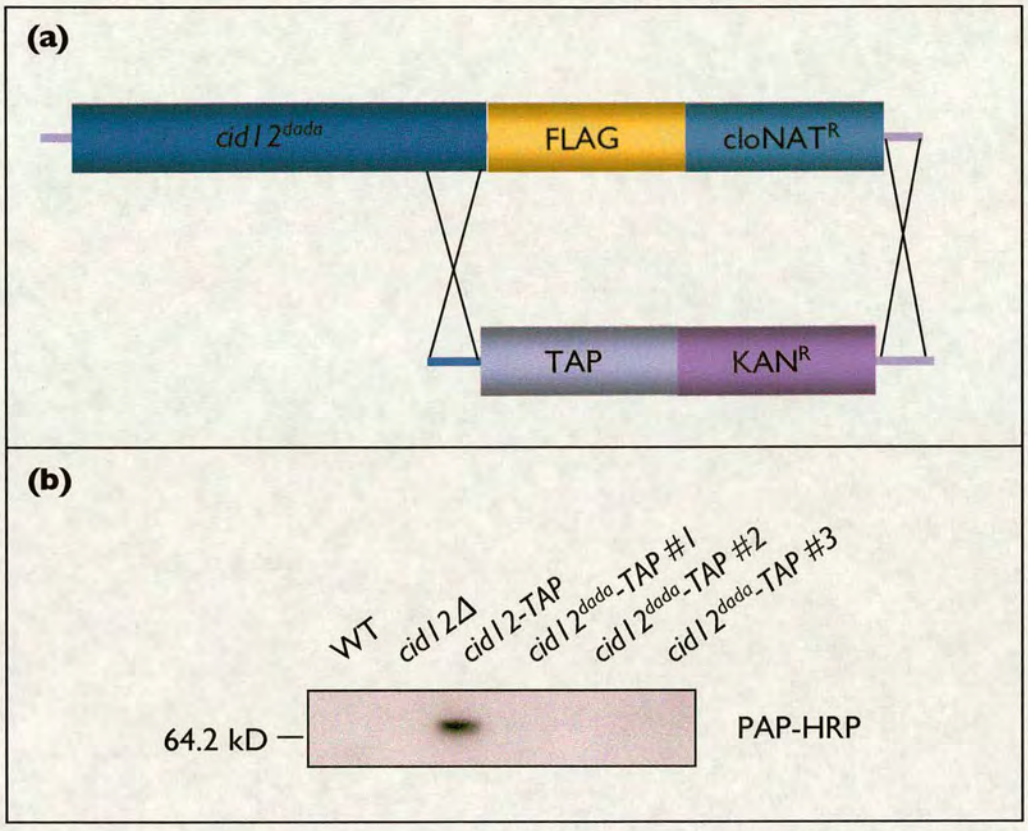


Figure 5.14. Tagging the *cid12*^{dada} mutant. (a) The FLAG tag of *cid12*^{dada} was replaced by a TAP tag. A PCR product was made using a vector containing the TAP tag and a kanamycin resistance gene as a template with 80 bp homology either side of the *cid12*^{dada} STOP codon. This was transformed into the FLAG tagged strain and colonies which were cloNAT sensitive and kanamycin resistant were selected. These colonies were then analysed as before by PCR to check for the presence of the TAP tag and the absence of the FLAG tag. **(b)** *cid12*^{dada}*-TAP* protein is not detectable by western blotting with peroxidase anti-peroxidase antibody compared to *cid12-TAP*.

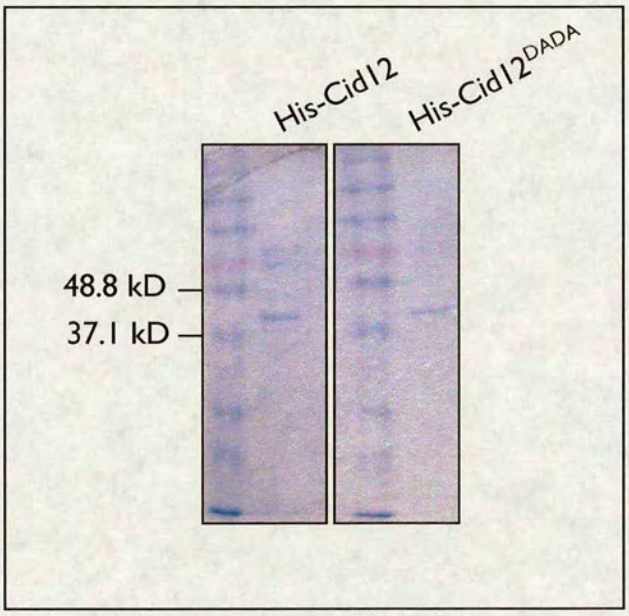


Figure 5.15. Expression of recombinant Cid12 proteins.
Coomassie stained gels showing Cid12 proteins His tagged at the C-terminus. Cid12 has a molecular weight of 38.5 kD. These proteins were expressed and purified from *E. coli* and their identity confirmed by mass spectrometry.

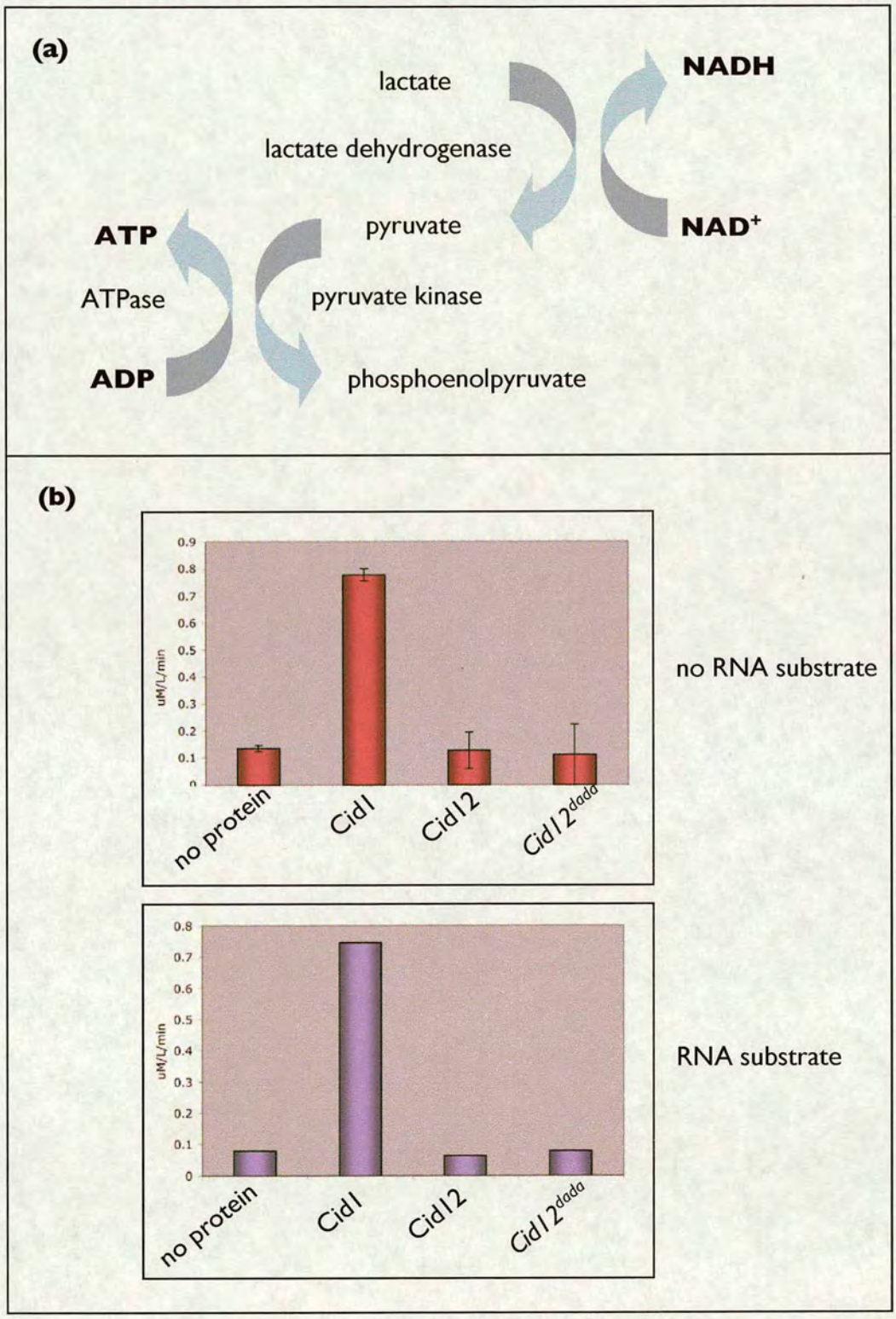


Figure 5.16. Cid12 does not display ATPase activity. (a) ATP hydrolysis and NADH oxidation are coupled. NADH oxidation causes a decrease in absorbance at 340 nm. **(b)** Cid1 shows a modest ATPase activity in the absence and presence of an RNA substrate. Cid12 proteins show no detectable activity under these conditions.

5.2.10 *In vitro* assays for poly(A) polymerase activity of recombinant Cid12 proteins

Several members of the Cid1-like protein family have been documented to display poly(A) polymerase activity (Wang et al., 2000; Win et al., 2006). To test whether recombinant Cid12 proteins have any poly(A) polymerase activity, a standard polymerase assay was used. In brief, recombinant proteins were incubated with a ^{32}P end-labelled RNA substrate in the presence of individual nucleotide triphosphates. To begin with, a synthetic 18-mer RNA oligo was used as a substrate. Reactions were incubated at 37°C for 1 hour, phenol/chloroform extracted, run on a denaturing urea gel and autoradiographed. Commercially available poly(A) polymerase (PAP from USB) and recombinant Cid1 protein were used as positive controls. However, using these conditions and a standard buffer in which Cid1 is known to display activity (see Materials and Methods 2.6.9), Cid12 proteins displayed no detectable activity (not shown).

A variety of parameters were altered in order to find experimental conditions that may be optimum for Cid12 activity. To begin with, all four nucleotide triphosphates were added to the reaction and compared to reactions where only ATP was removed (Figure 5.17a and b). As different buffers have been published with the other Cid1 family members which have shown poly(A) polymerase activity these buffers were also tested. A poly(A) polymerase assay using a buffer containing 0.7 mM $MnCl_2$ and 15 mM $MgCl_2$ is shown in Figure 5.17a. Cid1 shows polymerase activity both in the presence and absence of ATP (+ and - respectively) but Cid12 proteins display no detectable activity. Initially, Cid1 was characterised as a poly(A) polymerase but more recently it has been shown to form poly(U) tails which accounts for its activity in the absence of ATP (Rissland et al., 2007). Altering the buffer conditions to contain only 0.5 mM $MnCl_2$ causes Cid1 activity to become more robust than in the presence of magnesium but Cid12 again showed no activity.

Different polymerases have been shown to have varying requirements for

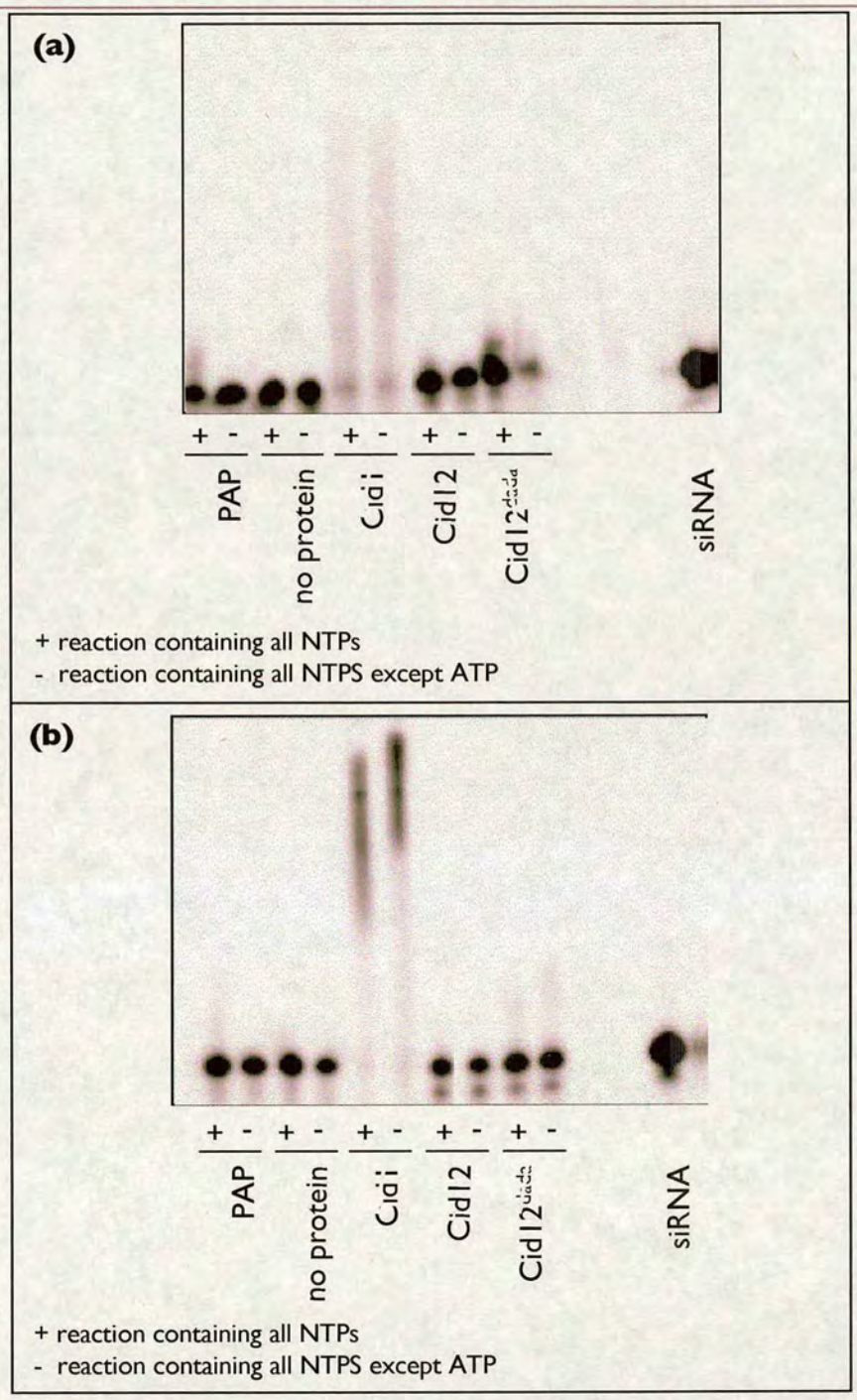


Figure 5.17. Poly(A) polymerase activity of Cid12. **(a)** ^{32} P end-labelled single-stranded siRNA was used as a substrate for poly(A) polymerase reactions. Cid1 shows modest poly(A) polymerase activity in buffer containing 0.7 mM $MnCl_2$ and 15 mM $MgCl_2$ but Cid12 shows no activity. + denotes reactions containing all NTPs, - denotes reactions containing all NTPs apart from ATP. **(b)** The same reaction set-up as above but buffer contains only 0.5 mM $MnCl_2$ and no magnesium. In this instance Cid1 activity appears more robust.

magnesium and manganese. However, manganese is known to affect the fidelity of DNA polymerases. A magnesium gradient was carried out using Cid1 and Cid12 to determine if Cid12 activity required different ion conditions from Cid1 (Beckman et al., 1985). Cid12 shows no poly(A) activity at the concentrations of magnesium tested, whilst Cid1 shows some activity at all tested concentrations (Figure 5.18). In addition, Cid1 is found to show most activity in buffer containing only manganese but this may be because manganese encourages promiscuous incorporation of NTPs (Goodman et al., 1983).

It has been shown in mammalian splicing assays that the yield of RNA products is greatly enhanced when potassium chloride is substituted by potassium glutamate or acetate (Reichert and Moore, 2000). This could be due to the relative physiological concentrations of the ions (Leirmo et al., 1987). A potassium glutamate gradient was performed in order to test the effect of this on the Cid proteins. To begin with, the gradient was tested on PAP and Cid1 as no activity had yet been observed with Cid12. This demonstrated that in buffer containing 80 mM potassium glutamate as opposed to 40 mM potassium chloride, Cid1 showed more robust activity (Figure 5.19a). PAP showed variable activity as has been seen previously when using any buffer other than that commercially provided. However, altering the potassium concentration had no effect on Cid12 and again no poly(A) activity was observed under the conditions tested (Figure 5.19b).

It is possible that Cid12 may require a specific type of RNA substrate in order to produce enzymatic activity. Therefore, in addition to altering the buffer composition in the poly(A) polymerase assays, a variety of RNA substrates were tested. Originally, a short single-stranded 18-mer synthetic oligo was used in all assays. A poly(A) polymerase assay using a short synthetic 20-mer blunt-ended dsRNA was performed (Figure 5.20). Cid1 shows some activity but Cid12 does not. A short synthetic dsRNA with 2 nt overhangs to mimic a naturally occurring siRNA was also used in the same assay and showed the same result (not shown).

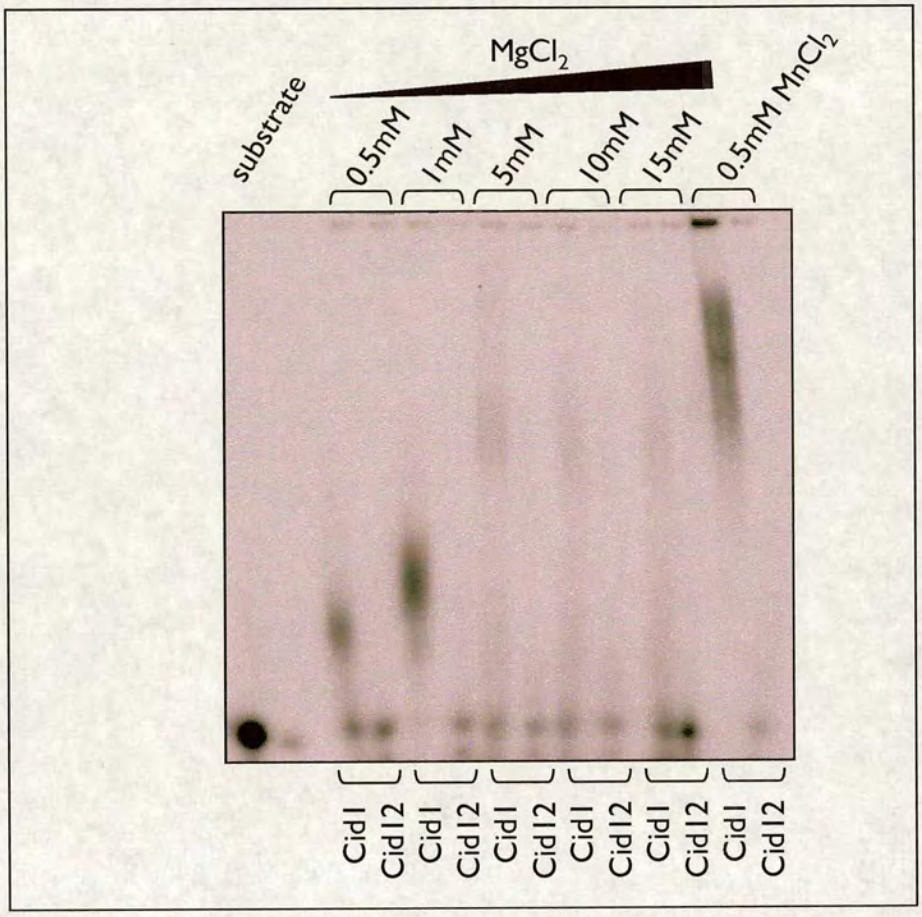


Figure 5.18. Altering the magnesium concentration does not produce poly(A) polymerase activity with Cid12. A magnesium gradient compared to manganese containing buffer using a short single-stranded RNA as a substrate. Cid1 shows robust poly(A) activity in the presence of 5 mM to 15 mM magnesium but the greatest activity is observed in buffer containing manganese. Cid12 fails to show detectable activity under any of the conditions assayed.

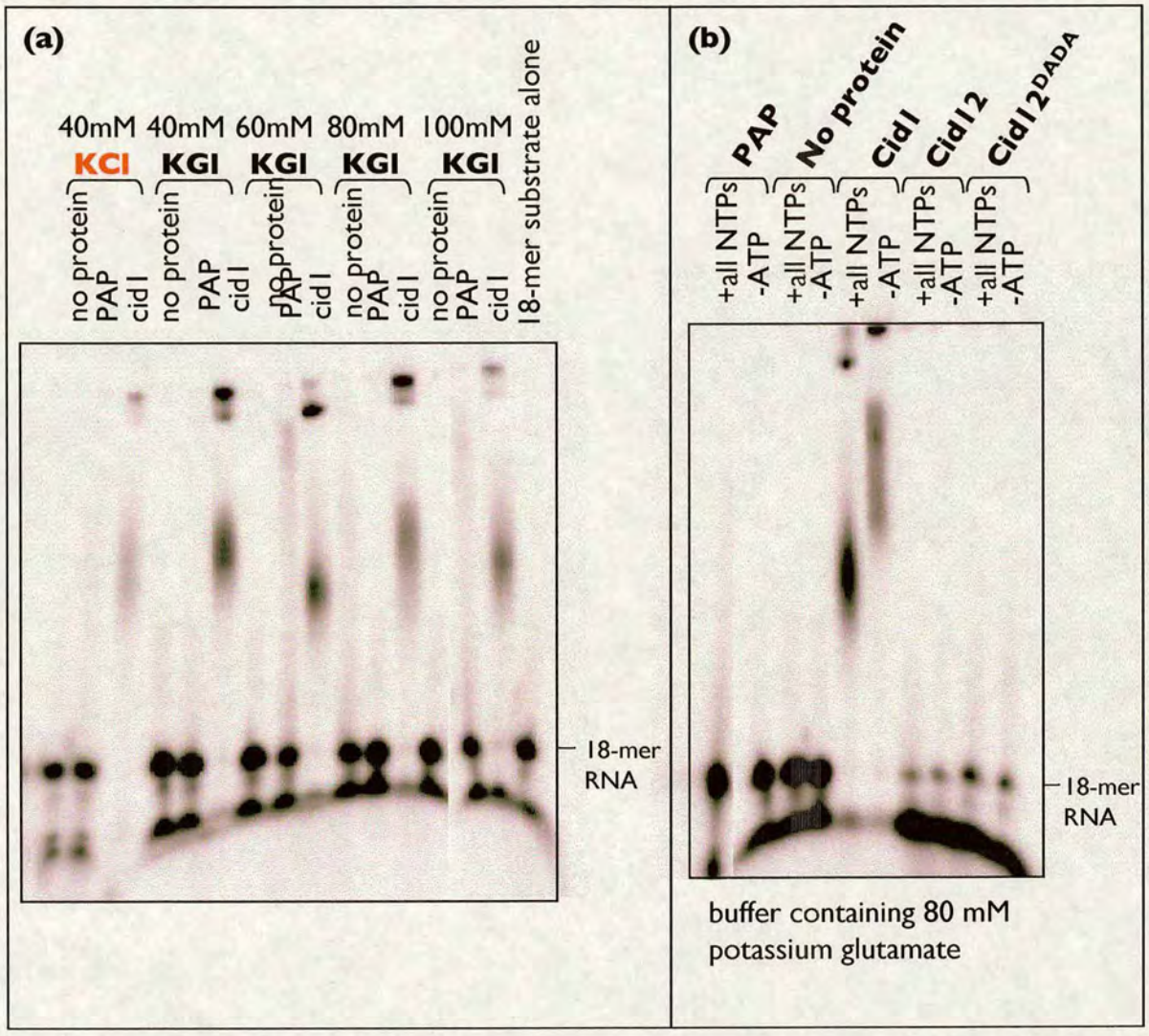


Figure 5.19. Substituting potassium glutamate for potassium chloride does not produce poly(A) polymerase activity with Cid12. Splicing assays demonstrate that potassium glutamate increases the yield of RNA substrates (Reichert and Moore, 2000). **(a)** shows a gradient comparing potassium chloride to potassium glutamate. This demonstrates that the poly(A) polymerase activity of Cid1 is increased when potassium glutamate is used. **(b)** A buffer containing 80 mM potassium glutamate was used in the same poly(A) assay as described previously. Cid1 shows robust poly(A) activity in this buffer but Cid12 proteins display no activity.

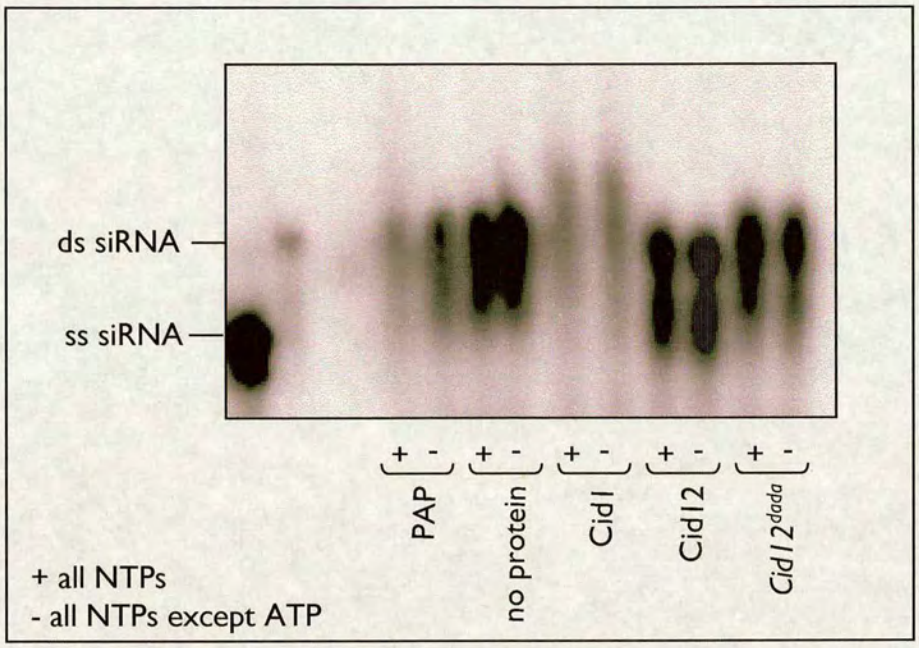


Figure 5.20. Altering the RNA substrate does not produce poly(A) polymerase activity with Cid12. Using a dsRNA substrate, Cid1 shows modest poly(A) activity. Cid12 shows no activity.

It is also conceivable that perhaps Cid12 requires some particularly modified RNA as a substrate and therefore complex mixtures of native RNA prepared from wild type *S.pombe* were used as substrates. These were split into two fractions; a fraction of total RNA including ribosomal RNA and transcripts above around 200 nts and a fraction of small RNAs including tRNAs and siRNAs. Data shown here used the small RNA fraction as the substrate mixture, although preliminary results with the large RNA fraction were similar. Poly(A) polymerase assays were carried out slightly differently, using ^{32}P labelled αATP and testing for incorporation both by running on an acrylamide gel to look for size differences and by scintillation counting. In this instance, preliminary data suggests that slightly increased incorporation of ATP was observed with Cid12 by scintillation counting but this activity was never robust and not consistently reproducible (Figure 5.21). This requires more rigorous testing.

In several of the assays shown, Cid12 appears to degrade the substrate rather than cause polyadenylation. This is characterised by smears or bands of degraded substrate and decrease in the substrate band. This may indicate that Cid12 may possess nuclease activity although as yet this has not been tested.

Cid12 has been shown to form a complex with Rdp1 and Hrr1. It is possible that Cid12 is only active when associated with these, or other, binding partners and thus no activity is seen with recombinant Cid12 alone. This is entirely plausible as Cid12 itself is not known to possess an RNA binding motif. It has been documented that other poly(A) polymerases such as *gld-2* in nematodes and mammalian cells cannot act alone and may require a partner to first bind the RNA substrate and thus allow polyadenylation (Kwak et al., 2004). To address this issue, a TAP purification of Cid12-TAP protein was undertaken in order to isolate the RDRC and any other binding partners of Cid12. The idea was to use this extract in a poly(A) polymerase assay and see if Cid12 indeed requires to be in a complex in order to produce activity. Cid12-TAP purifications yielded only a single band of the size of

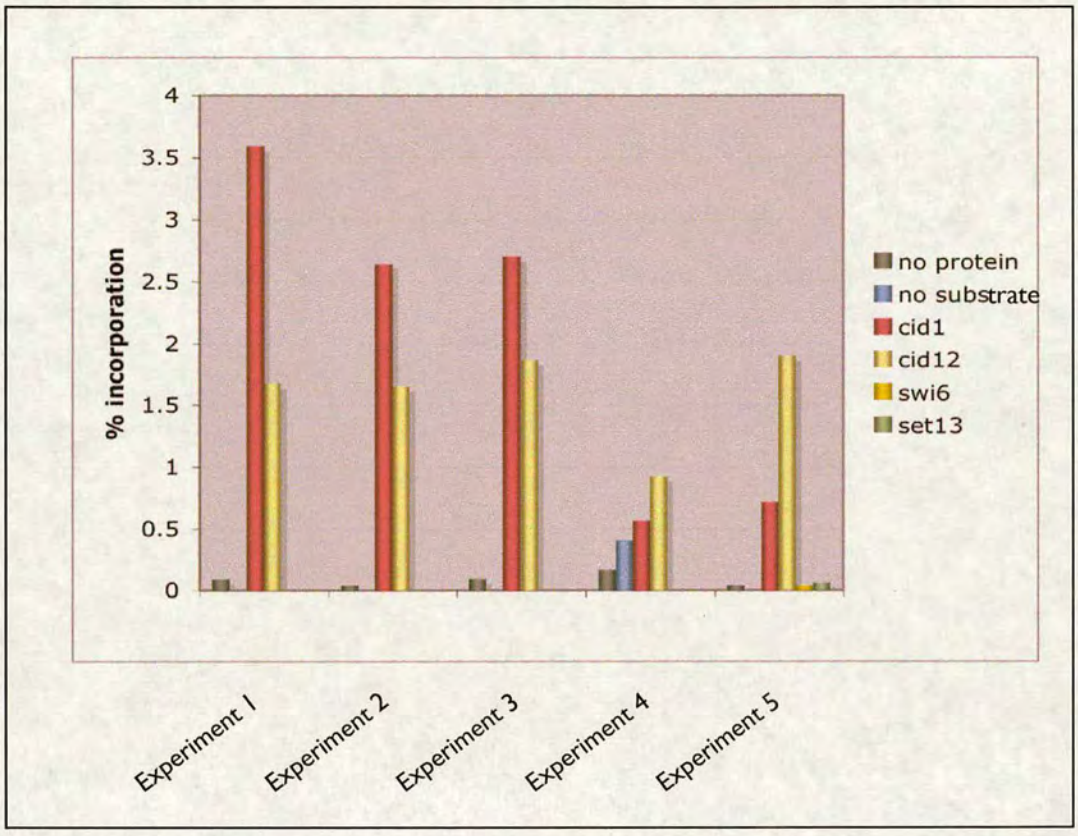


Figure 5.21. Poly(A) polymerase assay using a complex mixture of substrates. Incorporation of nucleotide was assayed by scintillation counting. A complex mixture of RNAs extracted from *S. pombe* were used as a substrate and ^{32}P-αATP was added. Reactions were then TCA precipitated and incorporation was counted. It appears here that Cid12 has some activity however, this was not consistently reproducible. No protein was included in all experiments as a control. In one experiment a no substrate and Swi6 and Set13-His tagged proteins were included as controls against promiscuous binding of nucleotide.

Cid12 protein by silver staining so strains which have Rdp1-TAP were utilised. A TAP purification was performed under conditions known to isolate the RDRC using a strain containing Rdp1-TAP and Cid12-HA and another strain with Rdp1-TAP but Cid12 deleted. These extracts were subsequently used to carry out poly(A) polymerase assays under a variety of conditions as described above. However, no activity was observed under any of the conditions tested although this has not been rigorously investigated.

5.2.11 Polyadenylation of centromere transcripts appears unaffected in *cid12Δ*

If Cid12 is indeed a poly(A) polymerase it seems reasonable to assume that one of its targets would be the centromere repeat transcripts. Centromere transcripts have previously been shown to be polyadenylated and Cid12 has been shown to associate with these transcripts (Djupedal et al., 2005; Motamedi et al., 2004). In order to address the function of Cid12 from another angle, poly(A) RNA was extracted from RDRC mutants to see if any gross changes could be observed by northern analysis as it may be that the poly(A) tails of centromere transcripts would be shortened in a *cid12Δ* mutant. Strand-specific RNA probes were used so as to distinguish between forward and reverse strands (Figure 5.22). As previously observed, wild type shows very little accumulation of centromere transcripts whereas *dcr1Δ* shows significant accumulation. In previous northern analysis using a DNA probe which recognizes both centromere transcripts, little difference in intensity of bands is observed between different RNAi mutants, apart from *hrr1Δ* which consistently appears to accumulate fewer transcripts than other mutants. It is apparent that *rdp1Δ* accumulates slightly more transcripts than a *dcr1Δ* mutant although this is more pronounced using the 5′ probe than the 3′ probe (Figure 5.22). However, these observations must be quantified in order to properly examine the levels of transcripts in different mutants. In addition, one can observe variations between accumulation of transcripts of different sizes. The reason for this is as yet unclear but may reflect the varying roles of the RNAi components within the pathway. No significant

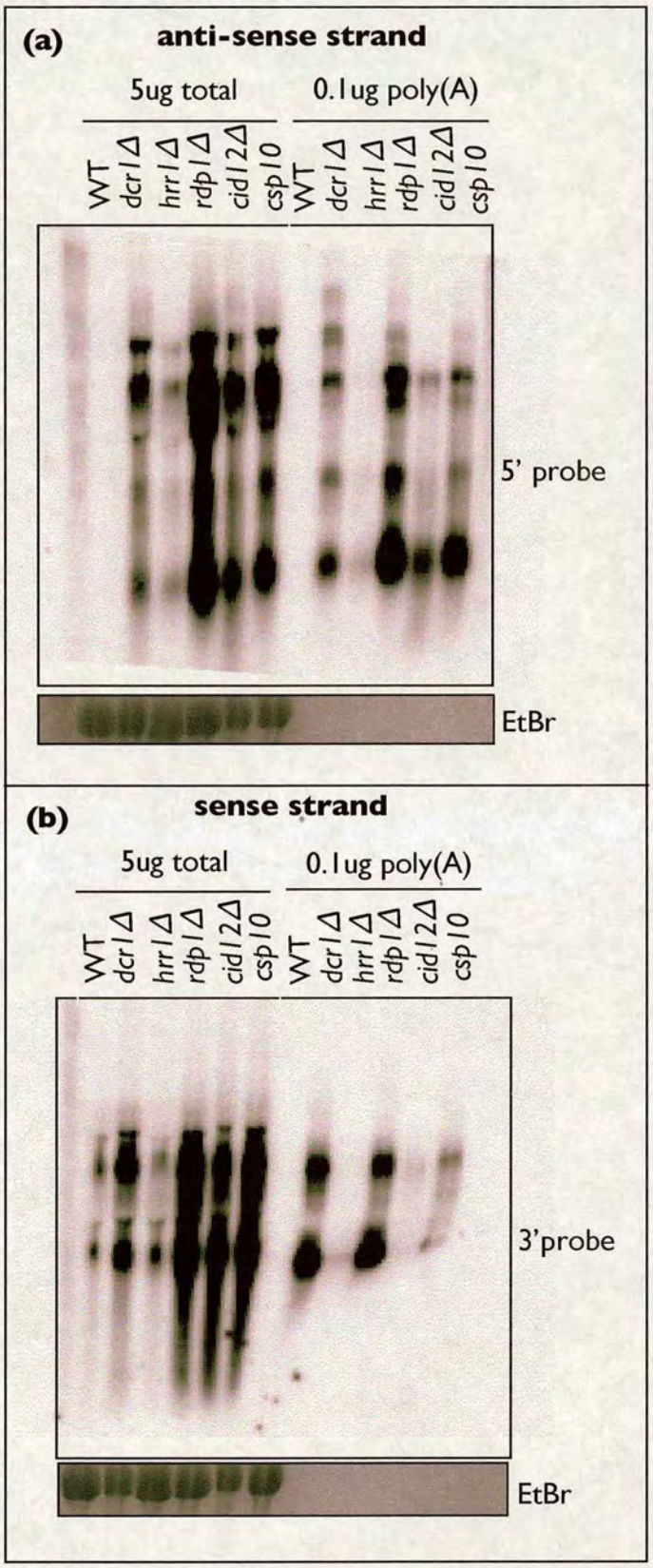


Figure 5.22. Poly(A) transcripts do not show any gross changes in *cid12Δ*. Strand-specific northern analysis demonstrates that *cid12Δ* does not show any gross changes in centromere transcripts with regards to polyadenlyation. However, this method shows that mutants may vary in the amounts of transcript they accumulate as *hrr1Δ* appears to accumulate much less than other components of the RDRC.

differences were seen in a *cid12Δ* mutant either in the total or the poly(A) RNA fraction as compared to other RDRC mutants which indicates that Cid12 may have little or no effect on polyadenylation of centromere transcripts (Figure 5.22).

5.2.12 Cid12 is unable to complement the *S. cerevisiae* mutant *trf4Δ*

Cid14 is the functional homolog of the *S. cerevisiae* poly(A) polymerases Trf4/Trf5 (Buhler et al., 2007). Trf4 is part of the TRAMP complex which is required to polyadenylate RNA transcripts in order to target them for degradation via the exosome (LaCava et al., 2005; Vanacova et al., 2005). Deletion of both Trf4 and Trf5 is lethal however, the enzymatic activity of Trf4 is not required for cell viability as *trf5Δ* cells which contain mutations within the catalytic domain of Trf4 are still viable (Castano et al., 1996; Wyers et al., 2005). Trf4 and Trf5 are thought to have redundant roles within the yeast cells. The same could also be true for Cid12 and Cid14.

To investigate whether Cid12 could complement a known poly(A) polymerase mutant, the following strategy was adopted. The Cid12 ORF was cloned into the *S. cerevisiae* vector pNOPpA which contains a LEU2 marker and an N-terminal TAP tag (a gift from Jon Houseley). This was transformed into a *trf4Δ* strain which expresses Trf5 from a galactose promoter which grows well on media containing galactose but fails to grow on media containing glucose. In addition, this strain was transformed with plasmids containing wild type Trf4 and catalytically dead Trf4, both of which should complement loss of viability when grown on glucose media. Both forms of Trf4 are able to complement the *trf4Δ* strain but Cid12 shows no such complementation (Figure 5.23a). Cid12 protein is expressed at comparable levels to Trf4 and therefore the lack of complementation is not due to lack of protein (Figure 5.23b). This indicates that Cid12 is unable to produce the poly(A), or indeed any, activity required for *S. cerevisiae* viability in the absence of Trf4 and Trf5.

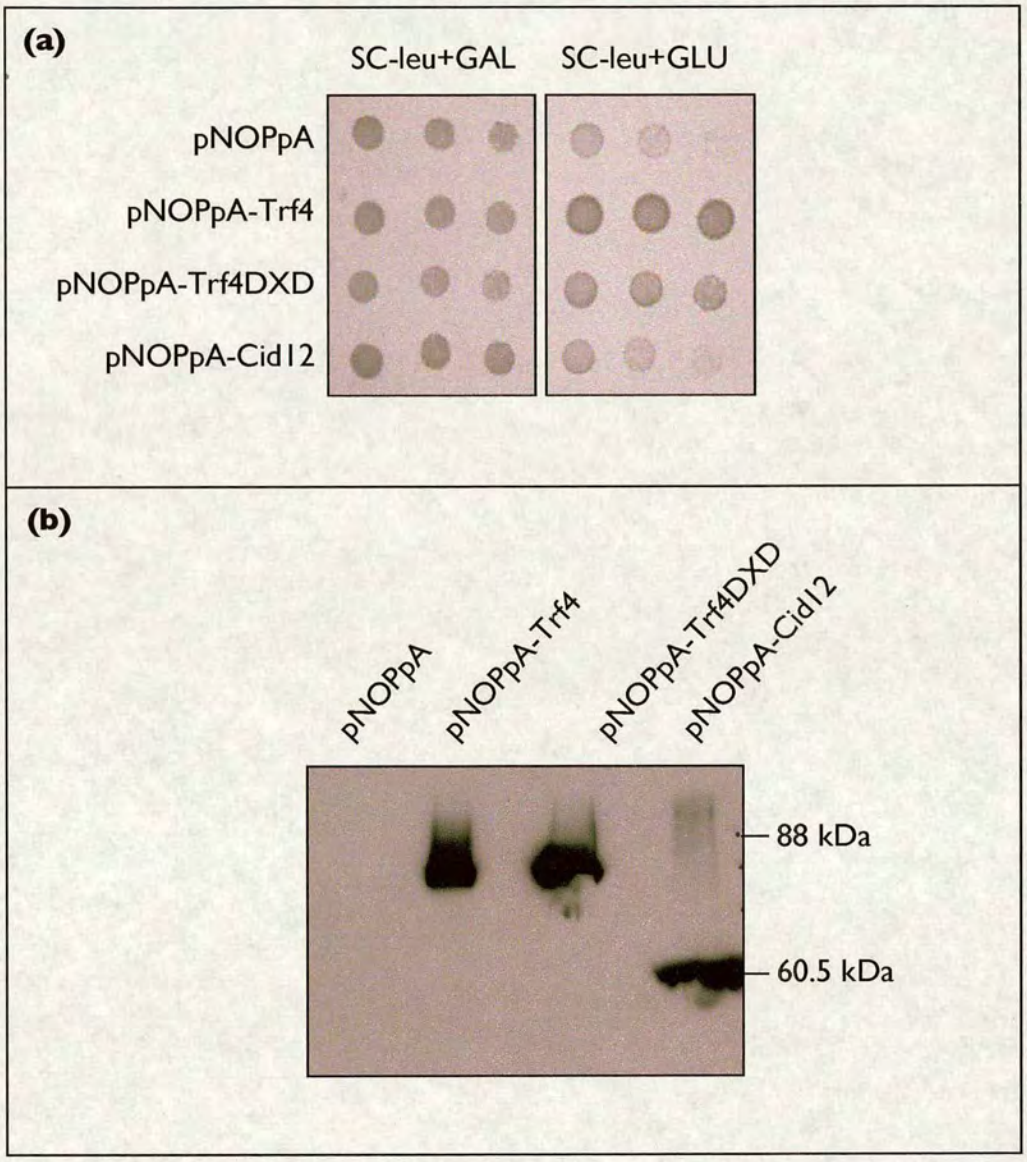


Figure 5.23. Cid12 cannot complement an *S. cerevisiae trf4Δ* strain. (a) Deletion of both Trf4 and Trf5 is lethal in *S. cerevisiae*. The strain used here has Trf4 deleted and Trf5 under the control of the galactose promoter and is therefore not viable when grown on media containing glucose. Transformation of wild type or catalytically dead Trf4 can rescue the loss of viability but Cid12 cannot. **(b)** Western analysis demonstrating that TAP-tagged Cid12 protein is produced to levels equivalent to TAP-tagged Trf4.

5.2.13 Microarray analysis

In order to attempt to identify additional potential RNA targets of Cid12 and to perhaps obtain further insight as to its role *in vivo*, microarray analysis was undertaken in collaboration with the lab of Karl Ekwall (Stockholm). RNA was made from wild type and *cid12Δ* strains which was used to probe the *S. pombe* Tiling 1.0FR array (Affymetrix) (Djupedal, I and Ekwall, K., Karolinska Institute, Stockholm). Unfortunately, no significant differences were observed other than the increased centromere transcripts previously observed by RT-PCR and northern analysis (Figure 5.24). This is surprising as it is known that Cid12 associates with numerous splicing factors and may have been expected to play a more general role in RNA processing. However, it could be that the differences in transcription may be too small to be deemed significant or that this technology is not sensitive enough to detect any changes. Nonetheless, it would seem from this data that the main role of Cid12 is at regulating the levels of centromere transcripts.

5.2.14 Mass spectrometry analysis of Cid12

Mass spectrometry analysis of Cid12-FLAG purifications has been undertaken in collaboration with Alexander Kagansky (Allshire Lab) and Juri Rappsilber (WTCCB, University of Edinburgh). The results of 3 such purifications are documented in Table 5.1. It has previously been documented that Cid12 interacts with splicing factors in an *rdp1Δ* background (Motamedi et al., 2004). In the analyses shown here, it is clear that Cid12 associates with numerous splicing factors. Co-immunoprecipitation experiments have been performed to try and confirm several of these interactions (pers com Elizabeth Bayne, Allshire Lab).

However, these have been unsuccessful which may indicate that the Cid12/splicing factor interactions are transient or may reflect the relative amounts of Cid12 to splicing factors within the cell. As well as splicing factors, Cid12 also displays interactions with various RNA-binding and processing factors. The fact that Cid12 interacts with several RNA binding proteins supports the idea that its activity may be dependant on its

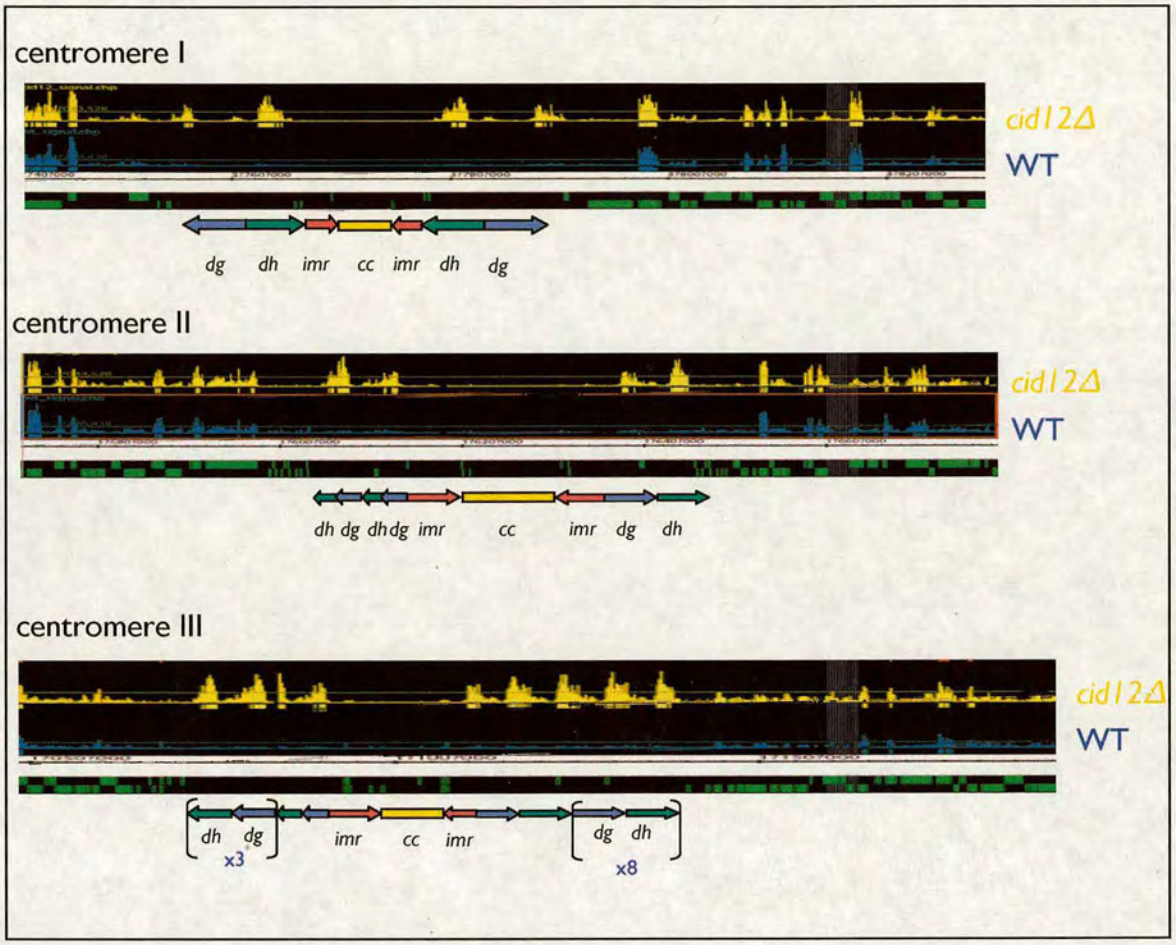


Figure 5.24. Microarray analysis of *cid12Δ*.
A comparison of the transcription of the centromeres between and *cid12Δ* (yellow) and wt (blue). This data shows the mean of the array performed 3 times with seperate samples. Green bars shows annotated genes. The position of the centromere domain bars are approximate.

Protein	Average peptide number
Splicing factors	
Brr2	22
Cwf11	20
Cdc5	17
Cwf4	11
Cwf3	9
Cwf13	7
Prp17	7
Cwf17	6
Cwf5	6
Cwf7	6
SPBC3E7.13 splicing factor, SYF2 family	6
Cwf2	5
Cwf12	5
Lea1	4
Cwf19	4
Prp22	3
Cwf21	3
Prp10 *	3
Cwf15	3
Prp43	2
Cwf18	2
RDRC	
Rdp1	16
Cid12	13
Heatshock proteins	
Hsp16	4
Ssa1	4
RNA	
Rsd1	3
Puf3	3
SPAC1486.03 RNA-binding	3
SPAC20H4.09 ATP-dependant RNA helicase	2
Other	
ilv1	4
gpd3	4
cka1	3
cyp1	3

Table 5.1 Mass spectrometry of Cid12-FLAG.
Mass spectrometry analysis of Cid12-FLAG purifications shows that Cid12 associates with many splicing factors as well as the RDRC. In addition, Cid12 also associates with proteins involved in RNA processing as well as several others. This table shows the average number of peptides identified in three experiments. Of particular interest is *prp10,* marked with an asterisk, as this have been shown to affect heterochromatic gene silencing at centromeres (Elizabeth Bayne, Allshire Lab).

association with a binding partner. The presence of RNA-helicases within the purifications also may support a role for Cid12 in more general RNA processing. These interactions must be confirmed and their relevance is still under scrutiny but nonetheless these analyses offers some insights into the role of Cid12 within the cell.

5.2.15 Cid12 may play a role at the central core

It has recently been documented that as well as transcripts originating from heterochromatic outer repeats at centromeres, a low level of transcription also occurs across the central core (ES Choi and R Allshire, pers. comm). Preliminary results from qRT-PCR analysis show that *cid12Δ* cells accumulate a significant amount of these central core transcripts compared to other Cid1-family mutants and a component of the nuclear exosome, *rrp6Δ* (Eun Shik Choi, Allshire Lab) (Figure 5.25b). These transcripts are not detectable by microarray and thus would not have been detected in the experiment detailed in 5.2.13.

To test whether Cid12 plays a role in central core silencing, *TM1::arg3* and *TM1::ura4* gene insertions were used. Initially, *cid12Δ* was compared to other Cid1-family member deletions containing the *TM1::arg3* insertion to test alleviation of silencing. It appears that *cid12Δ* is the only member of the Cid1-family to alleviate silencing at the central core (Figure 5.26a). This alleviation is comparable to *sim3*, a mutant isolated for its ability to alleviate silencing at the central core. To ensure that this is not specific to the *arg3*$^{+}$ gene and to examine if other members of the RDRC act in the same fashion, the *TM1::ura4* insertion was also used. It appears that with respect to *TM1::arg3*, *dcr1Δ*, *cid12Δ* and *csp10* alleviate silencing at the centromere although in this experimental set-up, the effect of *cid12Δ* is not so strong (Figure 5.26b). With respect to *TM1::ura4*, *dcr1Δ* does not appear to alleviate silencing but all of the RDRC mutants fail to grow on FOA and grow strongly on –ura plates (Figure 5.26b). However, this may simply reflect a slight alleviation at the central core as *clr4Δ*, *swi6Δ* and *rik1Δ* display this same phenotype but *ura4* transcripts are not seen to increase (Ekwall et al

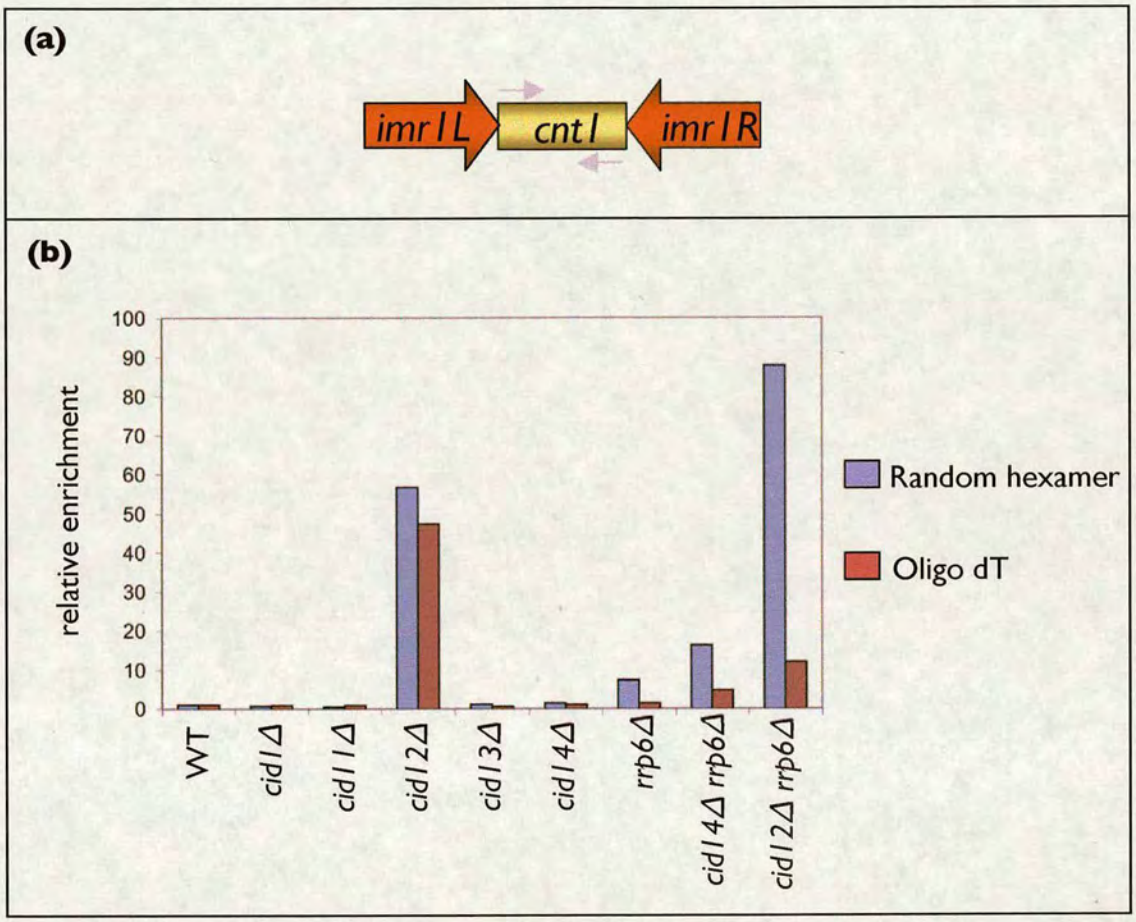


Figure 5.25. qRT-PCR analysis of central core transcripts.
(a) Schematic diagram showing the position of central core primers specific to centromere I used in qRT-PCR. **(b)** qRT-PCR analysis using RNA primer with both random hexamers and oligo dT. *cid12Δ* and *cid12Δ/rrp6Δ* both show a significant accumulation of transcripts originating from the central core (Eun Shik Choi).

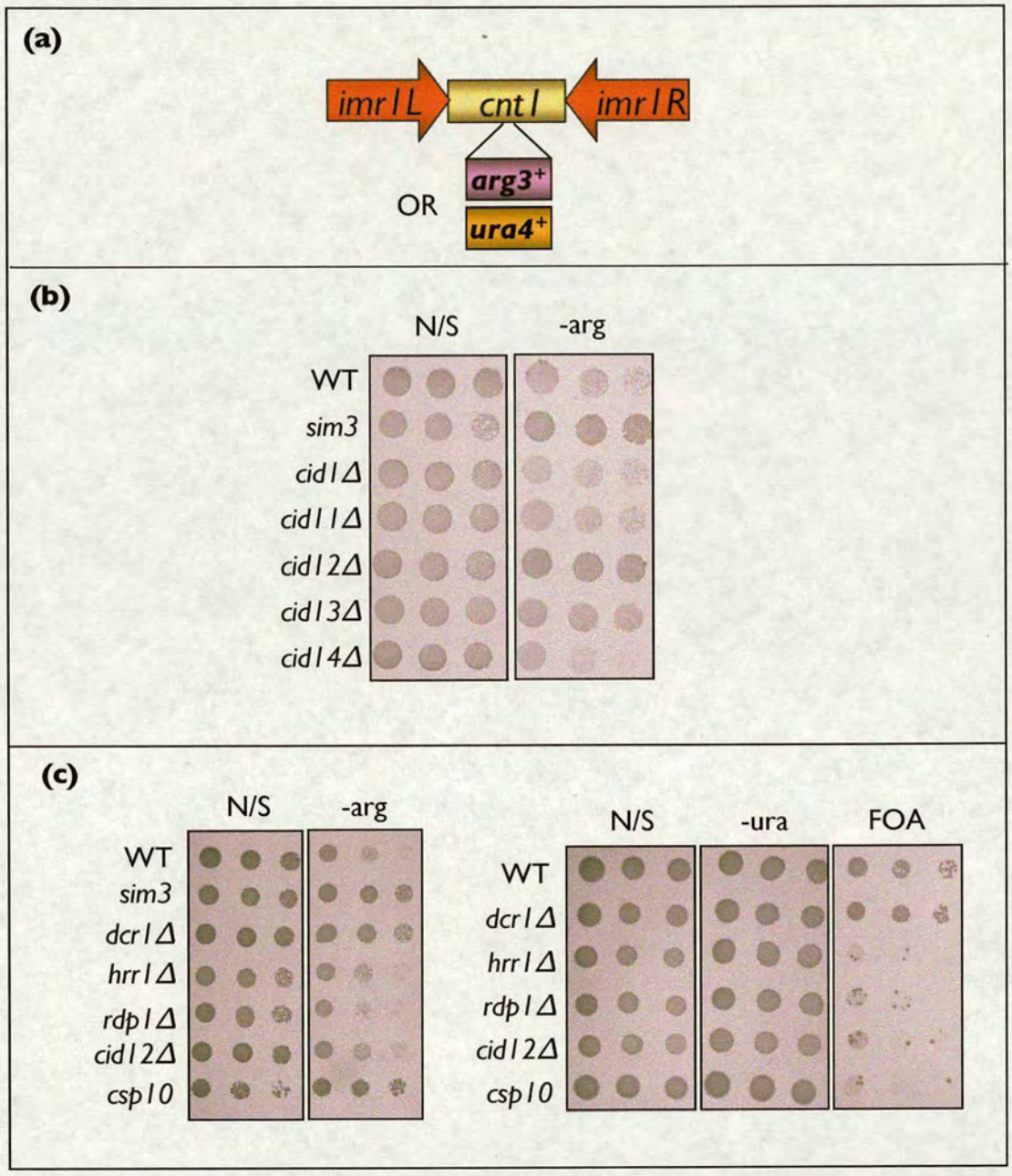


Figure 5.26. *cid12Δ* appears to alleviate silencing at the central core. **(a)** A schematic diagram showing the marker gene insertions, *TM1(Nco1):arg3* and *TM1(Ncol):ura4*. **(b)** *cid12Δ* appears to alleviate silencing of *TM1(Nco1):arg3* at levels comparable to *sim3*, a mutant known to affect central core silencing. **(c)** Components of the RDRC appear to alleviate silencing at the central core. This may be a non-specific effect as other heterochromatin components are known to appear to allevate silencing of *TM1::ura4* with no increase in *ura4* transcription.

1995). It may be that a very slight increase in *ura4* transcription is enough to allow increased growth on –ura plates.

Another way to test the integrity of the central core is to examine Cnp1 occupancy by ChIP. In many mutants which have defective central core silencing, Cnp1 fails to associate correctly. ChIP was carried out on the components of the RDRC to investigate the association of Cnp1 with the central core. No change in Cnp1 association is evident in any of the RDRC components which may be expected as Cnp1 has been shown to be localised correctly, at least in *rdp1Δ* and *cid12Δ* mutants by immunofluorescence (Figure 5.27 and Figure 4.13). Additionally, it is known that in mutants which show comparatively high levels of central core transcripts Cnp1 levels are unaffected, indicating that it may be transcription and not the transcripts themselves which is important for Cnp1 deposition (Eun Shik Choi, Allshire Lab).

5.3 Discussion

I have demonstrated that Cid12 is required to maintain silencing of marker genes inserted at centromeric outer repeats. Cid12 may also affect silencing at the central core but this must be investigated more fully. *cid12*Δ cells accumulate non-coding centromere transcripts and fail to generate centromeric siRNAs. In addition, *cid12*Δ cells display defective chromosome segregation. The enzymatic activity of Cid12 remains unknown and I have as yet failed to demonstrate any ATPase or poly(A) polymerase activity of the protein. Cid12 is unable to complement an *S. cerevisiae trf4*Δ/*trf5*Δ strain and therefore may lack poly(A) polymerase activity. Cid12 associates with splicing factors although its direct interactors and function with respect to splicing is not known.

Cid12 clearly contributes to RNAi-mediated heterochromatin formation in *S. pombe*. However, its exact role has yet to be identified. Cid12 may be required for the specific processing of non-coding transcripts originating

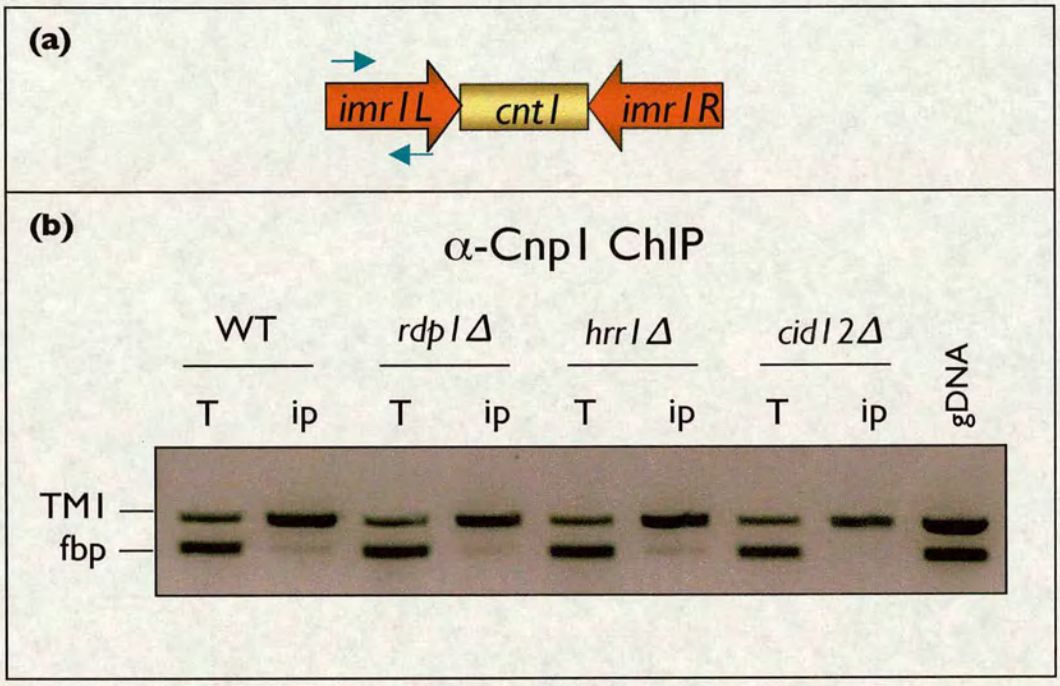


Figure 5.27. Cnp1 associates with the central core in RDRC mutants. **(a)** Schematic representation of primers used in this assay. **(b)** Cnp1 is able to associate with the central core in all of the RDRC mutants at a level comparable to that observed in wild type cells.

from heterochromatin or may aid with the provision of a template for Rdp1 (Figure 5.28). In light of recent evidence that Cid14 may act to degrade heterochromatic transcripts it is possible that these proteins perform overlapping roles within the cell (Bayne et al., 2007; Buhler et al., 2007).

So far, the enzymatic activity of Cid12 has proven elusive. It is possible that Cid12 may be a DNA polymerase or may possess nuclease activity, although these activities remain to be tested. Several poly(A) polymerase assays indicate that Cid12 may in fact degrade the substrate rather than polyadenyate it but it must be confirmed that this effect is due specifically to Cid12 rather than a contaminant of the protein extract. One explanation as to why no Cid12 activity has been found may be that it requires other proteins for its activity. Indeed, robust poly(A) polymerase activity of Trf4 requires other members of the TRAMP complex to bind the RNA substrate as do the mammalian GLD-2 homologs which require an RNA binding protein for their activity (Kwak et al., 2004; LaCava et al., 2005). However, the fact that catalytically dead Trf4 is still able to complement the *trf4Δ/trf5Δ* mutant suggests that Trf4 may possess another activity which can compensate for the loss of its poly(A) polymerase activity. It would be interesting to carry out eletrophoretic mobility shift assays to examine whether Cid12 alone or with its binding partners bind RNA.

Initial attempts to use a TAP-purified Cid12 complex for *S. pombe* in a poly(A) assay to look for elongation of an RNA oligo were unsuccessful although this approach is worth pursuing. It could be that conditions used for purifying Cid12-FLAG protein for mass spectrometry may be useful with respect to poly(A) and ATPase assays. It may be that the function of Cid12 differs when it is associated with splicing factors as opposed to the RDRC. Alternatively, it may be that Cid12 provides a link between the splicing machinery and the RNAi pathway as it has been demonstrated that splicing factors also affect siRNA production (Elizabeth Bayne, Allshire Lab).

The cellular RNA targets of Cid12 remain elusive. One can presume that it

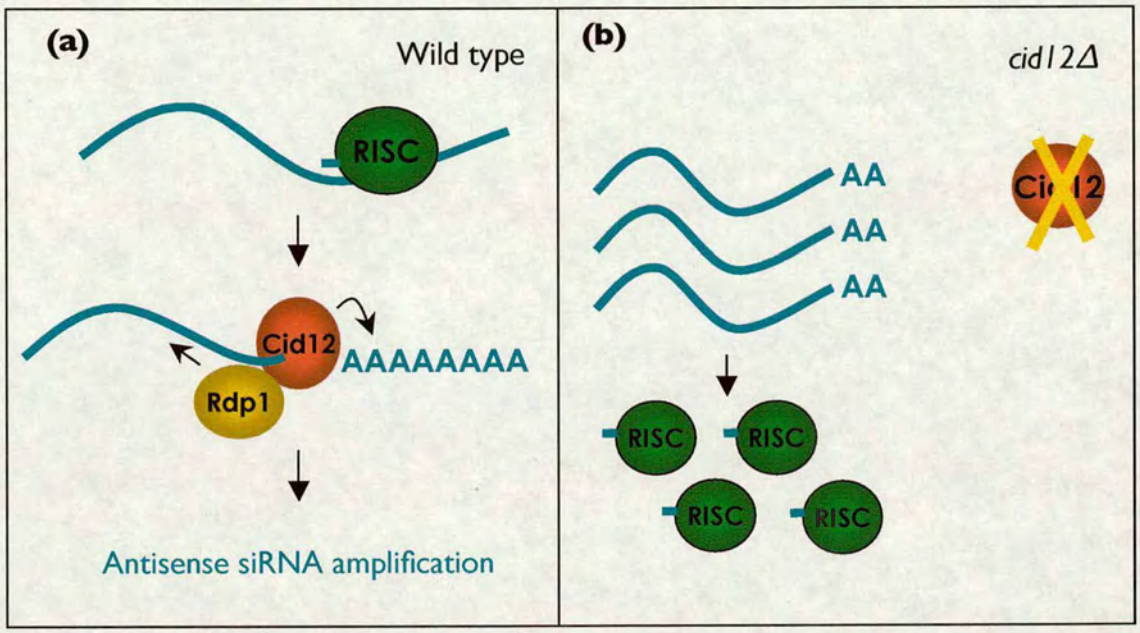


Figure 5.28. Possible roles of Cid12. **(a)** siRNAs target an initial round of mRNA cleavage. Ago1 'slices' the nascent transcript exposing the 3' end for polyadenylation by Cid12. This may stabilise the intermediate and allow siRNA amplification via Rdp1. **(b)** In the absence of Cid12, aberrant RNA accumulation could lead to activation of silencing pathways. Transcripts with short (or absent) poly(A) tails may allow production of 'aberrant' siRNAs thus titrating out factors required for efficient RNAi.

will act on the centromeric non-coding RNA but its action on these transcripts is still unknown. One way to investigate this would be to tether Cid12 to a piece of centromeric RNA and examine how/if the RNA changes with respect to polyadenylation and cleavage. Another way would be to pull down and identify the RNAs associated with Cid12, and/or the RDRC although given that Cid12 also interacts with splicing factors it may be difficult to observe any specific effect.

Cid12 has been demonstrated to interact with numerous splicing factors. It may be that Cid12 has several roles in the cell or acts on many RNAs as a general processing factor. If Cid12 acts on many transcripts, as one might expect from interactions with splicing factors, one would expect to see many transcripts either up or downregulated although this is not the case. Cid12 could act on non-coding RNA transcripts which are expressed at a very low level and are therefore undetectable in comparison to the high levels of centromere transcripts observed. However, it has been proposed that a sub-spliceosomal complex may act as a platform for siRNA amplification as mutations in specific splicing factors impairs processing of centromeric transcripts and siRNA production. Cid12 may interact with a specific pool of splicing factors to facilitate RNAi-mediated silencing.

There are many questions remaining about the role of Cid12 within the cell. For instance, does a 'catalytically dead' Cid12 protein still associate with the other members of the RDRC and RITS complexes? Is the RDRC complex still capable of RNA polymerase activity or is Cid12 activity also required? It may be that the complex is unstable when Cid12 is mutated. In light of the fact that the *Cid12*^{dada} mutant is unable to produce stable protein in *S. pombe*, it is possible that the stability of the protein depends upon its interaction with binding partners or it may simply be that tagging at the C-terminus renders the mutant protein, but not the wild type, unstable. One would assume that if the instability of *Cid12*^{dada} were due to gross misfolding that the protein would not be produced in *E. coli* but this is not the case. The production of a Cid12 antibody will undoubtedly be useful in solving some of these issues.

However, it would also be beneficial to try and tag Cid12 on its N-terminus. More work is required to investigate and solve these issues.

It would be interesting to investigate under which circumstances Cid12 associates with proteins other than those in the RDRC and whether these interactions also impact on heterochromatin formation. The role of several splicing factors in heterochromatic gene silencing is being investigated in the lab although efforts to identify with which factors Cid12 associates directly with have proven inconclusive (Elizabeth Bayne, Allshire Lab). This may be due to the fact that Cid12 may be expressed at much lower levels than the splicing factors, or may indicate that these interactions are transient. The answers to these questions will ultimately elucidate the role of Cid12 in RNAi-directed heterochromatin formation in fission yeast.

CHAPTER 6: DISCUSSION AND CONCLUSIONS

Fission yeast centromeres are similar to their more complex metazoan counterparts in composition and organisation and therefore provide an excellent model for the dissection of centromere structure and function. Metazoan centromeres appear to be epigenetically regulated in that they do not seem to require a defined DNA sequence for their formation and kinetochores can be formed on 'non-centromeric' DNA (Karpen and Allshire, 1997). However, it has been demonstrated in mammalians cells that *de novo* centromere formation requires the presence of α-satellite DNA containing binding motifs for the centromere protein CENP-B (Okada et al., 2007). Primary sequence may contribute in some way to define the site where centromeres are formed as centromere associated DNA is frequently AT-rich and highly repetitive (Sullivan et al., 2001).

Heterochromatin is associated with regions of highly repetitive DNA, such as centromeres and telomeres. The heterochromatin structure is thought to contribute to genome stability by reducing recombination and transcription across these important regions (Buhler and Moazed, 2007). However, it has been demonstrated in plants that transcription occurs across centromeric repeats and induces DNA methylation of these repeats (Ebbs et al., 2005). Furthermore, RNA transcripts from α-satellite DNA in human cells have been shown to be required for the correct localisation of centromere proteins (Wong et al., 2007). The outer repeats of fission yeast centromeres (and homologous regions) are transcribed and processed by the RNAi machinery which causes H3K9 methylation via Clr4 and effects silent chromatin formation at these regions (Buhler and Moazed, 2007). Paradoxically, it appears that transcription is a requirement for heterochromatin assembly and thus transcriptional repression.

RNAi, or post-transcriptional gene silencing (PTGS), is a conserved phenomenon which occurs in mammals, plants and fungi and plays important roles in diverse processes such as chromatin remodelling and

antiviral defence (Hannon, 2002). In fission yeast, it is clear that the RNAi pathway feeds back onto chromatin to induce H3K9 methylation (Buhler and Moazed, 2007). In plants it has been demonstrated that genes may be silenced both transcriptionally and post-transcriptionally (Matzke et al., 2001). It has been shown that siRNAs guide the methylation of homologous DNA sequences thus regulating both transgene and endogenous gene transcription (Matzke et al., 2007). Transcriptional gene silencing also occurs in *Drosophila* and *C. elegans* (Finnegan and Matzke, 2003). In human tissue culture cells it has been reported that siRNA can transmit DNA methylation, H3K9Me and transcriptional silencing to homologous promoter-CpG island/DNA (Bayne and Allshire, 2005).

A general model now exists whereby dsRNAs are processed by RNaseIII Dcr proteins into siRNAs which become incorporated into RISC (or RITS) and promote silencing via mRNA degradation, translational repression or chromatin remodelling (Filipowicz et al., 2005). However, there are many subtleties that are apparent in different organisms and in the way which distinct pathways induce gene silencing. Most organisms contain several copies of the key components of the RNAi pathway, Argonaute, Dicer and RNA-dependant RNA polymerase, and these different proteins are involved in specific pathways (Qi and Hannon, 2005). For instance, *Arabidopsis* has four Dicer-like proteins and two of these are known to produce siRNA populations of two discrete size classes (Qi and Hannon, 2005). *Drosophila* contains two Dcr proteins which are involved in generating siRNAs and miRNAs (Filipowicz et al., 2005). Fission yeast is an attractive model for dissecting events that occur during RNAi-mediated heterochromatin formation as it contains only one copy of each of the key components.

The heterochromatic element of fission yeast centromeres has allowed the development of screens which utilise the transcriptional repression across this region as a readout for chromatin integrity and thus centromere function. In this thesis I have detailed the identification and analysis of

several mutants produced using such a screen. In addition, I describe in-depth analysis of Cid12, a putative poly(A) polymerase required for effective RNAi-mediated heterochromatin formation at centromeres.

6.1 Identifying novel factors affecting centromere function

Silencing based screens to identify novel factors involved in centromere structure and function have proven to be highly successful. Previous screens for mutants that affect outer repeat silencing and silencing in the central domain (*csp* and *sim* mutants respectively) have resulted in the identification of genes that affect the integrity of these specialised regions within the centromere (Ekwall et al., 1999; Pidoux et al., 2003). The *csp* screen has uncovered previously unknown links between transcription, RNA processing as well as RNAi components, and heterochromatin formation (Djupedal et al., 2005; Volpe et al., 2003). However, as with many such screens, the identification of the affected genes has been a limitation. Several different approaches can now be employed to identify additional components involved in centromere function.

The Shimoda library used to identify the *csp* mutants was chosen because it has a relatively small insert size and high level of genome coverage (Tanaka et al., 2000). It is possible that the genes mutated in the uncloned *csps, 1, 2, 6, 11* and *13,* have been disrupted during construction of the library and therefore these *csps* will not be identified in this way. However, it is more likely that the high-copy number of the genes affecting the *csp* mutants interferes in the silencing pathway and therefore these plasmids do not display complementation. Several other libraries have been used to try and identify the *csps* but the transformation efficiency has always been low compared with the Shimoda library and no complementing plasmids were identified. Mating the *csp* mutants with likely candidates is not helpful in this instance as upon self-mating the *csp* mutants variegate making genetic analyses difficult. However, the recent advent of high throughput sequencing technology makes it possible to sequence the entire genome of a

mutant in order to identify the affected gene. This approach is currently underway to uncover the identity of the remaining *csps*. Hopefully this will reveal as yet uncharacterised genes.

Several other approaches can be applied and are indeed underway to identify additional factors involved in heterochromatin formation. A screen similar to the *csp* screen is currently being carried out (Femke Simmer and Alessia Buscaino, Allshire Lab). A tester strain containing a marker gene within the centromeric outer repeats has been mutagenised using UV irradiation and temperature-sensitive colonies alleviating silencing at 36°C, but not at 25°C, have been isolated. This provides a practical way of regulating silencing in a particular mutant to follow the order of events over time. These are being further characterised and efforts are being undertaken to identify the mutated genes.

Since the generation of the *csp* mutants, a commercial knockout library has become available (Bioneer). This library contains over 2700 non-essential genes knocked–out with a known marker gene. High throughput screening by mating of this library to a tester strain has identified a number of genes that affect centromere silencing to some extent. This includes expected genes such as *clr4* but did not isolate all previously known factors. As well as transcription factors and DNA binding factors, several sequence orphans were found. Obviously these are of particular interest as they may be novel factors involved in heterochromatin assembly. Several of these can be examined further and some may well correspond to the genes affected in the *csp11* and *csp13* mutants (Elizabeth Bayne and Dominika Bijos, Allshire Lab).

An alternative approach to identify additional factors that may be involved in centromere function has been to use comprehensive analyses of affinity purified proteins by mass spectrometry. This approach has been used with great success to identify members of a particular complex (Buker et al., 2007;

Hong et al., 2005; Horn et al., 2005; Jia et al., 2005; Motamedi et al., 2004; Verdel et al., 2004). Weaker interactions with other factors can also be identified using less stringent conditions (Alexander Kagansky, Allshire Lab and Juri Rappsilber, University of Edinburgh). Members of each of the complexes known to be important for heterochromatin assembly, RITS, RDRC, HULC, CLRC, are being purified in order to identify interactions between complexes as well as factors not previously known to affect silencing. It is anticipated that this will help to build up a network of interactions in parallel with screens of knockout libraries.

6.2 The *csp* genes are involved in RNAi-mediated heterochromatin formation

6.2.1 RDRC and transcription

The RNAi pathway in *S. pombe* has been subject to extensive investigation. In Chapter 4, I described the identification of several of the affected genes in *csp7, 9, 10* and *12*. Rdp1, Ago1 and Cid12, *csp7, 9* and *10* respectively, have all been shown to be essential for heterochromatin formation at centromeres. Rdp1 and Cid12 are part of the RDRC complex which has been shown to interact with the RITS complex and associate with the non-coding centromeric RNA transcripts (Motamedi et al 2004). The RDRC also contains the putative helicase Hrr1. All of the components of the RDRC are required for the correct processing of centromeric transcripts into siRNAs and therefore fail to form correct heterochromatin structures at centromeres (Motamedi et al 2004; Sugiyama et al, 2005). In addition, it has been shown that truncation of part of the conserved polymerase domain or point mutation in a predicted catalytic residue of Rdp1 causes loss of transcriptional silencing and heterochromatin formation (Motamedi et al 2004; Sugiyama et al, 2005). *csp7* and *csp10* also display loss of transcriptional silencing and heterochromatin formation and as such behave as the null alleles.

Rdp1 is required for efficient RNAi in *C. elegans*, plants and fungi (Hannon, 2002). Mammals and *Drosophila* lack any Rdp1 homologs, but *Drosophila* S2 extracts have been shown to possess RdRP activity (Lipardi et al., 2005; Stein et al., 2003). However, it may be that mammals can acquire such activity from viral RdRPs and it has been demonstrated in mouse oocytes that RdRP is not required for an effective RNAi pathway (Stein et al., 2003). In plants and *C. elegans*, the RdRP proteins act to amplify the siRNA signal by utilising the mRNA target as a template (Sijen et al., 2001). In *S. pombe*, surprisingly little is known about the action of Rdp1 and the RDRC compared to RITS. It has been documented *in vitro* that Rdp1 possesses RNA polymerase activity but the target of this activity, although assumed to be the centromeric transcripts, has not been defined (Sugiyama et al., 2005). It has been hypothesised that Rdp1 may use the noncoding transcripts as a template for dsRNA generation thus providing a source for Dcr1-mediated siRNA amplification. However, it is also possible that the sense and antisense noncoding transcripts may anneal and in this way provide a source of dsRNA.

The RDRC associates with both centromeric DNA and RNA (Motamedi et al, 2004; Volpe et al 2002). Many RNA processing events are linked to transcription, such as capping, splicing and polyadenylation (Neugebauer, 2002). It may be that the RDRC plays a role in the regulation of centromere transcript levels either by causing their degradation via the action of Cid12 (and splicing factors) or their amplification via the action of Rdp1. Cid12 is a putative poly(A) polymerase which associates with subunits of the spliceosome (Stevenson and Norbury 2006; Motamedi et al 2004, pers comm. Elizabeth Bayne and Alexander Kagansky, Allshire Lab). This may indicate a role for Cid12 in general RNA processing. The possible functions of Cid12 will be discussed later in this chapter.

As the RDRC also contains a helicase, Hrr1, it seems clear that the complex is somehow involved in RNA processing, at least of the non-coding

centromeric RNA transcripts. Hrr1 belongs to the DEAD/DEAH box family of helicases which are involved in all aspects of RNA metabolism from transcription to RNA decay and nucleocytoplasmic transport (Motemedi et al, 2004). Deletion of Hrr1 may cause accumulation of fewer centromere transcripts than observed in other mutants in components of the RNAi pathway (see Chapter 5 Figure 5.22). Hrr1 could aid transcription of the centromeric outer repeats by unwinding DNA and allowing RNAPII access. Alternatively, Hrr1 may act to induce conformational changes in the RNA substrate of Rdp1 and thus enhance its processivity. Furthermore, Hrr1 has been found to associate with Ago1, albeit in substoichometric amounts (Motamedi et al, 2004). In human cells, RNA helicase A has been shown to act as an siRNA loading factor for RISC, perhaps by unwinding the siRNA duplex (Robb and Rana, 2007). Also in human cells, the P68 helicase has been shown to be required for the unwinding of the let-7 microRNA precursor duplex but does not act upon a related siRNA duplex (Salzman et al., 2007). It may be that Hrr1 is required to unwind siRNAs prior to their loading into Ago1 in the RITS complex. More analysis of both the RDRC and centromere transcription is required to address these issues.

6.2.2 Argonaute complexes and small RNA

Argonaute proteins have been shown to mediate siRNA-directed RNA cleavage in RISC complexes and siRNA-directed transcriptional silencing in RITS (Hammond et al 2001; Verdel et al 2004). In addition, Argonaute proteins mediate miRNA-directed translational repression (Filipowicz et al., 2005). The outcome of these processes is ultimately the downregulation or silencing of the target mRNA message. This is the critical event in the RNAi pathway. Contrary to this, it has been shown that miRNAs are able to induce upregulation of translation during cell cycle arrest expanding their role in regulation of expression (Vasudevan et al., 2007)

Argonaute proteins are essential for efficient RNAi in *C. elegans*, plants, fungi and *Drosophila* (Hannon, 2002). Complex metazoans contain several

Argonaute proteins which have distinct functions. In *Drosophila*, which has five Argonaute proteins, Ago2 is required for siRNA-mediated RNAi and Ago1 is required for miRNA biogenesis and thus miRNA-mediated RNAi (Okamura et al., 2004). In *Arabidopsis*, 10 Argonaute proteins exist and eight of these contain a motif characteristic of the 'slicer' domain, suggesting that these Argonaute proteins may interact with different subsets of small RNAs which contribute to different RNAi related silencing pathways (Qi and Hannon, 2005).

In fission yeast, the single Ago1 is required for the correct processing of centromeric transcripts, siRNA production and heterochromatin formation. Argonaute proteins contain a PAZ domain, which binds siRNA, and a PIWI domain which is specific to the Argonaute family (Buker et al 2007). As yet, the small RNA species which have been identified in fission yeast all appear to be siRNAs, however the presence of miRNAs cannot be ruled out (pers. comm. Elizabeth Bayne, Allshire Lab). The PIWI domain of Ago1 has been shown to be required for its 'slicing' activity which mediates cleavage of its target mRNA (Irvine et al, 2006; Buker et al., 2007). This 'slicer' activity is required for efficient RNAi-mediated transgene silencing (Irvine et al, 2006 ;Buker et al., 2007). It is not known whether translational repression occurs in fission yeast. The RITS complex has been shown to localise to centromeric RNA transcripts and to be required for the localisation of RDRC to these transcripts (Motamedi et al, 2004). As expected, *csp9* also shows loss of processing of centromere transcripts and siRNA production and behaves like an *ago1*Δ mutant. More recently, Ago1 has been shown to be part of another complex, the ARC complex (Buker et al, 2007). This complex has been proposed to transfer double-stranded siRNAs from Dcr1 to the RITS complex and in this way perhaps play a role in the turnover and regulation of centromere transcripts (Buker et al, 2007).

The other components of the ARC complex are Arb1 and Arb2. Arb1 has homology with maturases which are involved in the self-splicing of introns

(Buker et al., 2007). I have shown that *csp12* contains a mutation in the Arb1 ORF. Both *csp12* and *arb1*Δ display similar phenotypes to the other RNAi and *csp* mutants. In mammalian cells and *Drosophila,* siRNAs are transferred into RISC as a duplex (Preall and Sontheimer, 2005). In *Drosophila,* the 'slicer' activity of Ago2 is required for the cleavage and release of the passenger siRNA and its ejection from RISC (Preall and Sontheimer, 2005). It has been proposed that this may form the basis of the activation of RISC. In fission yeast, the Arb proteins are thought to regulate the conversion of ds siRNA to ss siRNA and hence the transfer of siRNA into RITS (Buker et al, 2007). In keeping with this, Arb1 has been demonstrated to block the slicer activity of Ago1 and allows Ago1 to hold siRNAs in a duplex until transfer into RITS (Buker et al, 2007).

To summarise, the *csp* screen has provided a source of mutants involved in RNAi-mediated heterochromatin formation. These mutants have allowed the investigation of the RNAi pathway in fission yeast although disappointingly at the time of their identification novel factors were not discovered in the non-ts class. However, these mutants have allowed the analyses of phenotypes associated with defective RNAi and the two remaining unidentified non-ts mutants may yet provide more insight into this pathway, although this may be superseded by high throughput screens of non-essential genes.

6.3 Chromatin structure is linked to RNA processing

Much of the focus of this thesis has been to dissect the role of the putative poly(A) polymerase Cid12 in RNAi-mediated heterochromatin formation. There are outstanding issues relating to the mechanism by which Cid12 mediates RNAi-directed heterochromatin assembly as the activity of this protein has proven difficult to ascertain. However, attempts have by no means been exhaustive and there are many experiments which remain to be attempted. Despite this, I have demonstrated here that Cid12 is essential for proper transgene silencing at centromeres, correct chromosome segregation

and is required for the correct processing of centromere transcripts into siRNAs via the RNAi machinery.

The enzymatic activity of Cid12 continues to be elusive. Several poly (A) polymerases require their binding partners for their activity. The *C. elegans* GLD-2 has very low poly(A) polymerase activity alone but in association with GLD-3, an RNA binding protein with no poly(A) polymerase, this activity is significantly enhanced (Kwak et al., 2004). This is also true of the mammalian GLD-2/3 homologs which require to be associated for their activity (Kwak et al, 2004). Indeed, the budding yeast poly(A) polymerase Trf4 has virtually no activity alone but when incorporated into the TRAMP complex it displays robust poly(A) polymerase activity (LaCava et al 2005). It is entirely possible that Cid12 must be in a complex or associate with a binding partner to perform its cellular function. This problem can be addressed as we now have conditions where Cid12-FLAG can be purified with the RDRC and numerous splicing factors (Alexander Kagansky, Allshire Lab). These interactions may provide an environment required for the activity of Cid12 and such purifications can be utilised in assays similar to those described in Chapter 5 to examine if this is the case.

It is conceivable that Cid12 could have a function distinct from that of a poly(A) polymerase. Scrutiny of several poly(A) polymerase assays, where Cid12 specifically appeared to cause degradation of substrate rather than polyadenylation, suggest that Cid12 may possess a nuclease activity. This is further substantiated by evidence demonstrating that the 2′-5′ oligoadenylate synthetase family of enzymes, including the budding yeast Trf4 and fission yeast Cid1, has a conserved C-terminal domain which could have nuclease activity (Rogozin et al., 2003). Nuclease activity of Cid12 remains to be tested and a putative nuclease mutant has been generated that requires further analyses.

The possible role of a nuclease activity of Cid12 may be akin to that of the TRAMP complex (Buhler et al., 2007). In budding yeast, the TRAMP complex polyadenylates RNA and attracts the exosome to cause degradation of structured substrates (Haracska et al., 2005; LaCava et al., 2005). However, another member of the Cid1-family, Cid14, has been shown to form a fission yeast TRAMP complex together with homologs of budding yeast Air1, a RING finger protein which may bind RNA, and Mtr4, an ATP-dependant RNA 5′-3′ helicase (Buhler et al., 2007). It may be that instead of polyadenylating RNA substrates for their degradation, Cid12 may itself degrade noncoding centromere RNA transcripts and/or other unidentified substrates and thus provide an alternative way of regulating transcript levels.

The fission yeast homolog of Trf4 is Cid14 which is proposed to activate Rdp1 by polyadenylating centromere transcripts or their cleavage products (Buhler et al., 2007). However, Cid14 mutants display normal polyadenylation and have not been shown to associate with centromere RNA (Buhler et al, 2007; Wang et al, 2008). It could be that Cid12 and Cid14 perform overlapping roles within the cell similar to that seen in budding yeast with the Trf4 and Trf5 proteins. As yet no double mutants of Cid12 and Cid14 have been analysed but given that *trf4Δ/trf5Δ* mutation is lethal it would be interesting to investigate this.

The budding yeast poly(A) polymerase Trf4 was originally thought to be a DNA polymerase although this has now been refuted and it is apparent that Trf4 is required for polyadenylation (Haracska et al., 2005; LaCava et al., 2005; Wang et al., 2000). However, DNA polymerase activity has not been ruled out for Cid12. DNA polymerase assays should be performed with Cid12 to investigate this although it is unclear how DNA polymerase activity would contribute to heterochromatin assembly. Trf4 has been shown to be required for chromosome condensation, DNA replication and sister chromatid cohesion although exactly how its poly(A) polymerase activity

contributes to these processes is unknown (Wang et al 2000). *S. cerevisiae* Trf4 has been shown to associate with cohesin subunits but Cid12 does not display any such interactions by mass spectrometry analysis (Wang et al., 2000). Such interactions may be transient or occur at an undetectable level (Alexander Kagansky, Allshire Lab).

An alternative explanation as to why Cid12 has not shown activity in any of the assays performed is that it may have a very narrow substrate specificity which has not yet been fulfilled. siRNAs in plants and flies are known to be 2′-O-methylated at their 3′ terminus by methyltransferases such as Hen1 (Matranga and Zamore, 2007). As yet no such modification has been identified in fission yeast but it may be that Cid12 requires this or perhaps a specific length of 5′ or 3′ overhang for its activity on a substrate. This may now be addressed somewhat as Ago1-FLAG purifications which have recently become successful can provide a source of more physiological RNA substrates (Alexander Kagansky, Allshire Lab). In addition, purification of Cid12-FLAG and its associated RNAs may give a clue as to its cellular activities.

Although it has not been proven here that Cid12 has poly(A) polymerase activity, the idea that polyadenylation is important for heterochromatin assembly is still an attractive hypothesis. In *C. elegans*, a protein which shares homology with Cid12, RDE-3, is required for the efficient RNAi and siRNA generation (Chen et al, 2005). In addition, depletion of Ago2 in *Drosophila* results in stabilisation of transgene mRNA with a concomitant shortening of poly(A) tails (Siomi et al., 2005). Whatever its role within the cell it is clear that Cid12, along with the splicing factors, provides a link between RNAi, RNA processing, the formation of heterochromatin and thus centromere function in fission yeast.

6.4 Outlook

Although much is known about RNAi-mediated heterochromatin in fission yeast many issues remain unresolved. The initial discovery that the RNAi pathway directly contributes to heterochromatin formation and function in fission yeast was surprising. However, it is now clear that small RNAs direct chromatin and DNA modifications in a number of systems. Despite the identification of the RITS, RDRC and Rik1/Clr4 complexes our knowledge of how non-coding transcripts are processed to bring about chromatin modifications and heterochromatin assembly is still rudimentary. For instance, it is not known how RDRC contributes to RNA processing and siRNA production. In addition, we do not know how the key histone methyltransferase Clr4 or HDACs are recruited by RNAi factors to bring about methylation of histone H3 on lysine 9 on chromatin homologous to siRNA borne by RITS. It is not known why or how residual H3K9 methylation persists in cells lacking RNAi components. How much methylation is enough? It is known that Swi6 is required to "recruit" cohesin to centromere and other heterochromatin but it is not known exactly how this occurs. Dissection of the process is hampered by the fact the entire RNAi-mediated heterochromatin assembly pathway appears to collapse upon any intervention. New more subtle assays will be required to work out the intricate details of how endogenous transcripts from repetitive DNA elements are processed to siRNAs and how these siRNAs direct chromatin modification to induce silent chromatin assembly.

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RNA interference is required for normal centromere function in fission yeast

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Abstract

In plants, animals and fungi, active centromeres are associated with arrays of repetitive DNA sequences. The outer repeats at fission yeast (*Schizosaccharomyces pombe*) centromeres are heterochromatic and are required for the assembly of an active centromere. Components of the RNA interference (RNAi) machinery process transcripts derived from these repeats and mediate the formation of silent chromatin. A subfragment of the repeat (*dg*) is known to induce silencing of marker genes at euchromatic sites and is required for centromere formation. We show that the RNAi components, Argonaute (Ago1), Dicer (Dcr1) and RNA-dependent RNA polymerase (Rdp1), are required to maintain silencing, lysine 9 methylation of histone H3 and association of Swi6 via this *dg* ectopic silencer. Deletion of Ago1, Dcr1 or Rdp1 disrupts chromosome segregation leading to a high incidence of lagging chromosomes on late anaphase spindles and sensitivity to a microtubule poison. Analysis of *dg* transcription indicates that *csp* mutants, previously shown to abrogate centromere silencing and chromosome segregation, are also defective in the regulation of non-coding centromeric RNAs. In addition, histone H3 lysine 9 methylation at, and recruitment of Swi6 and cohesin to, centromeric repeats is disrupted in these mutants. Thus the formation of silent chromatin on *dg* repeats and the development of a fully functional centromere is dependent on RNAi.

Introduction

Arrays of repetitive DNA are found at active centromeres in many organisms and such repeats are frequently heterochromatic (Richards & Dawe 1998, Sullivan *et al.* 2001). In the fission yeast, *Schizosaccharomyces pombe*, centromeres are composed of outer repeat sequences (*dg* and *dh*) that flank the central kinetochore domain (Matsumoto *et al.* 1990, Takahashi *et al.* 1992, Steiner *et al.* 1993) (see Figure 4C). Deletion analyses and testing of various plasmid constructs

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Loss of Dicer fowls up centromeres

Sharon A. White and Robin C. Allshire

Centromeres, specialized regions on chromosomes, are essential for accurate chromosome segregation during cell division. In fission yeast, the RNA interference machinery has a pivotal function in the assembly of centromeric heterochromatin, which mediates sister centromere cohesion. Studies in vertebrate cells now suggest that many aspects of this process are conserved.

RNA interference (RNAi) is an evolutionarily conserved mechanism whereby double-stranded RNAs (dsRNAs) target homologous transcripts for degradation. Long dsRNAs are cleaved by the endonuclease Dicer to produce small interfering RNAs (siRNAs) of around 21 nucleotides in length. These siRNAs become incorporated into the RNA-induced silencing complex (RISC), which directs them to their homologous RNA target. RNAi functions in several cellular processes, such as gene silencing, protection of genome stability from viruses and transposable elements[1] and the assembly of silent chromatin, including heterochromatin at centromeres[1–3].

Centromeres are sites on chromosomes where the kinetochore complex assembles and mediates interactions with spindle microtubules, thus ensuring accurate chromosome segregation. In many eukaryotes, the kinetochore is embedded in heterochromatin[4]. Human centromeric DNA is primarily composed of α-satellite repeats arranged in tandem arrays of 1,000–5,000 kb[4]. Arrays of perfect alphoid repeat DNA provide a good substrate for the *de novo* assembly of active centromeres. This correlates with the presence of intact binding sites for CENP-B, an α-satellite-binding protein[5]. Remarkably, human centromeres maintain their activity when transferred into chicken DT40 cells by somatic cell fusion to form hybrid cell lines. This suggests that host chicken centromere proteins can recognize and propagate a pre-existing kinetochore on introduced human chromosomes[4].

In fission yeast, the assembly of heterochromatin at centromeres is vital for tight physical cohesion between sister centromeres, and is therefore critical for accurate chromosome segregation[6]. Observations in metazoans also suggest a link between heterochromatin formation and sister chromatid cohesion[6]. Evidence in fission yeast, plants and flies suggests that siRNAs function to recruit histone-H3-Lys-9 methyltransferases, and DNA methyltransferases, to specific loci to direct the chromatin- and DNA modifications required for heterochromatin formation[1–3,7–9]. In fission yeast and in flies, mutations in components of the RNAi pathway result in defective heterochromatin as seen by loss of silencing, loss of specific histone methylation and delocalization of the heterochromatin-associated proteins HP1 or Swi6 (refs 2, 3, 7–11). In fission yeast, defects in the RNAi pathway also cause loss of Rad21 and thus cohesion at centromeres, resulting in aberrant chromosome segregation[5,10,11].

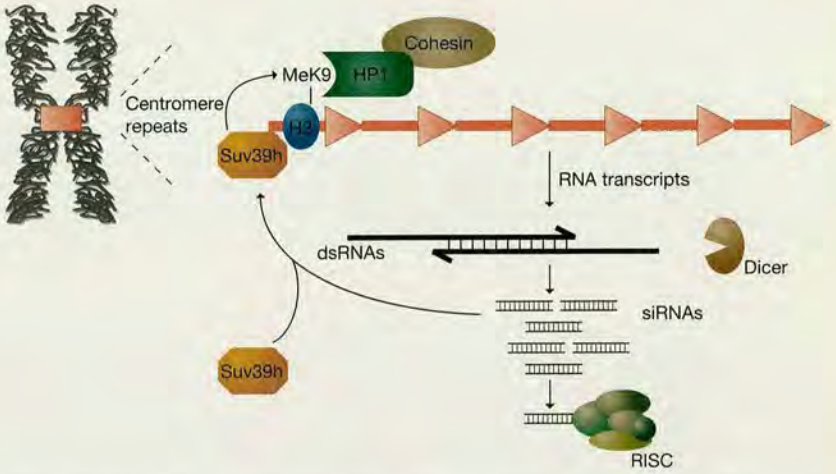

Figure 1. A model for RNAi-mediated heterochromatin assembly in vertebrates. Centromeric non-coding transcripts form dsRNAs that are processed by Dicer to produce siRNAs. Incorporation of siRNAs into RISC recruits the histone methyltransferase Suv39h, which methylates histone H3-K9 resulting in HP1 binding. The formation of heterochromatin results in recruitment of cohesin and ensures accurate chromosome segregation.

It is not known whether RNAi mediates the assembly of centromeric heterochromatin in vertebrates. On page 784 of this issue[12], Fukagawa and colleagues demonstrate that the link between RNAi, heterochromatin formation and centromeric cohesion is conserved in vertebrates. They show that a conditional loss-of-function mutant of Dicer in chicken DT40 cells causes defects in heterochromatin formation and chromosome segregation, similar to those observed in fission yeast. Depletion of Dicer ultimately results in cell death, with an accumulation of abnormal mitotic cells.

Previous analyses demonstrated that a heterochromatin-related structure at mammalian centromeres is RNase sensitive, consistent with a role for RNA in heterochromatin formation[13]. In agreement with this idea, non-coding transcripts homologous to centromeric repetitive DNA elements have been observed in both mouse embryonic fibroblasts and fission yeast[2,14]. In their studies, the authors used a chicken DT40 hybrid cell line carrying human chromosome 21. Consistent with observations in other systems, transcripts homologous to the centromeric repeats of human chromosome 21 clearly accumulate in Dicer-deficient DT40 cells. Extremely low levels of these human centromeric transcripts are also seen in Dicer$^+$ hybrid cells, in accordance with data from fission yeast suggesting that centromeric transcripts are rapidly turned over in wild-type cells[12]. As chicken centromeric DNA has not been characterized, the authors were unable to test if transcripts are also produced from endogenous chicken centromeres.

In fission yeast, centromeric transcripts are processed into siRNAs by the RNAi pathway. The detection of short RNAs (~30

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generations[6]. *A priori*, one might think that these alleles have residual CDC-14 activity. However, the *he118* allele introduces a nonsense mutation that is predicted to eliminate 60% of the protein length[6]. The *he141* allele, which was isolated in a separate polymerase-chain-reaction-based screen, is a deletion that removes 1.2 kilobases of the *cdc-14* gene[6], including critical catalytic residues of the phosphatase domain[2]. The *he141* allele is a null by genetic criteria and produces no protein (as assessed by western blot analysis), suggesting that it is a molecular null[6]. Interestingly, *cdc-14* RNAi in the hands of Saito *et al.* produces a larval phenotype similar to the *cdc-14* mutants with no embryonic lethality or mitotic defects[6].

The studies differ in their conclusions about whether CDC-14 is required for central spindle formation and embryonic viability, and more curiously they even vary in the phenotype produced by *cdc-14* RNAi when the same clones are used to prepare dsRNA[5,6]. Gruneberg *et al.* observed 100% embryonic arrest upon injection of *cdc-14* dsRNA[5]. Saito *et al.*, on the other hand, observed a convincing concurrence in the phenotypes of the null allele, a truncation allele, and RNAi, all of which produced extra postembryonic cells but no embryonic lethality[6]. Two possibilities could account for the divergent *cdc-14* RNAi results. First, cross-RNAi may have inactivated other genes required for mitosis. In *C. elegans*, the RNAi effect is amplified through the action of an RNA-directed RNA polymerase, so that only a few dsRNA molecules per cell can eliminate a vast excess of mRNA[14]. Although there are no *C. elegans* genes closely related to *cdc-14*, cross-RNAi would only require a match of around 22 nucleotides[15]. The potential for cross-RNAi may have been exacerbated by the high level of dsRNA that Gruneberg *et al.* used for injection, that is, 3–5 mg ml^{-1} compared with the more conventional concentration of 0.5–1 mg ml^{-1} (ref. 5). The second possibility is that the two groups used *C. elegans* strains that differ in their susceptibility to loss of CDC-14. Part of the *cdc-14* RNAi analysis by Gruneberg *et al.* and all of the *cdc-14* RNAi analysis in Mishima *et al.* used a temperature sensitive *zen-4*-mutant strain that contains a rescuing *zen-4::GFP* transgene to allow the observation of ZEN-4 localization[5,13]. Recent experiments in the Glotzer laboratory indicate that *cdc-14* RNAi is more effective at producing the mitotic phenotype in this strain: multiple non-overlapping regions of *cdc-14* dsRNA can elicit the RNAi response in this strain, but not in wild-type (V. Pavicic and M. Glotzer, personal communication). Therefore, strain differences and the use of high dsRNA concentrations, potentially producing cross-RNAi effects, may have independently contributed to the mitotic phenotype. Nevertheless, the increased RNAi effect in the temperature-sensitive *zen-4*/ZEN-4–GFP background suggests that CDC-14 functions in a ZEN-4-dependent process[13]. Coupling this with the ability of CDC-14 to dephosphorylate ZEN-4 *in vitro*[13], the observation that *cdc-14* RNAi works at the level of ZEN-4 dephosphorylation[13], and the coincident localization of CDC-14 and ZEN-4 to the central spindle[5], provides considerable evidence that CDC-14 functions *in vivo* to dephosphorylate ZEN-4.

In summary, it appears that these seemingly opposed studies have in fact identified two distinct functions for CDC-14 in *C. elegans*: preventing extra cell divisions during periods of cell quiescence by stabilizing CKI-1; and promoting ZEN-4 localization to microtubules to create the central spindle, athough there seems to be genetic redundancy for the latter function. Thus, these two contradictory results have enhanced our understanding of two central cell-cycle processes. □

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nucleotides) homologous to human centromeric α-satellite repeat DNA in Dicer$^+$ cells, which decline in Dicer-deficient cells, suggests that Dicer and the RNAi machinery are required to cleave these human centromere repeat transcripts. However, these short RNAs persist to some extent in Dicer-depleted cells, perhaps due to some residual Dicer activity[12].

Fission yeast centromeres contain a clearly demarcated central domain where kinetochores assemble, which is flanked by heterochromatin[4]. In contrast, vertebrate centromeres lack a clear boundary between heterochromatin and the CENP-A-kinetochore-specific chromatin that marks the site of kinetochore assembly[4]. Therefore, it is possible that centromeric α-satellite transcripts in vertebrate cells have a function in kinetochore assembly. Perhaps only a subset of the α-satellite array repeats are transcribed, triggering patches of heterochromatin assembly with kinetochore proteins being loaded in between; but in this scenario it is unclear how the boundaries for this would be established and maintained. Regardless, the localization of the kinetochore-specific proteins CENP-A and CENP-C seems to be normal in Dicer-deficient cells, suggesting that defective RNAi does not completely disrupt kinetochore assembly and that the presence of kinetochore proteins does not impede transcription of the centromeric repeats in vertebrate cells. However, the authors found that heterochromatin formation is perturbed in Dicer-deficient cells. In Dicer$^+$ cells, HP1 proteins colocalize to discrete nuclear foci in the vicinity of CENP-C at both endogenous and human centromeric heterochromatin. In contrast, although HP1 signals are still detectable in Dicer-deficient cells, HP1 becomes diffusely distributed and seems to associate non-specifically with all chromatin, similar to the redistribution of HP1 and HP2 observed in RNAi fly mutants[3]. It is probable that specific histone modifications associated with centromeric heterochromatin, such as H3-K9 and H4-K20 methylation, are also disrupted, but this was not tested.

Further analyses revealed that the Rad21 cohesin does not concentrate at endogenous or human chromosome 21 centromeres, but displays a more dispersed localization in Dicer-deficient cells. This suggests that Dicer-dependent heterochromatin is required for cohesion between sister centromeres, as found in fission yeast[5,10,11]. Consistent with this, these cells exhibit premature sister centromere separation and arrest in mitosis. As HP1 localization and cohesion seems to be defective at all centromeres in these DT40 Dicer-deficient cells, this suggests that RNAi is also required for heterochromatin assembly and cohesion at endogenous chicken centromeres[12].

The data presented in this study suggest a general model for heterochromatin formation, and suggest that RNAi-mediated heterochromatin assembly has a direct and conserved role in recruiting cohesin (Fig. 1). Surprisingly, the budding yeast genome does not encode any known RNAi components and it also lacks centromeric heterochromatin, although cohesin is enriched around centromeres[6]. So why is RNAi-dependent heterochromatin required in higher eukaryotes? In metazoans, cohesin is removed from along chromosome arms early in mitosis. It is possible that heterochromatin functions in the protection of centromeric cohesin from this removal process until it is cleaved at anaphase[6]. However, there must be other roles for this centromeric heterochromatin as, similarly to budding yeast, all cohesion between sister chromatids is lost simultaneously at anaphase in fission yeast. Centromeres in fission yeast, plants and animal cells in mitosis are contacted by multiple spindle microtubules, whereas in budding yeast, mitotic centromeres only attach to one spindle fibre. Therefore, it is possible that centromeric heterochromatin has a structural role in organising and orienting mutiple microtubule-binding sites at sister centromeres[4]. In this case, RNAi-dependent chromatin modifications would probably be required for the formation of such a structure.

This and other studies raise many questions. First, it is unclear precisely how siRNA nucleates heterochromatin assembly. Observations in plants suggest that siRNA may participate in RNA–DNA interactions that result in chromatin modifications. Alternatively, it is possible that nascent chromatin-associated transcripts are targeted by siRNAs and a RISC-like complex in the nucleus to form heterochromatin. Interestingly, in plants, the silent state can be propagated in the absence of a dsRNA trigger, suggesting that RNAi functions to nucleate and establish silent chromatin, and also that it can be subsequently propagated by chromatin-associated factors[15]. Second, how often centromere repeats are transcribed is not known. They may be transcribed occasionally in a stochastic manner, allowing re-establishment of heterochromatin when required, or, transcription may occur at a particular stage of the cell cycle allowing reformation of robust heterochromatin in conjunction with, or following, replication. Finally, exactly how centromere repeats are transcribed, and whether transcription occurs across the entire centromere repeat region, is not known. It is possible that centromere-repeat-binding proteins such as CENP-B promote repeat transcription, but this and other issues await further investigation.

The ablation of Dicer function in chicken cells underscores the conserved role of RNAi in heterochromatin assembly and centromere function. Further analyses may ruffle feathers regarding how RNAi promotes heterochromatin assembly. □

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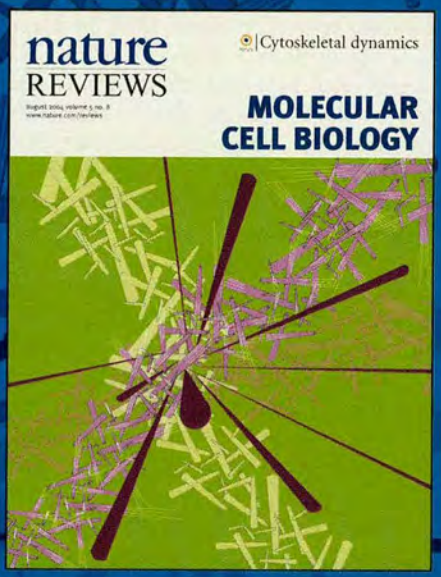

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DegrAAAded into Silence

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In fission yeast, RNA interference (RNAi)-dependent heterochromatin formation silences transgenes inserted at centromeres. In this issue, Bühler et al. (2007) demonstrate that the RNAi machinery directly targets transgene transcripts. Furthermore, they link transgene silencing to a protein complex resembling the TRAMP complex of budding yeast, which promotes transcript degradation via the exosome. Thus, RNAi-independent transcript degradation may also contribute to heterochromatin gene silencing.

The packaging of chromosomal DNA into heterochromatin is important for cellular processes such as regulation of gene expression and accurate chromosome segregation. In the fission yeast, *Schizosaccharomyces pombe*, heterochromatin is found at the mating-type locus, telomeres, and centromeres. Regions of heterochromatin are generally associated with transcriptional repression, and consistent with this finding, marker genes inserted into fission yeast heterochromatin are silenced.

Heterochromatin assembly involves an ordered series of events in which lysine 9 on histone H3 becomes methylated by the histone methyltransferase Clr4 (equivalent to metazoan Suv39), creating a binding site for chromodomain proteins such as Swi6, Chp1, and Chp2 (HP1-related proteins). RNAi is required to establish and maintain heterochromatin at centromeres but is dispensable for maintenance of heterochromatin at the mating-type locus (Grewal and Jia, 2007). In mutants of the RNAi pathway centromeric small interfering (si)RNA production is defective and homologous centromeric repeat transcripts accumulate. This has led to a model whereby siRNAs generated from centromere transcripts are required to target chromatin-modifying machinery to the centromere, resulting in tran-

scriptional repression (Grewal and Jia, 2007). However, it is paradoxical that transcriptional "silencing" should require transcription itself. Recent evidence suggests that levels of transcription of centromere repeats are largely unaffected by their assembly into "silent" heterochromatin (Buhler et al., 2006; Volpe et al., 2002). In this issue of *Cell*, Bühler et al. (2007) shed new light on the mechanism of heterochromatin silencing in fission yeast, presenting evidence for post-transcriptional silencing by an RNAi-independent pathway.

Bühler et al. (2007) set out to address an outstanding question: How do marker genes inserted into regions that surround heterochromatin become silenced? Although siRNAs corresponding to centromere repeat sequences are abundant, siRNAs from a *ura4*$^+$ marker gene inserted within centromere repeats have previously been undetectable. Consequently, it has been unclear whether the RNAi-dependent silencing machinery is recruited directly to the *ura4* gene or whether heterochromatin simply spreads into this gene from the surrounding centromeric sequence. To enrich for siRNAs, Bühler et al. (2007) exploit the siRNA-binding activity of the RNAi component Ago1 (Argonaute) and also delete the ribonuclease *eri1*—which is normally required to suppress high levels of siRNAs. In this way the authors could detect a low level of siRNAs corresponding to centromeric *ura4*$^+$. This result demonstrates that transcripts from this *ura4*$^+$ gene are processed into siRNAs and therefore that the transgene could be a direct target of the RNAi machinery.

The authors hypothesized that these *ura4* siRNAs alone are insufficient to direct silencing of the transgene for two reasons: (1) the very low abundance of siRNAs from *ura4* relative to those from centromeric sequences and (2) the siRNAs detected are predominantly of sense orientation and therefore unable to target Ago1 to the *ura4* mRNA. These observations led the authors to investigate the role of an additional RNA degradation pathway in heterochromatin silencing. In the budding yeast *Saccharomyces cerevisiae*, polyadenylation can stimulate RNA degradation by the exosome. This polyadenylation is mediated by the TRAMP complex, which contains the poly(A) polymerases, Trf4 and Trf5 (LaCava et al., 2005). Fission yeast has six members of the Cid1 family of noncanonical poly(A) polymerases. One of these, Cid12, has previously been implicated in heterochromatin silencing (Motamedi et al., 2004; Stevenson and Norbury, 2006). Cid12 is found in the RNAi complex RDRC (RNA-dependent RNA polymerase complex) along with Rdp1 and Hrr1. Moreover, *cid12* mutants alleviate silencing in a similar way to other RNAi mutants, although the exact role of Cid12 in heterochromatin silencing has yet to be elucidated (Motamedi et al., 2004). In their new work, Bühler et al. (2007) investigate the role of a second member of the family, Cid14, which is the *S. pombe* functional homolog of *S. cerevisiae* Trf4/Trf5. The authors find that functional Cid14 is required for intact silencing at centromeres and for the generation of centromeric siRNAs. Unlike RNAi mutants, deletion of the *cid14*$^+$ gene also alleviates silencing at the mating-type locus. Curiously, the *cid14* mutant shows a less marked effect on the levels of H3K9 methylation and Swi6 associated with heterochromatin than do RNAi mutants. Bühler et al. (2007) also confirm that Cid14 has poly(A) polymerase activity in vitro and that mutations in the catalytic residues of the enzyme alleviate silencing in vivo. Thus, the authors propose a model in which Cid14-mediated polyadenylation of heterochromatin transcripts is required for silencing by the RNAi machinery, the exosome, or both (Figure 1).

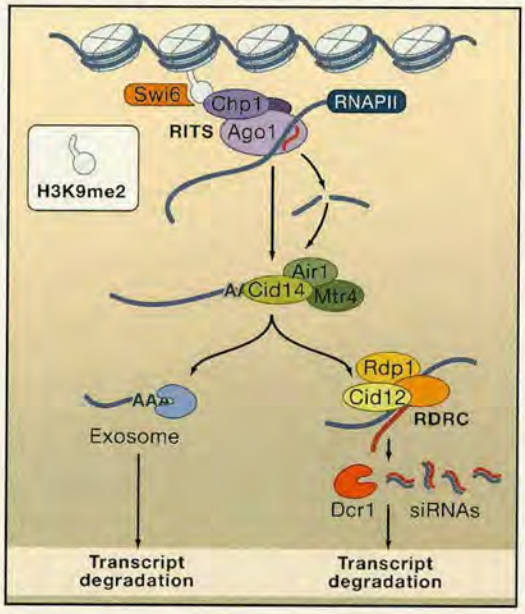

Figure 1. Silencing Heterochromatin in Fission Yeast
Noncoding RNA transcripts originating from heterochromatin repeats, or transgene insertions within heterochromatin, may be polyadenylated by Cid14. The transcript may be polyadenylated directly upon termination of transcription, or alternatively transcripts may be first sliced by Ago1 and then polyadenylated by Cid14. This may "mark" the transcript for degradation and/or processing via the RNAi machinery, RDRC/Dcr1, or the exosome.

Biochemical purification of Cid14 did not reveal any association with known RNAi or heterochromatin components. Instead Cid14 associates with fission yeast homologs of other TRAMP complex components, Mtr4 and Air1, as well as ribosome synthesis factors, consistent with the known role of Cid14 in rRNA polyadenylation (Win et al., 2006a). These findings suggest that *cid14* might act as part of a fission yeast TRAMP complex (*sp*TRAMP) to target heterochromatin transcripts for degradation. Deletion of *air1*$^+$ shows no effect on heterochromatin silencing, whereas mutation of *mtr4* alleviates silencing at the mating-type locus but not at centromeres, indicating that the components of *sp*TRAMP play varying roles in heterochromatin silencing.

To further investigate the possibility that Cid14 directs degradation of heterochromatin transcripts by the exosome, the authors also tested heterochromatin silencing in the absence of a component of the nuclear exosome, Rrp6. Deletion of *rrp6* alleviates silencing both at centromeres and at

the mating-type locus. This is consistent with recent findings that Dis3, an exosome-associated ribonuclease, is also required for silencing at centromeres and the mating-type locus (Murakami et al., 2007). Unlike *cid14*, neither of these exosome components is required to generate siRNAs that are homologous to heterochromatin regions, suggesting that the role of *cid14* may be more complex than simply exosome recruitment.

The findings of Bühler et al. (2007) strongly suggest that Cid14 is involved in targeting centromere transcripts for degradation. However, it remains to be determined whether heterochromatin transcripts are bona fide substrates for Cid14 polyadenylation. Centromere transcripts are known to have poly(A) tails, and these tails are unchanged in cells lacking Cid12, so it might be revealing to check their status in a *cid14* mutant (Win et al., 2006b). It would also be informative to examine whether Cid14 or the whole TRAMP complex associates with centromeric transcripts. Another outstanding question is the relationship between Cid14 and Cid12. Based on their observation of a large RNA species associated with Ago1 in cells lacking Cid14, the authors suggest that Cid14 may be required to convert single-stranded precursor RNA into dsRNA. This is a role also proposed for the RDRC complex raising the possibility that Cid12 and Cid14 may have some functional redundancy, analogous to Trf4 and Trf5. Such an effect might explain how Cid14 can be intimately associated with the RNAi pathway despite having a distinct mutant phenotype.

Clearly much remains to be revealed about the mechanisms underlying RNAi-directed heterochromatin formation and silencing. Nevertheless, the analyses by Bühler et al. (2007) reveal that siRNAs are made from transgene insertions at centromeres, and expose intriguing connections between heterochromatin silencing and general RNA turnover mechanisms.

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Report

The Chromatin-Remodeling Factor FACT Contributes to Centromeric Heterochromatin Independently of RNAi

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Summary

Centromeres exert vital cellular functions in mitosis and meiosis. A specialized histone and other chromatin-bound factors nucleate a dynamic protein assembly that is required for the proper segregation of sister chromatids. In several organisms, including the fission yeast, *Schizosaccharomyces pombe*, the RNAi pathway contributes to the formation of silent chromatin in pericentromeric regions. Little is known about how chromatin-remodeling factors contribute to heterochromatic integrity and centromere function. Here we show that the histone chaperone and remodeling complex FACT is required for centromeric-heterochromatin integrity and accurate chromosome segregation. We show that Spt16 and Pob3 are two subunits of the *S. pombe* FACT complex. Surprisingly, yeast strains deleted for *pob3+* are viable and alleviate gene silencing at centromeric repeats and at the silent mating-type locus. Importantly, like heterochromatin and RNAi pathway mutants, Pob3 null strains exhibit lagging chromosomes on anaphase spindles. Whereas the processing of centromeric RNA transcripts into siRNAs is maintained in Pob3 mutants, Swi6-association with the centromere is reduced. Our studies provide the first experimental evidence for a role of the RNA polymerase II cofactor FACT in heterochromatin integrity and in centromere function.

Results and Discussion

FACT Is an Evolutionarily Conserved Nuclear Complex

Centromeres are composed of specialized chromatin in which the histone H3 variant CENP-A underpins the kinetochore and is flanked by heterochromatic regions. This heterochromatin is known to attract cohesin and contribute to centromere function by ensuring physical cohesion between sister chromatids [1]. Significant progress has been made in dissecting the connections between heterochromatin and centromere function. It is known that specific histone modifications [2] and RNAi-related processes [3] contribute to an "epigenetic" mechanism that defines the heterochromatic nature of centromeric DNA.

We wish to further investigate how the correct nucleosomal structure is established and maintained over centromeric repeats. In fission yeast, Pol II is also required for centromere function because it transcribes complementary regions of the centromeric outer repeats [4], but it is not known whether Pol II requires coactivating factors. Evidence already implicates the transcriptional cofactors and chromatin-remodeling complexes RSC (remodeling and spacing complex) and PBAF (polybromo, brahma-related gene 1-associated factor) in centromere-related functions [5, 6]. Pol II transcription is stimulated by the chromatin-remodeling complex FACT (facilitates chromatin transcription) [7]. In order to investigate the possible role of this factor in centromeric chromatin, we first identified the fission yeast FACT complex.

FACT from a variety of organisms contains two core proteins (Spt16 and Pob3/SSRP1). We identified a single set of closely related sequences for these subunits in the *S. pombe* genome (Figure S1 in the Supplemental Data available online). A strain was constructed to express *S. pombe* Pob3 fused to a FLAG-PreScission-HA epitope (FPH-Pob3). Western blots reveal expression of the functional FPH-Pob3 fusion protein. To check whether FPH-Pob3 forms a FACT-like complex with Spt16, we coexpressed green fluorescent protein (GFP)-tagged Spt16 (Figure S2). α-HA antibodies immunoprecipitate Spt16-GFP (Figure 1A). Thus Pob3 and Spt16 associate in vivo.

We next biochemically purified *Sp*FACT from cellular extracts. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis revealed two specific bands in the FPH-Pob3 extracts (Figure 1B). Mass-spectrometry analysis identified the two bands as Pob3 and Spt16. To verify whether the two FACT subunits interact, we performed glutathione S-transferase (GST) pulldowns. This demonstrated that Spt16 and Pob3 associate in vitro and that the interaction requires the Spt16-M domain (Figure 1C). Our biochemical data show that *S. pombe* contains a FACT complex similar to other eukaryotes.

To determine whether *Sp*FACT localizes to the nucleus, we imaged functional Spt16- and Pob3-GFP fusions (Figure S2B). Pob3-GFP and Spt16-GFP are nuclear factors (Figure 1D). Together, our biochemical and localization data are consistent with nuclear functions of the *Sp*FACT complex.

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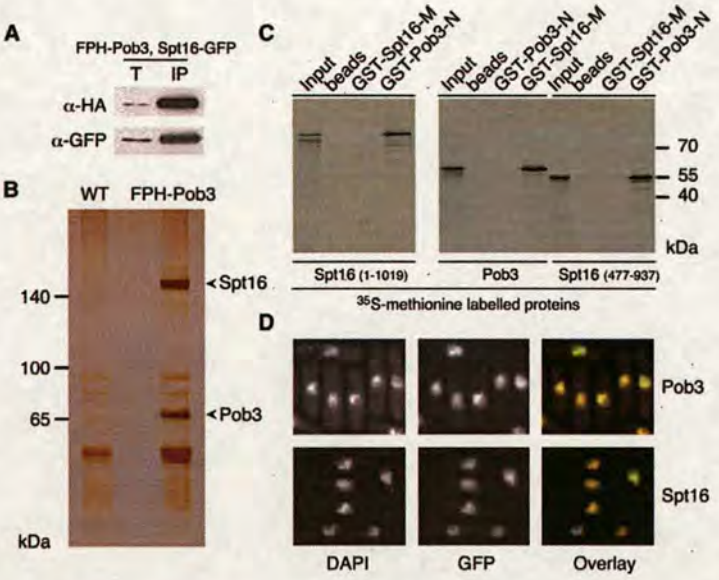


Figure 1. *S. pombe* FACT Is a Heterodimeric, Nuclear-Protein Complex

(A) Coimmunoprecipitation between tagged *S. pombe* Pob3 and Spt16. Extracts prepared from FPH-Pob3 Spt16-GFP strains were incubated with HA-antibody-coupled agarose. Fractions were analyzed by western blotting with either HA polyclonal or GFP monoclonal antibody. Lanes labeled "T" show the equivalent of 10% extract used in the IP.

(B) Biochemical purification of FPH-Pob3 and identification of copurifying proteins. IPs from WT and FPH-*pob3+*-tagged strains were performed with FLAG-epitope affinity purification. Silver staining resolves two specific bands. Peptide sequencing by tandem MS identifies Pob3 and Spt16.

(C) GST pulldowns map necessary interaction domains within *Sp*FACT. Immobilized GST-fusion proteins were incubated with in vitro translated Pob3 and Spt16. GST-Pob3-N (1-448) and GST-Spt16-M (477-937). The input lane contains 10% of the ^{35}S-proteins.

(D) Nuclear localization of Pob3-GFP and Spt16-GFP. The overlay shows the merge between DNA (4',6-diamidino-2-phenylindole [DAPI]) and *Sp*FACT (GFP).

The Small FACT Subunit Pob3 Is Not Essential for Viability in *S. pombe*

In *S. cerevisae*, both subunits of the FACT complex are essential, and mutant alleles with phenotypes in genome stability have been described [8]. As expected, the *S. pombe* ortholog for the large FACT subunit *spt16+* is essential (Figure S3). Surprisingly, a strain bearing the deletion of *pob3+* is viable (Figure 2A, Figures S4 and S5), though it shows temperature sensitivity. No paralog that might account for genetic redundancy can be identified with genomic basic local alignment search tool (BLAST) searches (data not shown). Because the *pob3Δ* strain is viable, we tested its role in distinct chromatin-based events. Cells lacking Pob3 are sensitive to hydroxyurea (HU), camptothecin (CPT), ultraviolet (UV), and (mildly) to 6-azauracil (6AU), suggesting DNA replication, DNA repair, and transcription phenotypes (Figure S6). The sensitivity of *pob3Δ* to these stress agents indicates that *Sp*FACT is involved in multiple chromatin-based cellular functions and participates in genome stability. Yet, its deletion in *S. pombe* is viable.

Deletion of *pob3* Results in Transcriptional-Silencing Defects

In vitro observations have shown that the FACT complex aids Pol II to overcome the nucleosome barrier to transcription [7]. In fission yeast, it is known that Pol II is required to transcribe centromeric, noncoding outer repeats (*otr*) and to form silent heterochromatin [4].

We therefore checked whether the loss of Pob3 affects transcriptional silencing within centromeres. Fission-yeast strains with reporter genes inserted at distinct locations within the centromere (Figure 2) allow the assessment of the repressive state of chromatin at these locations [9]. We tested two mutant *pob3Δ* strains with *ura4+* inserted either at the *imrIR*(*Nco*I)::*ura4+* or at *otrIR*(*Sph*I)::*ura4+* repeats. Strains with an active *ura4+* gene grow well in the absence of uracil (−Ura) but are unable to grow on counterselective plates containing 5-fluoro-orotic acid (FOA) [9]. Plating assays show that *pob3Δ* strains grow slower than does a wild-type (WT) strain on FOA medium (Figure 2B). Contrary to FACT's known roles as a transcriptional elongation factor, our results reveal that the loss of Pob3 function allows higher levels of *ura4+* gene expression relative to that of the WT (Figure 2B), with a strong effect at *imr1R* and a weaker one at *otr1R*. This suggests that Pob3 has a novel, repressive role in centromeric transcription. In comparison, mutant strains *clr4Δ* and *tas3Δ* display the complete alleviation of silencing at both loci. Further, *ade6+* reporter assays show that *pob3Δ* loss of silencing is not a *ura4+* gene-specific phenotype (Figure S2). Only a mild effect of *pob3+* deletion is observed when *arg3+* is inserted at the central core region (*cnt1*::*arg3+*; Figure 2B). The silencing assays show that the *pob3Δ* mutation distinctly affects the expression level of centromeric reporter genes, depending on their location. Importantly, these data reveal an unexpected in vivo role for *Sp*FACT in heterochromatin integrity.

We next determined whether *pob3+* deletion affects the silencing of marker genes placed in other transcriptionally silent regions [10, 11]. The results show that *pob3Δ* causes derepression of reporter silencing at the mating-type locus (Figure 2C). In contrast, the *pob3Δ* mutation does not affect the silencing of rDNA and telomeric reporters. Pob3 thus has a role in the formation or maintenance of heterochromatin at the mating locus and at centromeres.

FACT is a general transcription and remodeling factor. Recently, the human Pob3 ortholog, SSRP1, was shown to regulate the expression of a specific subset of genes [12]. The *pob3Δ* loss-of-silencing phenotype could therefore be indirect, altering the expression of specific heterochromatin factors. Thus, we performed gene-expression profiling on *pob3Δ* cells. The results reveal that no such genes were up- or downregulated

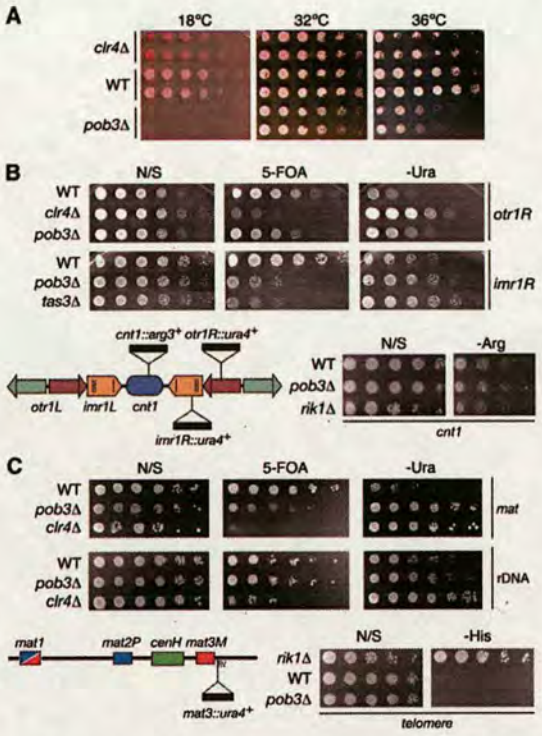


Figure 2. Viability and Heterochromatic Silencing Phenotype of the *S. pombe pob3+* Deletion Strain

(A) Deletion of the small subunit of the chromatin-remodeling complex FACT does not affect *S. pombe* viability.

(B) *pob3+* deletion alleviates heterochromatic silencing at *imr1R* and mildly at *otr1R* centromeric repeats and at the central core (*cnt1*). Mutants in the histone methyl-transferase (*clr4Δ*), in the *rik1+* gene (*rik1Δ*), and in a RITS subunit (*tas3Δ*) serve as positive controls.

(C) Gene-silencing phenotype of the *pob3+* deletion in other heterochromatic regions, such as the mating loci (*mat*), ribosomal DNA repeats (*rDNA*), and the telomere.

significantly (Table S1). Importantly, the few genes whose expression is altered are similarly affected in mutants that play a role in heterochromatin integrity, such as *clr1+*, *clr3+*, *clr6+*, and *rpb7+* (Table S2) [13]. For example, a significant fraction of *pob3Δ* upregulated genes are also upregulated in *rpb7-G150D* and in *clr3Δ* (Table S2). Because Clr3, Clr6, and Rpb7 are required for heterochromatin formation at centromeres, it is likely that FACT cooperates with these histone deacetylase (HDAC) enzymes and Pol II in centromere function.

Our data show that the *Sp*FACT complex has a new role in gene silencing at centromeric heterochromatin. Also, the transcriptional phenotype of *pob3Δ* significantly overlaps that of known heterochromatin mutants. Together, our data strongly suggest that Pob3 plays a specific and direct role in the establishment and/or maintenance of heterochromatin.

Pob3 Is Required for Accurate Chromosome Segregation

The observed centromeric-silencing defects in *pob3Δ* cells suggest that centromeric heterochromatin is disrupted. It is well established that mutants affecting heterochromatin integrity at fission-yeast centromeres also exhibit specific defects in mitotic segregation [14, 15]. We therefore conducted three types of test to identify mitotic defects. First, we checked for lagging chromosomes on anaphase spindles. Immunofluorescence staining shows that *pob3Δ* cells display a high incidence of lagging chromosomes (10%) in anaphase (Figure 3A). This represents a more than 200-fold increase over that of the WT (Figure 3B).

Second, we determined whether *pob3+* is required for minichromosome maintenance over several cell divisions. We measured the fidelity of chromosome segregation with two distinct minichromosome-loss assays [14]. In WT cells, the 530 kb linear *Ch16* minichromosome is mitotically stable [16]. Removal of Pob3 function increases the rate of minichromosome loss by more than 20-fold (Figure 3C). Because this phenotype could be due to defective telomere function in linear minichromosomes, we also tested the mitotic stability of the 30 kb circular minichromosome *CM3112* [16]. The loss rate of CM3112 is increased by approximately 30-fold in *pob3Δ* compared to that of the WT (Figure 3C). Thus, the mitotic segregation of both minochromosomes is severely affected in cells lacking *pob3+*.

pob3+ deletion might affect chromosome segregation by altering mitotic spindle function, as is seen in heterochromatin mutants [15]. We thus examined the growth and viability of *pob3Δ* in the presence of the microtubule-destabilizing drug thiabendazole (TBZ) [15]. The plating assays clearly reveal that, compared with the WT, *pob3Δ* cells are TBZ-sensitive (Figure 3D), although to a lesser extent than are *clr4Δ* cells.

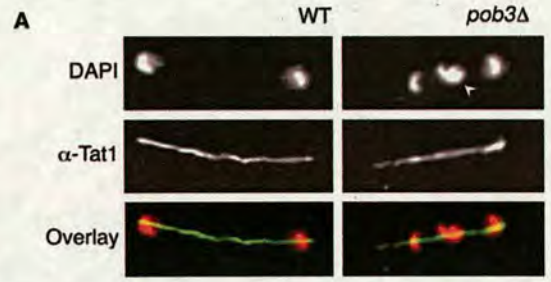
Together, these assays demonstrate that *pob3+* plays a new and important role in accurate chromosome segregation. Pob3's role in centromeric silencing might account for its mitotic functions by contributing to heterochromatin integrity.

Loss of Pob3 Does Not Affect the RNAi Pathway

To investigate the molecular mechanism underlying FACT's novel repressive function, we analyzed the effect of *pob3Δ* on the RNAi-mediated heterochromatin formation at centromeres. In fission yeast, the RNAi pathway directs transcriptional gene silencing to the centromeric outer repeats and is required to assemble intact centromeric heterochromatin [3]. In this pathway, RNA transcripts are generated from *otr* regions and are processed into siRNAs by Dcr1. These siRNAs are incorporated into the ribonucleic acid-induced transcriptional silencing (RITS) effector complex, which is required to establish heterochromatin [3].

Mutants in the RNAi pathway, such as *dcr1Δ* and *ago1Δ*, are defective in processing noncoding centromeric outer-repeat transcripts to homologous siRNA molecules. As a consequence, unprocessed *otr* transcripts accumulate [3]. In principle, a *pob3Δ* strain could display altered levels of the primary transcript and/or show changes in siRNA accumulation. Any of these phenotypes could explain the centromere-silencing and chromosome-segregation defects.

Northern blots show a clear accumulation of unprocessed *otr* transcripts in *dcr1Δ* cells, but they are not detected in *pob3Δ* or WT cells (Figure 4A). This could



B

	WT	pob3Δ
Total # of cells (late anaphase)	307	347
Lagging chromosomes	1	35
Percentage	<0.05	10.1

C

	WT	pob3Δ
Loss of 530 kb linear minichromosome	0/2199 (<0.05%)	26/2493 (1.04%)
n-fold increase over WT		>21x
Loss of 30 kb circular minichromosome	54/2664 (2.0%)	1299/2240 (58%)
n-fold increase over WT		29x

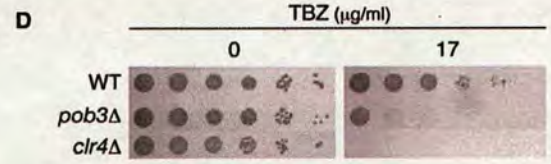


Figure 3. *S. pombe* FACT Subunit Pob3 Is Required for Accurate Chromosome Segregation

(A) *pob3*Δ mutant cells display lagging chromosomes in late anaphase. Cells grown at 25°C were subjected to anti-tubulin (Tat1) immunodetection and DAPI staining. The arrowhead indicates a lagging chromosome in the midzone of the microtubule spindle in *pob3*Δ mutants.

(B) *pob3*Δ deletion increases the frequency of abnormal anaphases. The percentage indicates the fraction of anaphase cells with lagging chromosomes.

(C) Enhanced minichromosome loss in *pob3*Δ mutant versus WT strains.

(D) *pob3*+ deletion strains display a pronounced sensitivity to the tubulin-depolymerizing drug TBZ. *clr4*Δ serves as a positive control.

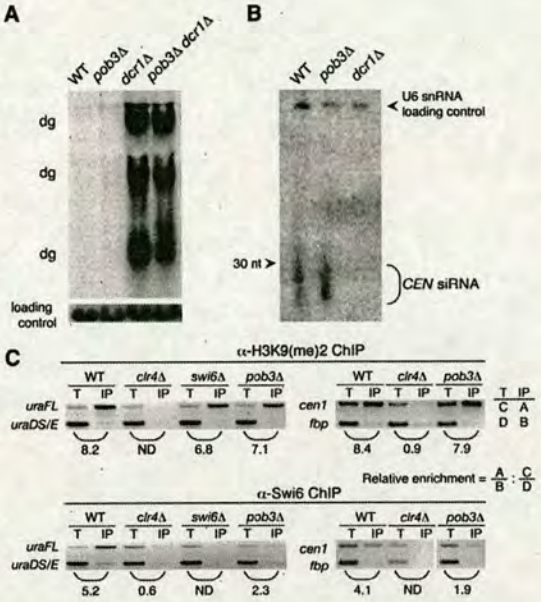


Figure 4. Pob3 Probably Acts Downstream of Dicer and Influences the Deposition of the Heterochromatic Swi6 Protein on Silenced Chromosomal Regions

(A) Northern analysis of noncoding centromeric *dg-dh* transcripts detects no measurable changes in the accumulation of these transcripts in *pob3*Δ mutant strains, in contrast to *dcr1*+ deletion. The *pob3*Δ *dcr1*Δ double mutant does not alter the noncoding *dg* RNA levels. Centromeric transcripts were detected with a probe specific for the *dg-dh* repeat (top). RNA loading controls indicate the total RNA added (ethidium bromide staining, bottom).

(B) Centromeric siRNAs are unaffected by *pob3*+ deletion. A northern blot of small RNAs extracted from WT, *pob3*Δ, and *dcr1*Δ strains was probed with a centromeric (*dg-dh*) probe. U6 snRNA serves as a loading control.

(C) In *pob3*Δ mutants, Swi6 association is altered at centromeric outer repeats, whereas histone H3K9 me2 levels are maintained. ChIP of H3K9 me2 and Swi6 in WT, *pob3*Δ, and heterochromatin mutants *clr4*Δ and *swi6*Δ detects a significant loss of Swi6 both at the *uraFL* transgene and at the endogenous *cen1* locus (*uraDS/E* and *fbp* serve as euchromatic control regions, respectively). The figure shows a representative example of three independent biological experiments. The relative enrichment of IP/Input is calculated as shown.

also be because of an important role of Pob3 in transcript generation. We therefore tested cells lacking both Dcr1 and Pob3. The results show that these transcripts appear to accumulate to the same extent as observed in *dcr1*Δ single mutants (Figure 4A). Outer-repeat transcripts thus accumulate in a *dcr1*Δ strain independently of Pob3. Consistently, centromere-repeat-homologous siRNAs are detected at similar levels in WT and *pob3*Δ cells, but not in *dcr1*Δ cells (Figure 4B). Taken together, our results indicate that Pob3 function at the centromere does not appear to affect the production or accumulation of both unprocessed transcripts and siRNAs. FACT may thus affect centromeric silencing through changes in the integrity of silent chromatin itself and by acting either downstream of (or parallel to) the RNAi machinery.

The Swi6 Heterochromatin Mark Is Altered in the *pob3*Δ Mutant

To further dissect FACT-mediated heterochromatinization, we analyzed chromatin structure at the centromeric repeats in the *pob3*Δ strain. Other fission-yeast mutants, such as *clr3*Δ and *sir2*Δ, also affect RNAi-directed silent chromatin without affecting the production or abundance of centromeric-repeat siRNAs (unpublished data). In such mutants, the H3K9 me2 levels are reduced, and consequently less Swi6 associates with centromeric repeats [17]. We therefore used chromatin immunoprecipitation (ChIP) assays to determine the levels of H3K9 me2 and Swi6 at centromeric outer repeats. In cells lacking Pob3, the ChIP assays reveal normal levels of histone H3K9 me2 on both the *otrIR::ura4*+ marker gene

and directly on the outer repeats (Figure 4C). Thus, Pob3 is not required to maintain normal levels of H3K9 me2 methylation at centromeres. Because the H3K9 me2 mark is unaffected, we expected the Swi6 protein levels to be maintained in *pob3*Δ. Surprisingly, we find that Swi6 association shows a moderate but reproducible decrease over both the reporter gene and the endogenous centromeric region (Figure 4C). This shows that although centromeric Swi6 association still occurs to some level, its association is disturbed in *pob3*Δ strains. Consistent with this partial effect, we observe that Swi6-GFP remains localized to heterochromatic loci in *pob3*Δ cells, as is seen in several RNAi-pathway mutants (Figure S7) [18]. Thus, although the key histone H3K9 me2 mark is retained on centromeric repeats in cells lacking Pob3, Swi6 association is reduced. *Sp*FACT might thus play a role in assembling or retaining Swi6 on centromeric heterochromatin.

Conclusions

Here we have identified and characterized the *Sp*FACT complex. Surprisingly, we show that deletion of the *Sp*FACT subunit Pob3 is viable. This has allowed us to critically assess Pob3's functions in vivo. Our experiments reveal a conserved biochemical protein assembly that functions in chromatin-based processes. Importantly, we provide the first biological evidence that Pob3 is required for accurate chromosome segregation. We find that this might be because of a novel role of the *Sp*FACT complex in heterochromatin integrity at centromeres. *pob3+* deletion does not affect H3K9 me2 levels, but it leads to decreases in Swi6 association at *otr* repeats. Together, our genetic and biochemical data implicate the chromatin-remodeling complex FACT in forming functional centromeres.

FACT is known to facilitate transcription through chromatin. It has been proposed that FACT can disassemble H2A-H2B from nucleosomes in front of an advancing Pol II enzyme and reassemble H2A-H2B in its wake [19]. At centromeres, mutations in the *Sp*FACT histone chaperone might affect H2A/H2B dimer incorporation and thus change the structural integrity of heterochromatin. Any alterations in the positioning or composition of nucleosomes could interfere with Swi6 association and/or spreading. This could alleviate silencing without noticeably changing H3 K9 methylation, as we observe. Alternatively, FACT might recruit Swi6 directly to *otr* regions. Decreased binding of Swi6 to heterochromatic would be expected to impair sister-chromatid cohesion, resulting in defective chromosome segregation [1].

In summary, our results show that the small subunit of the *Sp*FACT complex is required to form normal silent chromatin on the centromeric repeats and for accurate chromosome segregation. Recent studies have shown that both subunits of the human FACT complex biochemically interact with centromeric CENP-A nucleosomes [20, 21]. Although the biological role of this interaction is unclear, our in vivo data now suggest that FACT might use its histone chaperone activity to assemble and maintain the structural integrity of centromeric heterochromatin. Given the high degree of conservation in FACT subunit sequences and in biochemical functions, it is likely that our data point to an important and evolutionarily conserved role for FACT in maintaining centromere integrity.

Experimental Procedures

Strains, Media, Transformation, and Genetic Techniques

Strains are listed in Table S3. Standard genetic techniques were used [22]. Cells were grown in yeast extracts supplemented with adenine (YES) or in synthetic minimal medium [Piperazine-1,4-bis(2-ethanesulfonic acid), $MgSO_4$, glycerol (PMG)]. When required, phloxin B, 6AU, CPT, HU, or TBZ was added. Damage assays [23], minichromosome loss rates [14], silencing assays, comparative plating, and 5-fold serial dilution experiments [9] were performed as described.

Expression Profiling and ChIP Assays

Microarrays were carried out as described [13]. RNA was extracted with a standardized acid phenol protocol. cDNA was generated with *S. pombe*-specific primers and random hexamers and labeled with Cy3 or Cy5. Dye swaps were done for all experiments. Hybridized slides were scanned (Biorad scanner), quantified (ImageQuant 4.2 [Imagene]), and analyzed (Gene Spring [Silicon Genetics]). Similar gene lists were identified with hypergeometric distribution tests (Table S2). Swi6 and H3K9 me2 ChIP assays were performed as described [4]. Bands were quantified with the Eastman Kodak EDAS 290 system and 1D image-analysis software.

Immunofluorescence Microscopy

Cell-growth conditions, TAT1 immunofluorescence, and staining protocol have been described [15]. Images were collected on a Carl Zeiss MicroImaging Axioplan 2 IE fluorescence microscope. Image acquisition was controlled with Metamorph (Universal Imaging).

Northern Blots

RNA was extracted from log-phase cells by acid phenol protocol, and polyethylene glycol (PEG) precipitation to separate high- (HMW) from low-molecular-weight (LMW) RNA followed. Twenty micrograms of HMW RNA and 40 μg of LMW RNA were resolved on 6% formaldehyde gels containing 1% agarose and on 8% urea-denaturating PAGE, respectively. Gels were blotted overnight to a Hybond-XL membrane (GE). DNA probes, complementary to centromeric *dg-dh* repeats, and U6 snRNA were generated with High-Prime labeling (Roche) and T4 polynucleotide kinase (Promega), respectively. HMW and LMW RNA blots were hybridized overnight in a rotating oven at 65°C and 42°C, respectively. Phosphorscreens or films were exposed for between 3 hr and 3 days.

Protein Methods

Immunoprecipitations (IPs) with anti-HA agarose (Sigma) on whole-cell extracts were performed as recommended (Sigma). For GST pulldowns, ^{35}S-Met proteins were expressed by TnT Quick-coupled in vitro transcription and translation (Promega). 20 μl of reaction and 160 μl buffer (1x HEMG, 0.15 M KCl, 1 mM dithiothreitol [DTT], 0.1% NP40) were added to 10–30 μg of immobilized GST fusions, incubated for 1 hr at 4°C, and washed 5×. Gels were exposed on Kodak X-Omat AR. *Sp*FACT was purified with yeast-cell extracts [24]. Lysates were incubated with anti-FLAG M2 agarose (Sigma) for 6 hr at 4°C and washed in ice-cold phosphate buffered saline (PBS), elution was performed with FLAG peptide (Sigma), and SDS-PAGE or mass spectrometry (MS) analysis (Innova Proteomics) followed.

Supplemental Data

Seven figures and three tables are available at http://www.current-biology.com/cgi/content/full/17/14/1219/DC1/.

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